



2020 Physical Biosciences Research Meeting

September 14-15, 2020

2020 Physical Biosciences Research Meeting

Program and Abstracts

Zoom Virtual Meeting, Anywhere USA
Sept. 14-15, 2020

Chemical Sciences, Geosciences, and Biosciences Division
Office of Basic Energy Sciences
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FOREWORD

This volume provides a compendium of abstracts submitted to the 7th biennial meeting of investigators funded by the DOE-BES Physical Biosciences (PB) program. PB is one of the two bio-oriented programs within BES (the other being Photosynthetic Systems), and together with a robust program in Solar Photochemistry we comprise the Photochemistry and Biochemistry Team. Our mission is to fund the best fundamental basic science in areas that align with our programmatic objectives, and quite frankly, our investment in your lab is a bet that you will be able to help us reach this goal.

The year 2020 has been a challenging one for all of us, but at the same time I hope most of us realize how very fortunate we still are. I doubt any of us worry about where our next meal is coming from or whether there will be a roof over our heads when we go to sleep at night. I remind myself of these things when I'm feeling less than my usual optimistic self, while at the same time I try to look after others and help out where and when I can. As Maya Angelou said, "Try to be a rainbow in someone else's cloud." I like that!

Speaking of rainbows in cloudy times, I think we have a great meeting planned for you! Though "virtual" this year, on the positive side for the first time your co-PIs, postdocs, grad students and others in your lab were all invited to participate. There are close to 300 registrants - wow! And though truncated, I'm really excited about our speaker line-up. As always, we'll start off with Divisional and program updates from Gail McLean (our Team Lead) and me. Then it's on to our Keynote Speaker, Dr. Dieter Soll from Yale University, whose talk is provocatively titled "*The Genetic Code: from Synthetic Chemistry to Synthetic Biology.*" After a break, a session titled "Electron Bifurcation and Extracellular Electron Transfer" will showcase five exciting new projects funded in FY19 or FY20 and then we'll end Day 1 after a brief closeout session. There will be short, moderated Q&A after each talk – I'll discuss the logistics of that in my opening talk.

Day 2 will kick off with a short update on the DOE Office of Science from our Division Director Bruce Garrett. Following that, I have the pleasure of introducing Jan Keereetawee from BNL, who received the 2020 Paul K. Stumpf Award from the International Symposium on Plant Lipids (given to a distinguished young investigator in the field). Jan will give us a nice update on the complex biochemical processes that regulate plant lipid biosynthesis, and you'll see why we are proud to support yet another rising star in this program. Jan's talk will be followed by a short session called "Spotlight on Membrane-Bound Oxidoreductases" with three talks – again all from newly funded PIs. If two of those names look familiar to you, why there's a reason – and I'll explain when I introduce them. ☺ After the break that follows, we'll have one more science session called "Metallocluster Biosynthesis and 'Insanely Great' Plant Biology!" with four talks – and again, all from newly funded investigators. As you should know by now from my many emails, the focus this year is on introducing our new PIs and their projects to our more well-established PIs, always hoping that many sparks of collaboration will be created from all this talk of electron flow that will take everyone's project to the next level. We'll end Day 2 with a slightly longer close-out session, and you'll have the opportunity to ask the entire Photochemistry and Biochemistry team any question you'd like, with or without the beverage of your choice in hand. We may not have an immediate answer for the toughest of them, but we're good at finding the answers and promise to get back to you as soon as we do.

It's really unprecedented that there are twelve new projects funded over the last two years in PB, and gratifyingly, there is at least one in every current major focus area of the program. I'll explain more about how we were able to support this large number of new projects in my opening presentation, so stay tuned for that!

Alas no poster sessions this year, but I encourage you to skim through this program book and acquaint yourself with the amazing projects that your colleagues in this program are working on. PB is not defined by discipline, but rather by a focus on a relatively small number of programmatic priorities that we feel are best tackled with multidisciplinary approaches. Thus everyone from physicists to archaeal geneticists to computational chemists to plant biologists – and even a few hard core biochemists – find a collaborative environment in which to pursue their research on some of the toughest challenges in energy science. It seems that the most successful PIs in this program over time are the ones that take advantage of this environment and reach out to others, or are open to others who reach out to them, so pay close attention and make it a priority to read the “ideal collaborator” wanted by your colleagues. That description might sound like you!

In closing, I want to express my appreciation to Connie Lansdon and Linda Severs from ORISE for their valuable assistance in helping us plan this meeting, and handling most of the logistics and Zoom issues (hopefully we won't have any!). I also want to give a major shout-out to Kate Brown at NREL, who has been a fantastic help all year, but right now she's done a fabulous job getting all of the info you submitted compiled and organized into this volume. Of course I'm also extremely grateful to my three “teammates” - Steve Herbert, Chris Fecko and Gail McLean – for their mutual support, integrity, and dedication to public service. It may sound a bit corny, but it's 100% true. And of course we would not have a meeting like this one without researchers like you – so thank you!

Robert J. Stack, Program Manager, Physical Biosciences, DOE-BES

Agenda

AGENDA

7th Biennial Physical Biosciences Research Meeting
Zoom Virtual Meeting, Anywhere, USA
September 14-15, 2020

Note: All Times Below are Eastern!

Monday September 14, 2020

12:45 – 1:00 Log-in to Zoom and “settle in.” Having trouble? Call 865-951-3259

Session I: Welcome and Team and Physical Biosciences Program Updates

1:00 – 1:15 [Welcome and Photochemistry and Biochemistry Team Update](#)
Gail McLean, Team Lead, Photochemistry and Biochemistry, DOE-BES

1:15 – 2:00 [Welcome and Physical Biosciences Program Update](#)
Robert Stack, Program Manager, Physical Biosciences, DOE-BES

Session II: Keynote Lecture

2:00 – 3:00 [The Genetic Code: from Synthetic Chemistry to Synthetic Biology](#)
Dieter Soll, Yale University

3:00 – 3:40 Break (Coffee, Bio, Lunch, or Whatever – Your Choice!)

Session III: Electron Bifurcation and Extracellular Electron Transfer

3:40 – 4:00 [Elucidating the Mechanistic Determinants of Flavin-Based Electron Bifurcation](#)

Cara Lubner, National Renewable Energy Laboratory (NREL)

4:00 – 4:20 [Gating Electron Transfer in Biological Energy Storage and Conversion](#)
Anne-Frances Miller, University of Kentucky

4:20 – 4:40 [Electron Bifurcation Theory](#)
David Beratan, Duke University

4:40 – 5:00 [Electron Transport in Polymerized Cytochrome Appendages](#)
Allon Hochbaum, University of California, Irvine (Collab. Project)
Daniel Bond, University of Minnesota
Edward Egelman, University of Virginia

5:00 – 5:20 [Identifying a Novel Pathway for Extracellular Electron Uptake in *Methanosarcina barkeri* using Shotgun Proteomics](#)
Annette Rowe, University of Cincinnati

5:20 – 5:30 Close-out session, Day 1

Tuesday September 15, 2020

12:45 – 1:00 Log-in to Zoom and “settle in.” Having trouble? Call 865-951-3259

Session IV: CSGB Divisional Update and Invited Talk on Plant Lipid Biosynthesis

1:00 – 1:20 [DOE-BES and CSGB Divisional Update](#)

Bruce Garrett, Division Director, Chemical Sciences, Geosciences, and Biosciences (CSGB), DOE-BES

1:20 – 2:00 [Understanding the Biochemical Processes that Limit Plant Lipid Accumulation](#)

Jan Keereetawee, Brookhaven National Lab

Session V: Spotlight on Membrane-Bound Oxidoreductases

2:00 – 2:20 [Structural Mechanism of Energy Conservation in Hyperthermophiles](#)

Huilin Li, Van Andel Research Institute

2:20 – 2:40 [Defining the Mechanism of Proton Pumping in Heme Copper Oxidases](#)

Matthew Kieber-Emmons, University of Utah

2:40 – 3:00 [Bioinorganic Chemistry of Nitrification: Structure and Function of Ammonia Monooxygenase](#)

Kyle Lancaster, Cornell University

3:00 – 3:40 Break (Coffee, Bio, Lunch, or even Sanity – Your Choice!)

Session VI: Metallocluster Biosynthesis and “Insanely Great” Plant Biology!

3:40 – 4:00 [Probing Novel Pathways of Iron Sulfide Acquisition and Trafficking in Model Biocatalytic Systems](#)

Eric Boyd, Montana State University

4:00 – 4:20 [Primary and Secondary Sphere Effects on the Valence Isomerism of Fe-S Clusters](#)

Daniel Suess, Massachusetts Institute of Technology

4:20 – 4:40 [Atomic Resolution of Lignin-Carbohydrate Interactions in Native Plant Tissues from Solid-State NMR](#)

Tuo Wang, Louisiana State University

4:40 – 5:00 [Understanding the Mechanism and Properties of Catalytic Thi4 Proteins](#)

Andrew Hanson, University of Florida

Session VII: Closing Session and Q&A with DOE-BES Program Staff

5:00 – 5:30 [What’s Next and Where Do We Go From Here?](#)

Robert Stack, Program Manager, Physical Biosciences, DOE-BES

5:30 – 6:00 [Open Discussion and Q&A with Entire Photochem. and Biochem. Team](#)

Robert Stack, Steve Herbert, Chris Fecko and Gail McLean

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Abstracts

Oral Presentations

Sept. 14, 2020

Session II

Keynote Lecture

The Genetic Code: from Synthetic Chemistry to Synthetic Biology

Dieter Söll

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The genetic code, initially thought to be universal and immutable, is now known to contain many variations, including biased codon usage, codon reassignment, ambiguous decoding and recoding. As a result of advances in the areas of genome sequencing, biochemistry, bioinformatics and structural biology, our understanding of genetic code expression and flexibility has advanced substantially in the past decade. This presentation will highlight the diversity of mechanisms ensuring the faithful interpretation of the genetic message in the living world, as well as the status of genetic code evolution *in vivo* and *in vitro*.

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*Mukai T, Englert M, Tripp HJ, Miller C, Ivanova NN, Rubin EM, Kyrpides NC, Söll D (2016) Facile recoding of selenocysteine in nature. *Angew Chem Int Ed Engl* **55**, 5337

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* Publications supported in part by the Department of Energy grant DE-FG02-98ER20311 are indicated by an asterisk

Oral Presentations

Sept. 14, 2020

Session III

*Electron Bifurcation and
Extracellular Electron Transfer*

Elucidating the Mechanistic Determinants of Flavin-Based Electron Bifurcation

Carolyn E. Lubner, Principal Investigator

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Overall research goals:

In this program, we are developing a fundamental understanding of how electrons are controlled temporally, spatially, and energetically in novel flavin-based electron bifurcating enzymes. Flavin-based electron bifurcation is a process where an electron pair is separated into individual electrons by a special flavin cofactor and used to drive two distinct chemical reactions (**Figure 1**). These systems utilize the free energy generated from an exergonic oxidation-reduction reaction to drive a coupled endergonic reaction. Flavins are used extensively throughout biology to catalyze both one- and two-electron transfers, however the atomic level determinants that impart them the ability to bifurcate are largely unknown. The objective of this work is to delineate the physical and electronic determinants of flavin electron bifurcating sites to generate a detailed, mechanistic framework that leads to a robust understanding of how biocatalysts transform electrochemical potential into chemical bonds.

This is being addressed in three aims to:

- I. Elucidate the physical features responsible for tuning of bifurcating flavin cofactors.
- II. Investigate the impact of spatial configuration and coupling between bifurcating flavin cofactors and the initial acceptors of bifurcated electrons.
- III. Understand how bifurcating enzymes impart independent control of the two bifurcated electrons.

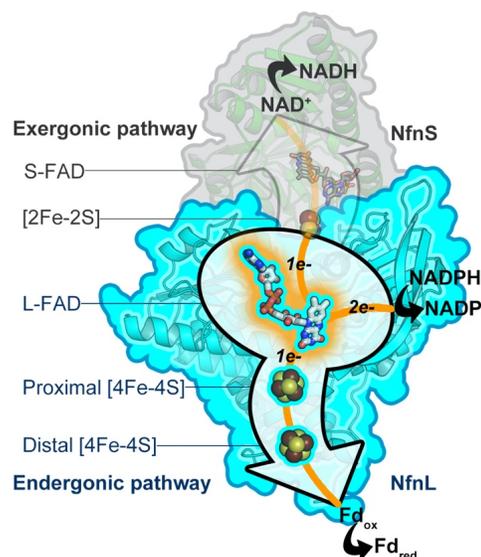


Fig. 1. Nfn from *Pyrococcus furiosus* (Pf) (PDB 5JFC) is comprised of a large subunit (NfnL, blue) and a small subunit (NfnS, grey). Following oxidation of NADPH, the bifurcating flavin, L-FAD (center), performs two one-electron transfers, first to the site-differentiated [2Fe-2S] of NfnS, then to the site-differentiated proximal [4Fe-4S] cluster of NfnL. The [2Fe-2S] is oxidized by S-FAD, which catalyzes the reduction of NAD⁺ to NADH following two rounds of bifurcation. The proximal NfnL cluster reduces the distal [4Fe-4S], which subsequently reduces one equivalent of ferredoxin (Fd) for each NADPH oxidized.

Significant achievements (2019-2024):

Our previous work on the Nfn enzyme revealed an inferred energy landscape with features unprecedented in biochemistry and with novel energetic challenges, the most intriguing being a large thermodynamically uphill step for the first electron transfer from the bifurcating flavin. However, ambiguities in the energy landscape at the bifurcating site, deriving from overlapping flavin spectral signatures, have impeded a comprehensive understanding of the specific mechanistic contributions afforded by thermodynamic and kinetic factors. To address this limitation, we developed a system to

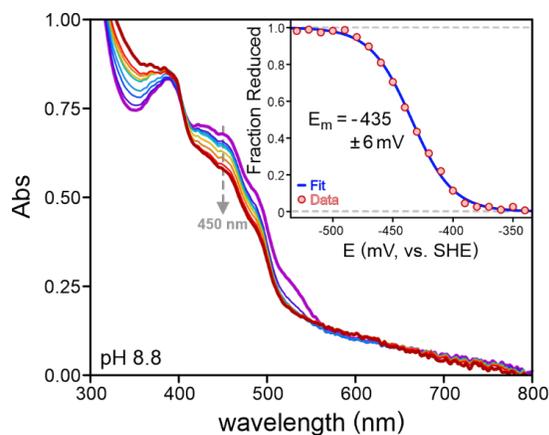


Fig. 2. Spectroelectrochemical measurements showing the two-electron Ox/HQ couple of L-FAD to be -435 ± 6 mV at pH 8.8 and room temperature. Flavin reduction was monitored using the decrease in absorbance at 450 nm (gray arrow), where the Fe/S cofactors of NfnL have little spectral interference. The fraction of reduced L-FAD (inset, red) was derived from the absorbance change at this wavelength and plotted as a function of the applied potential, then fit to a Nernst function (inset, blue). Reported values represent the average of four replicate measurements.

negative relative to other bifurcating enzymes, Nfn may have an increased plasticity to place unproductive electron transfer events into disfavored regimes; in contrast with other systems that utilize conformational movements to prevent unproductive reactions. Ultimately, such differences enhance our understanding of the breadth of mechanisms that can be exploited to perform challenging, endergonic transformations. The subtle variations are expected to illuminate the more intricate phenomena involved in the control of bifurcated electrons, such as coupling of electron transfer with proton transfer or alterations in flavin orientation and environment.

Science priorities for the next year (2020-2021):

- Studies on the individual NfnS construct to inform on how electrons are arranged and controlled within the exergonic branch between the first and second rounds of bifurcation.
- Investigate the extent by which unique site-differentiated ligation of the [4Fe-4S] cluster proximal to the bifurcating flavin influences the reaction coordinate.
- Assess how specific atomic and molecular interactions from the surrounding protein environment affect the thermodynamics and stability of L-FAD and the binding interaction with NADPH.

My major scientific area(s) of expertise is/are: ultrafast optical spectroscopy, biophysical and biochemical analysis of redox enzymes and photosynthetic systems.

To take my project to the next level, my ideal collaborator would have expertise in: electron transfer theory and computation, MCD, RR.

Publications supported by this project 2019-2020:

1. C. E. Wise, D. W. Mulder, K. J. Chou, J. W. Peters, P. W. King, C. E. Lubner, "An uncharacteristically low-potential flavin governs the energy landscape of electron bifurcation." *J. Am. Chem. Soc.*, in revision (2020).
2. J. L. Yuly, P. Zhang, C. E. Lubner, J. W. Peters, D. N. Beratan, "Universal free-energy landscape produces efficient and reversible electron bifurcation." *Proc. Natl. Acad. Sci. U. S. A.* (2020). DOI: 10.1073/pnas.2010815117

specifically probe the bifurcating flavin without complication from the additional chromophore. We expressed and purified the Nfn large subunit, NfnL, containing only the bifurcating flavin (L-FAD) and two [4Fe-4S] cluster cofactors. Electrochemical and spectroscopic experiments were performed to assess the midpoint potential of the two-electron couple of L-FAD, which was found to be uncharacteristically low at $E_m \text{ Ox/HQ} = -435$ mV (vs. SHE) (**Figure 2**). We were also able to measure the $E_m \text{ SQ/HQ}$ couple at +40 mV, in excellent agreement with our calculated value of +41 mV. Nfn represents the only system where all three redox couples of a bifurcating flavin site were experimentally measured. This has led to a considerable change in how we view the energy landscape of Nfn, since the first electron transfer event is now shown to be favorable, and has implications for understanding how bifurcation is initiated and how short circuit reactions are prevented. Nfn appears to achieve these two actions differently from all other studied bifurcating enzymes. By encompassing a sufficiently large energy gap, which is more

Gating electron transfer in biological energy storage and conversion.

Anne-Frances Miller, Principal Investigator

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Overall research goals:

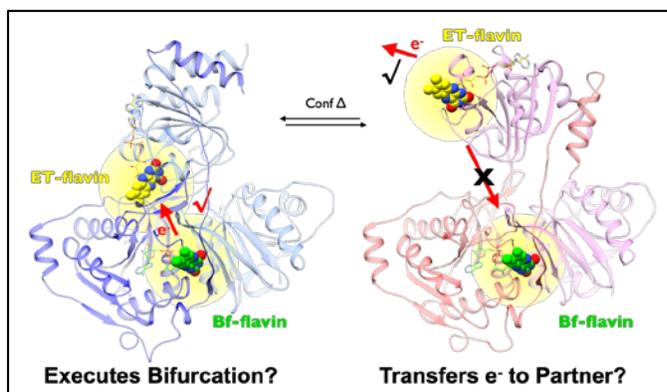
The urgency of replacing fossil fuels cannot be overstated. Thanks to investments by D.O.E. and other agencies, America has technologies for generating renewable energy. However implementation continues to be hampered by socioeconomic inertia, energy storage challenges, and costs of certain critical materials. As EPSCoR/DOE recipients, we work to address all three of these issues. In support of the EPSCoR mission, our outreach efforts seek to convince Kentuckians that they have a personal stake in the future, via participation in STEM. Meanwhile, our research in Basic Energy Science seeks deployable energy solutions that already work, are renewable, and are cheap.

We study the electron transfer bifurcation strategies used by Nature, to extract physico-chemical principles that can be incorporated into man-made materials and devices, and do not require the use of rare metals. Electron bifurcation enables microbes to exploit abundant low-grade electrochemical fuels to drive critical high-demand reactions. In essence, bifurcating electron transfer proteins (BfETFs) redistribute energy among electrons, thereby optimizing efficiency and versatility in electrochemical energy use. Crucially, they also interface single-electron reactions (as driven by photosynthetic electron transfer), with pairwise redox chemistry yielding stable closed-shell products wherein the energy is stored for future use and is portable.

Flavins are ideally suited to execute electron bifurcation by virtue of their tunable redox reactivity on the cusp between one-electron ($1e^-$) and $2e^-$ activity. The protein environment is critical in determining what redox activity is expressed by flavins, and protein *motions* are proposed to underpin efficiency, by directing the transfers of electrons to different acceptors at different points in the catalytic cycle. For BfETFs, two very different conformations have been captured crystallographically. In one, the flavin that executes bifurcation (the Bf-flavin) is near the flavin that accepts one electron and transfers it to a high- E° acceptor (the electron transfer, or ET-flavin, see Figure). This conformation is believed to model one in which electron transfer occurs between the two flavins in the BfETF. In the other conformation, the 'head' domain containing the ET-flavin is rotated by 80° , placing the ET-flavin on the exterior of the ETF, accessible to partner proteins but far from the Bf-flavin (Figure). This conformation is believed to prevent reverse electron flow back to the Bf-flavin, and to instead mediate electron transfer to a partner enzyme. In order to play its proposed role in gating electron transfer, the conformation change must be tightly coordinated to other elements of turnover. Moreover in order to support efficient electron transfer between the two flavins in the ETF, the head domain should in fact rotate further than has yet been observed in crystal structures. Thus, we propose studies in solution to probe for a third conformation, or a greater amplitude of motion than has yet been documented, and we propose experiments to identify elements of catalysis that are coupled to conformational change. In particular we will test for a conformational response to (1) substrate binding / product release, (2) association with partner protein, and (3) oxidation state of the Bf- and ET-flavins.

We will use small-angle neutron scattering (SANS) to determine how the conformational change is coordinated with elements of turnover. SANS is one of very few structural techniques that does not result in flavin photoreduction, because it does not expose the sample to energetic electromagnetic radiation. Thus, SANS will enable us to compare the conformations of Bf-ETF containing different oxidation states of the two flavins. SANS will be implemented in close collaboration with experts at the Oak Ridge National Laboratory. We will exploit the distinct scattering properties of ^1H vs. ^2H , in conjunction with specific isotopic labeling of individual subunits of the protein complex, to render partner proteins 'invisible'. SANS of such samples will highlight the domain

reorientation that is proposed to occur upon binding with partner proteins, and/or release of NAD^+ . Computation of SANS predicted by existing crystal structures, and comparison with experimental data, will reveal whether the ETF solution structure can be described in terms of the known conformations, and establish -1- the extent to which both contribute and -2- the possible significance of additional conformation(s). Comparisons of SANS when samples are prepared with different oxidation states of the two flavins, and \pm substrate analogs bound, will reveal whether -1- flavin oxidation state is coupled to domain-scale protein conformation and/or -2- whether binding of nicotinamide is coupled to protein conformation. Finally, we will compare SANS \pm partner protein to learn whether the internal structure of the ETF changes upon binding of FixX. Our studies conducted in solution will permit observation of unconstrained conformational responses to events involved in turnover. Thus we will identify factors that tip conformational equilibria and enable the protein to gate electron transfer to minimize backflow, and maximize efficiency of energy deployment.



Significant achievements (Funding is to begin on 1 Sept. 2020):

Measurement of flavin E° 's, production of series of mutants addressing specific amino acids' roles, experimental attribution of individual activities to particular flavins within BfETF, computation of electronic spectra of flavins reproducing experimental differences between them.

Science priorities for the next year (2020-2021):

- Generation of domain-scale isotopomers of the electron transfer flavoprotein (ETF) target.
- Optimize conditions for production of partner proteins.
- SANS trials to establish contrast matching and conformational landscape of the ETF alone.

My major scientific area(s) of expertise is/are: Flavin spectroscopy, electrochemical titrations, protein manipulation including anaerobic and photochemical, computation (nascent), 'strategic' NMR applicable to enormous badly-behaved systems.

To take my project to the next level, my ideal collaborator would have expertise in: Structure determination under anaerobic and other defined non-standard conditions, HDX and related mass spectrometric methods, synthesis of flavin variants, low-temperature EPR.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years] : (Supported by NSF CLP CHE-1808433, and DOE-BES EFRC DE-SC0012518.)

1. Duan, H. D., Lubner, C. E., Tokmina-Lukaszewska, M., Gauss, G. H., Bothner, B., King, P. W., Peters, J. W., **Miller, A. F.** (2018) "Distinct properties underlie flavin-based electron bifurcation in a novel electron transfer flavoprotein FixAB from *Rhodospseudomonas palustris*." *J. Biol. Chem.* 293 (13):4688-4701 DOI: 10.1074/jbc.RA117.000707. PMC5880144
2. Mohamed-Raseek, N., Duan, H. D., Hildebrandt, P., Mroginski, M.-A. and **Miller, A.-F.** (2019) "Spectroscopic, Thermodynamic and Computational Evidence of the Locations of the FADs in the Nitrogen Fixation-Associated Electron Transfer Flavoprotein". *Chem. Sci.* 10:7762-7772. DOI 10.1039/C9SC00942F
3. Duan, H. D., Mohamed-Raseek, N. and **Miller, A.-F.** (2020) "Spectroscopic evidence for direct flavin-flavin contact in a bifurcating electron transfer flavoprotein." *J. Biol. Chem.* 295 <https://www.jbc.org/content/early/2020/07/13/jbc.RA120.013174.full.pdf>

Electron Bifurcation Theory

David N. Beratan, Principal Investigator

Prof. Peng Zhang, Research Associate Professor

Mr. Jonathon Yuly, Graduate Student

Departments of Chemistry, Biochemistry, and Physics, Duke University, Durham, NC 27708

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Overall research goals:

The overarching objective of this proposal is to examine the consequences of the privileged free energy landscape that we postulate is essential for high-efficiency and reversible electron bifurcation (EB)/confurcation (EC) reactions. We will explore how this landscape informs the design of synthetic bioinspired bifurcating systems. The following are the project's specific objectives:

- *Determine how biological EB avoids short-circuiting reactions during the electron refilling step.* When two electrons “refill” the fully oxidized bifurcating cofactor, in order to initiate the next cycle of EB, it is possible that the reaction intermediated may short-circuit, dissipating energy, and leading to failure of the overall EB machinery. Design constraints on the EB/EC machinery must assure that high energy-efficiency refilling is realized. We will explore whether or not inverted potentials are required for high-efficiency refilling by modeling the multi-particle kinetics of refilling.
- *Develop a general procedural approach to generate (computationally) the requisite kinetics equations to describe electron bifurcations and to explore key factors that lead to dysfunction or enhanced function of the privileged EB landscape.* Our assessment of candidate EB energy landscapes required the elaboration of a kinetic model that enumerated every possible microstate of the system (cofactors in every possible oxidation/reduction state). Having the capacity to evaluate candidate landscapes will enable the design of synthetic bioinspired EB constructs. We will also expand kinetic modeling to describe larger EB networks, describe the coupled (refilling) bimolecular ET reactions and the proton coupled reactions with improved realism.
- *Explore design schemes for synthetic EB systems and assess design constraints and principles for architectures based on non-biological constructs.* Possible platforms for bioinspired EB assemblies include synthetic organic and organometallic structures, *de novo* proteins, DNA origami, and redox active nanostructures including quantum dots. We will examine the opportunities and constraints surrounding these synthetic bioinspired designs.

Significant achievements (Funding begins September 2020)

Science priorities for the next year (2020-2021):

- Our research priority focuses on the physical principles that govern the electron refilling process in electron bifurcating enzymes. We will examine whether or not thermodynamic efficiencies of refilling dictate characteristics of the redox potential landscape of the EB machinery.
- We will also begin to develop the generalized computational machinery needed to model multi-particle hopping networks, a capability that will enable us to explore diverse bioinspired EB schemes (as well as other charge transport networks in bioenergetics).

My major scientific area(s) of expertise is/are: Theoretical biophysical chemistry; electron tunneling pathways; electron transfer theory; theory of multi-step and multi-particle hopping processes; electron bifurcation; transport in bacterial “nanowires.”

To take my project to the next level, my ideal collaborator would have expertise in: Chemical and biochemical design/engineering of synthetic, semisynthetic, and bioinspired structures that bifurcate electrons and couple to redox catalysts; expertise in studying the flow of *more than one electron or hole* through a multi-cofactor network; ability to manipulate the proton-coupled events in electron bifurcates; ability to study how transport characteristics may change in multi-cofactor chains as the electron (or hole) number are varied (probing redox “traffic jams”).

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years]:

1. This project begins in September 2020.

Electron Transport in Polymerized Cytochrome Appendages

Allon Hochbaum, Principal Investigator

Daniel Bond, Edward Egelman, Co-PI(s)

Komal Joshi, Zahra Nazemi, Fengbin Wang, Postdoctoral Research Associates

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Overall research goals:

In anoxic environments, from aquatic sediments to the human gut, respiring bacteria routinely transfer electrons micron-scale distances beyond their outer membranes to distant and insoluble terminal electron acceptors. Despite its central role in global geochemical cycling, and unique bioelectronic potential, the mechanism enabling such long-distance electron transfer and between-cell conductivity remains a topic of active debate. One organism long studied as a model of this process, *Geobacter sulfurreducens*, uses filamentous, electrically conductive appendages to transfer reducing potential from cells to extracellular terminal oxidants. These filaments have been extensively studied by a range of indirect methods and their molecular identity was recently revealed to be polymerized cytochromes of the *G. sulfurreducens* OmcS. Such well-ordered cytochrome filaments are novel structures in biology, and polymerized OmcS is the best-characterized example of protein supramolecular aggregates that support long-range electronic conduction.

Our long-term research goals are to:

- (1) Reveal the mechanism facilitating long range electron transport in polymerized cytochrome filaments.
- (2) Define biosynthetic pathways enabling assembly of conductive extracellular cytochrome filaments.
- (3) Identify homologous structures enabling redox conduction in *G. sulfurreducens* and other environmental bacteria.

This first 3-year project will focus on the distinctive electron transport mechanisms and structures inherent to newly discovered filaments composed entirely of the cytochrome OmcS, using a cross-disciplinary combination of biochemistry, molecular biology, spectroscopy, electrochemistry, solid-state transport, and cryo-electron microscopy (EM) methods.

Significant achievements (2019-2022):

- Identified two phenotype variants in a commonly used culture collection “strain” which produce different amounts of OmcS appendages, potentially explaining disparities in literature reports that used inconsistent strain backgrounds.
- Constructed markerless deletions of *omcS*, homologs thereof, and physiologically related cytochromes in the new *G. sulfurreducens* background, representing the first clean deletions of these cytochromes in the field.
- Developed an efficient, single-step mating protocol to construct strains of these *G. sulfurreducens* mutants with genomic expression of the selected cytochromes under control of tunable strength promoters. These efforts generated a new over-expression strain for appendages exhibiting all molecular characteristics of the wild-type OmcS fibers.
- Cryo-EM imaging and analysis confirmed the presence of filaments with structural features consistent with OmcS polymers characterized in our previous work.
- Developed a new fiber isolation protocol for higher-yield and purity extraction of OmcS filaments and clean monomers, and characterized their spectroscopic absorption features.

Science priorities for the next year (2020-2021):

- Biochemical characterization of knockout and single-amino acid mutants
- Cryo-electron microscopy imaging and structure of knockout and single-amino acid mutant filaments
- Electron transport characterization of knockout and single-amino acid mutants

Our major scientific area(s) of expertise are: Functional genomics, molecular biology and analytical chemistry, cryo-electron microscopy and computational image analysis, charge transport processes, electrochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: analytical or numerical modeling of charge transfer over long distances; methods for studying charge transfer dynamics.

Identifying a novel pathway for extracellular electron uptake in *Methanosarcina barkeri* using shotgun proteomics

Annette Rowe, PI

Department of Biological Sciences, University of Cincinnati

731F Rieveschl Hall, Cincinnati, OH, 45221-0006

Email: annette.rowe@uc.edu ; Website: www.electromicrobiology.org

Overall research goals:

The biochemical diversity of electron transfer reactions in microbial systems is reshaping our understanding of the metabolism, ecology, and utility of microbes for energy related applications. Recently, members of the *Methanosarcinales*, a lineage of the cytochrome-containing methanogens, were shown to participate in direct interspecies electron transfer (DIET) with organisms such as *Geobacter metallireducens*. This work highlighted an electron transfer mode between species that required the extracellular electron transfer (EET) pathways known in *Geobacter*, and pointed to an unidentified mode of extracellular electron uptake in *Methanosarcina barkeri*. To uncover a potential mechanism for DIET from the methanogen perspective, our recent work has focused on characterizing the electrochemical activity of *Methanosarcina barkeri*. We were able to confirm the ability of *M. barkeri* to perform electron uptake from electrodes, and link cathodic current to quantitative increases in methane production. The underlying mechanisms identified in this work include: 1) a recently proposed association between cathodes and non-cell associated extracellular enzymes (e.g., hydrogenases) that can facilitate current generation through the formation of reduced and diffusible methanogenic substrates (e.g., hydrogen) and 2) a lower voltage hydrogen-independent electron uptake pathway that facilitates cathodic activity coupled to methane production. This second feature remained in electrochemical experiments after minimizing the contributions of non-cell associated extracellular enzymes (via washing cells) and using a mutant that lacked methane-linked hydrogenases (provided by William Metcalf, UIUC). This points to a novel and extracellular-enzyme-free mode of electron uptake able to facilitate cathodic current generation at redox potentials lower than -498 mV vs. a standard hydrogen electrode (over 100 mV more reduced than the observed hydrogenase midpoint potential under these conditions). At present, we lack insight into the biophysical basis of this process (i.e., what are the key enzymes or pathways involved?), as well as the role this process plays during DIET.

To identify the proteins involved in *M. barkeri*-EET, we propose to utilize shotgun proteomic techniques to: 1) identify the extracellularly exposed proteins of electrode grown *M. barkeri*, and 2) compare differential protein abundances between electrode and traditional methanogenic growth conditions (e.g., methanol). Understanding the extracellular proteome will highlight redox active proteins in contact with the extracellular environment. Coupling this knowledge to identification of proteins that are present and/or function uniquely during *M. barkeri*-EET, we will highlight the potential bioenergetic pathway for EET driven methanogenesis. We predict that *M. barkeri*-EET generates cellular reducing power that can be utilized by the known CO₂ reduction pathway, though we have shown hydrogenases can be eliminated from this pathway under EET conditions. Stemming from the low voltage measured for electron uptake, we hypothesize that these proposed experiments will point to a direct electron uptake mechanism that results in reduced ferredoxin. The ability of this low voltage EET pathway measured in *M. barkeri* to operate during DIET will also be investigated electrochemically. Specifically, electrochemical gating measurements and interdigitated electrode arrays will be used to quantify the formal potential for electron flow through the co-culture network to confirm relevance of *M. barkeri*-EET to DIET.

Significant achievements (2020-2022, etc.):

Funding not currently started on this project, however we are in the processes of establishing co-cultures with various *M. barkeri* mutants that will be utilized for proteomics and electrochemistry. Graduate student Linda Vu, is also working on protein extraction methods from different electrode materials.

Science priorities for the next year (2020-2021):

- The main priority for the coming year is to characterize the extracellular proteome of *M. barkeri*
- We will also continue developing co-culture electrochemical techniques for *M. barkeri* and *G. metallireducens*
- Practice experimental set up for iTRAQ experiments.

My major scientific area(s) of expertise is/are: Microbiology, Anaerobic Microbiology, Electromicrobiology, Physiology, Proteomics, Electrochemistry .

To take my project to the next level, my ideal collaborator would have expertise in: My ideal collaborators would be biochemists/biophysicists that could help us reconcile protein and electrochemical data. Additionally people who focus in methanogen genetics would be great once we get a sense of the proteins we should be targeting.

Oral Presentations

Sept. 15, 2020

Session IV

Invited Talk:

Plant Lipid Biosynthesis

Understanding the Biochemical Processes that Limit Plant Lipid Accumulation

Jantana Keereetaweep, Associate Biochemist; John Shanklin, Principal Investigator

50 Bell Avenue, Upton, NY 11973

Email: Keereetaweep@bnl.gov; Website: <https://www.bnl.gov/staff/Keereetaweep>

Overall research goals:

The first committed step in fatty acid synthesis is mediated by Acetyl-CoA carboxylase (ACCase). By using *Arabidopsis* cell suspension cultures, our recent study showed that ACCase can be regulated by short-term (reversible) and longer-term (irreversible) inhibition by the oversupply of fatty acids (FA) upon feeding with Tween80. Inactive analogs of biotin carboxyl transfer proteins (BCCPs), Biotin-Attachment-Domain-Containing (BADC), can displace BCCP subunits within ACCase complex and downregulate its activity. While the reversible phase of ACCase inhibition was similar for cells derived from *badc1badc3* and wild-type, the irreversible phase of inhibition was reduced by 50% in *badc1badc3* relative to wild-type. We present the two important homeostatic roles for BADC proteins in the regulation of ACCase activity: firstly, during normal growth and development, and secondly, by contributing to its long-term irreversible feedback inhibition from oversupply of FA. **The major thrust going forward is to understand how BADC proteins contribute to the long-term irreversible inhibition of ACCase using a proteomic approach to identify potential post-translational modifications (PTMs) of BCCP or BADC subunits.**

Another study demonstrated the involvement of a catalytic α -subunit of the SUCROSE-NON-FERMENTING1-RELATED PROTEIN KINASE1 (SnRK1) in the phosphorylation-dependent proteasomal degradation of WRINKLED1 (WRI1). We recently showed that trehalose-6-phosphate (T6P) an inhibitor of SnRK1, directly binds to KIN10, weakening its association with GEMINIVIRUS REP-INTERACTING KINASE1 (GRIK1), required to activate KIN10, thereby stabilizing WRI1. We tested the hypothesis that the phosphate group on T6P can preferentially bind to a site on KIN10 in an area of positively charged residues i.e., lysines or arginines. Upon the inspection of the surface of a KIN10 homology model, several such potential T6P binding sites were identified. Lysines and arginines within these sites were substituted by alanine residues and T6P binding assays were performed. Equilibrium dissociation constants (K_d) derived from microscale thermophoresis between KIN10 K63A-R65A-R66A and T6P was significantly increased relative to that of WT. KIN10 K63A-R65A-R66A-K69A-L73A also showed weakened association with GRIK1 compared to WT. Therefore, we propose the potential binding site(s) for T6P on KIN10 and rationalize how it weakens the interaction between KIN10 and GRIK1 blocking its activation. **Various complementary approaches including protein crystallography, small-angle X-ray scattering (SAXS) of proteins in solution and protein footprinting will be utilized to identify structural details of the interactions in order to obtain a deeper understanding to how T6P regulates SnRK1 activity.**

Significant achievements ([Click to Enter Years of Current Funding, e.g. 2018-2021; 2019-2022; 2020-2022, etc]): New investigator.

[Click to enter the scientific achievements so far for the current 2 or 3 year grant/FWP. Text can be a bulleted list or multiple paragraphs. Feel free to add figures and figure legends as needed.]

Science priorities for the next year (2020-2021):

- Identify possible post-translational modifications of Acetyl-CoA subunits that contribute to its long-term irreversible inhibition during excess fatty acids
- Solving crystal structures of KIN10 and the complex between KIN10, GRIK1 and T6P to gain the understanding the molecular level to how T6P disrupt association of KIN10 and GRIK1 using x-ray crystallography and/or cryo-EM.
- Using protein footprinting to identify residues involved in interactions between KIN10 and T6P

My major scientific area(s) of expertise is/are: Biochemical regulation of lipid biosynthesis, plant growth and development, plant lipid metabolism.

To take my project to the next level, my ideal collaborator would have expertise in: proteomics, and bioinformatics.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years]:

1. Liu, H., Zhai, Z., Kuczynski, K., **Keereetaweep**, J., Schwender, J. and Shanklin, J. (2019) WRINKLED1 regulates BIOTIN ATTACHMENT DOMAIN-CONTAINING proteins that inhibit fatty acid synthesis. *Plant Physiology*, **181** (1), 55-62.
2. Zhai, Z., **Keereetaweep**, J., Liu, H., Feil, R., Lunn, J.E. and Shanklin, J. (2018) Trehalose 6-Phosphate Positively Regulates Fatty Acid Synthesis by Stabilizing WRINKLED1. *The Plant Cell*, **30**, 2616–2627.
3. **Keereetaweep**, J., Liu, H., Zhai, Z. and Shanklin, J. (2018) Biotin Attachment Domain-Containing Proteins Irreversibly Inhibit Acetyl CoA Carboxylase. *Plant Physiology*, **177**(1), 208–215.

Oral Presentations

Sept. 15, 2020

Session V

*Spotlight on Membrane-Bound
Oxidoreductases*

Structural mechanism of energy conservation in hyperthermophiles

Huilin Li, Principal Investigator

Xiang Feng, Postdoctoral Research Associate

Structural Biology Program, Van Andel Research Institute

Email: Huilin.Li@vai.org; Website: <https://HuilinLiLab.vai.org>

Overall research goals:

Our overarching goal is to understand at the molecular and chemical level the mechanism of electron transport and energy conservation in the biological world. Our strategy is two pronged – to forge close collaborations within the DOE Physical Biosciences Program, in particular with Dr. Michael Adams; and to use the cryo-EM based structural biology approach to solve atomic or near atomic resolution structures of key protein complexes involved in electron bifurcation and energy conservation. The enzyme complexes to be studied are derived from hyperthermophilic microorganisms such as *Pyrococcus furiosus* and *Thermotoga maritima*, that thrive at temperatures from 80°C to 100°C. We investigate two types of enzyme complexes, one involved in the conversion of low potential reducing equivalents into ion gradients across the membrane, the other involved in electron bifurcation, which is considered the third mechanism of energy conservation. Specific protein complexes we are studying include the membrane-bound hydrogenase and membrane-bound sulfane sulfur reductase and the electron bifurcating enzyme complex Fix/EtfABCX.

Significant achievements (2019-2022):

1) We have solved the structure of the membrane-embedded 13-subunit MBS and have elucidated its polysulfide reduction mechanism. A comprehensive manuscript reporting the structure and mechanism of MBS has been submitted for publication.

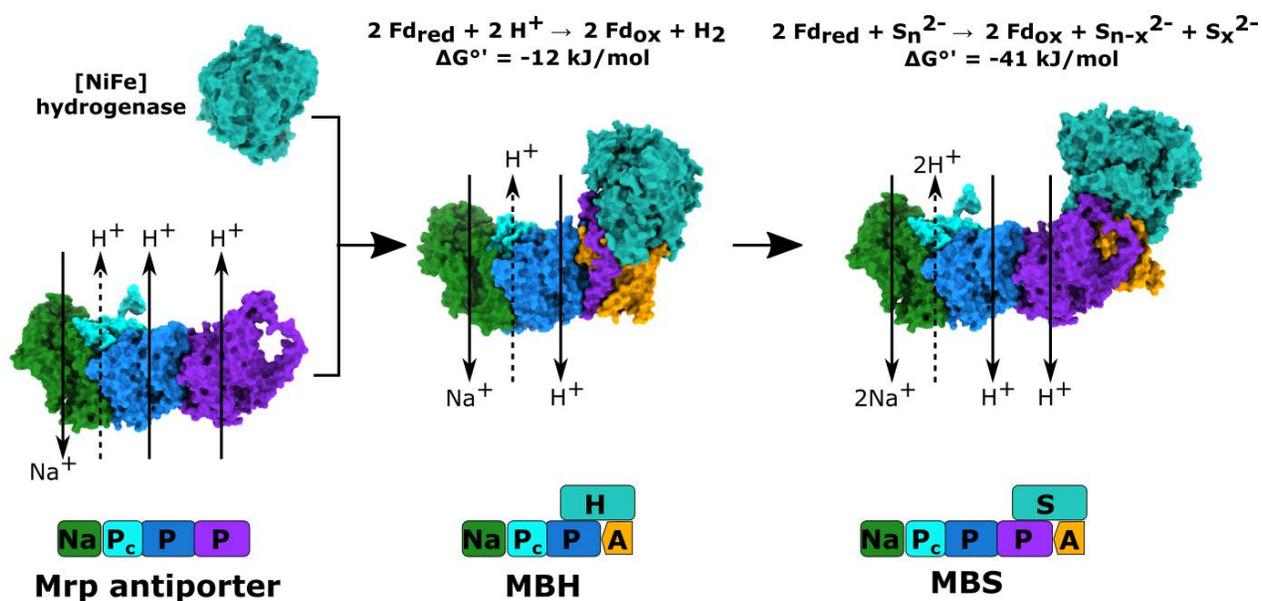


Figure 1. Our structures of MBH and MBS, along with the computational model of Mrp, reveal the evolution of energy conservation systems that eventually led to the modern-day Complex I.

2) We have determined the cryo-EM structure of Fix/EtfAB-CX quaternary complex, revealing the unexpected electron bifurcation pathway. A manuscript has been submitted for publication.

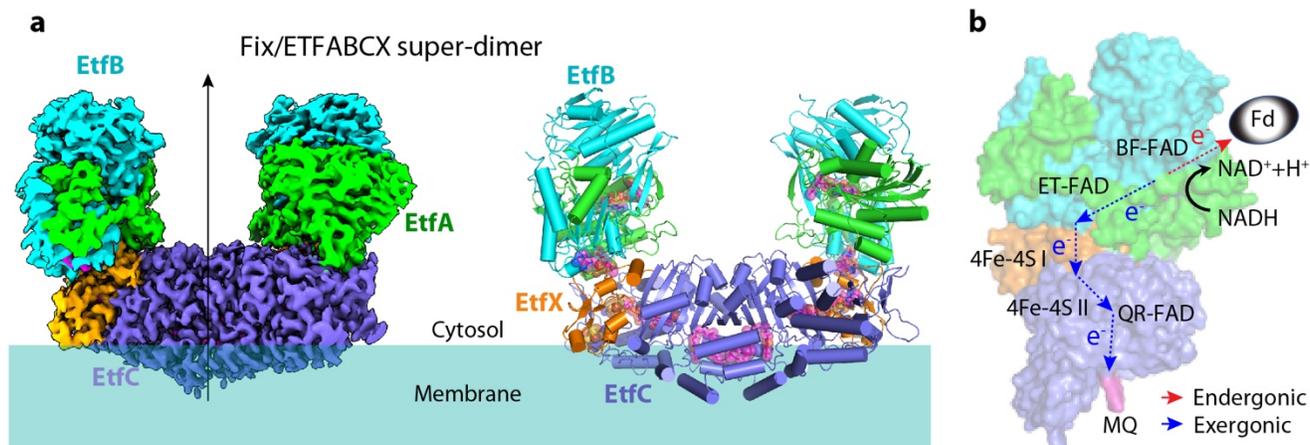


Figure 2. (a) Cryo-EM map at 2.8 Å and the atomic model in cartoon view. (b) Path of electron bifurcation in EtfABCX as determined by the structure.

Science priorities for the next year (2020-2021):

- We will focus on several metal-containing electron bifurcation protein complexes as their mechanisms are potentially novel and highly interesting.

My major scientific area(s) of expertise is/are: Cryo-EM-based structural biology and related protein biochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: biochemistry and microbiology and works in the areas of bioenergy, electron bifurcation, or other energy conservation mechanisms..

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over the Past 3-5 Years]:

Yu H, Wu CH, Schut GJ, Haja DK, Zhao G, Peters JW, Adams MWW, Li H. Structure of an Ancient Respiratory System. Cell. 2018, 173, 1636-1649. doi: 10.1016/j.cell.2018.03.071.

Defining the Mechanism of Proton Pumping in Heme Copper Oxidases

Matthew T. Kieber-Emmons, Principal Investigator

315 S 1400 E RM 2020, Salt Lake City, UT 84112-0850

Email: matthew.kieber-emmons@utah.edu; Website: www.kieber-emmons.com/group

Overall research goals:

The purpose of this proposal is to define proton transfer sites, protein motions that gate proton transfers, and underlying thermodynamic drivers of proton transfers in heme-copper oxidase (HCO) enzymes to understand how HCO enzymes vectorially drive proton transfers to create an ion gradient. HCO enzymes couple cellular metabolism to energy transduction by reduction of dioxygen. The excess free energy from this process is efficiently used to transfer or “pump” protons across the inner mitochondrial membrane. While many molecular level details have been uncovered, uncertainty in the identity of the critical sites and molecular level details on how these sites are modulated to drive proton transfers have remained elusive. Understanding how nature drives proton transfers is foundational to all redox processes in biology. Thus, this problem is of *high fundamental significance* because i) proton pumping in HCOs is the central biological energy conversion process in aerobic life that has relevance for fuel cell and battery technologies, ii) this problem is part of a broader class of problems on long-range proton transfer and proton-coupled electron transfer (PCET) reactions in biology that often appear in multi-proton, multi-electron redox processes related to energy transduction, and iii) requires development of new methods to define how protein motions are related to function. Proton transfer processes are perhaps the most frequently occurring reactions in biological systems with well-known examples beyond respiration that include water oxidation in photosynthesis and nitrogen fixation. This problem is important because basic mechanistic understanding of how nature generates ion gradients has promise in manipulation of biology to more efficiently generate end products and to guide development of bioinspired approaches in batteries and fuel cells.

The historical challenge in defining a mechanism of proton translocation is that while it is relatively easy to observe the electron transfers by optical changes in the redox cofactors of HCOs, it is a challenge to resolve the protons, and thus the key pumping sites, spatially and temporally. This information is the starting point for revealing a step-wise mechanistic scheme for proton pumping and identifying structural factors that control pumping. Our approach is to use genetically incorporated unnatural amino acids as vibrational Stark effect reporters to define the electrostatic topology within an HCO enzyme as a function of pumping. One aspect that is particularly novel in these studies is development of time-resolved resonance Raman as a detection method for these vibrational shifts. In the current cycle, our major objectives are to:

- 1) **Map the background electrostatic variations as a function of electron transfers** to define how the redox states of the heme cofactors and protein conformation modulate the internal protein electrostatic environment
- 2) **Identify electrostatic variations during a “pump” event** (a particular sequence of intermediates called PM, PR, and F) to enable identification of critical proton transfer sites.

In our preliminary studies, we genetically incorporated infrared probes and measured two states of the enzyme which differed in the oxidation state of the electron transfer heme. The shifts were smaller than would be predicted by simple electrostatic models. This begs the question; *how does the porphyrin attenuate the change in the e-field upon oxidation?* My presentation will describe recent developments along these lines.

Significant achievements (2020-2022):

- We have finished collection of a vibrational dataset on R and MV-CO states of HCO

Science priorities for the next year (2020-2021):

- Complete definition of the role of the porphyrin in modulating its surrounding e-field.
- Evaluate R, MV, O, and fluoride bound states to define redox induced conformational changes.
- Use in situ infrared spectroscopy with electron deficient states of the protein to arrest turnover at specific intermediates as a function of pH.
- Benchmark light throughput versus resolution in interferometric UV Raman spectroscopy.

My major scientific area(s) of expertise is/are: inorganic electronic structure, mechanistic bioinorganic chemistry, spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: Building Markov models of MD trajectories, other advanced sampling strategies.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years]:

[Click here to enter text.](#)

Bioinorganic Chemistry of Nitrification: Structure and Function of Ammonia Monooxygenase

Kyle M. Lancaster, Principal Investigator

Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca NY 14853

Email: kml236@cornell.edu; Website: Lancaster.chem.cornell.edu

Overall research goals:

Ammonia monooxygenase (AMO) is an integral membrane copper-containing metalloprotein that initiates the environmental defixation of nitrogen by the selective hydroxylation of ammonia to hydroxylamine. This reaction is carried out as the first step in the primary metabolism of nitrifying bacteria and thaumarchaea. The hydroxylamine that is produced undergoes subsequent multi-electron, multi-proton oxidation to yield reducing equivalents for cellular respiration. Enzymes capable of selective oxidation of recalcitrant substrates such as ammonia represent a pinnacle of chemical control achieved through eons of evolution. Thus, interrogating the as-yet unexplored mechanism of AMO offers insights toward design of next-generation fuel cell technology as well as catalysts for challenging molecular functionalization. Our long-term goal is to establish the mechanism by which AMO selectively hydroxylates ammonia to hydroxylamine using earth-abundant Cu and “green” dioxygen.

AMO has never been purified in an active form due to several factors including its complexity and its integral membrane nature. Moreover, native organisms that bear AMO grow slowly and to low cell densities. What is known about AMO comes from analogy to the related copper membrane monooxygenase particulate methane monooxygenase: AMO consists of three distinct subunits amoA, amoB, and amoC thought to assemble into a trimer of heterotrimers. Cu is believed to be the metal in the active site responsible for O₂ activation and subsequent substrate hydroxylation. The goal of our project during this funding period is to obtain biophysically-useful quantities of active AMO using a multi-pronged approach that involves recombinant and/or cell-free expression of AMO's subunits, refolding, and solubilization using membrane mimics.

Significant achievements ([2020-2022]):

While our project has not technically started at the point of writing this abstract, we have nevertheless managed to confirm recombinant expression of full-length amoB, albeit in the insoluble fraction of *E. coli* lysate. This has been confirmed by SDS-PAGE as well as mass spectrometry. SDS-PAGE of cells bearing expression vectors for amoC suggest that we have also expressed this subunit, but as of writing this abstract we await confirmation by mass spectrometry.

Science priorities for the next year (2020-2021):

- Recombinantly express amoA and amoC using various protein-expression strains of *E. coli* as either unaltered peptides or as conjugates with proteins to aid detection and solubilization such as green fluorescence protein or maltose binding protein.
- Carry out cell-free protein expression of amoA and amoC.
- Establish conditions for solubilization and purification of individual AMO subunits.
- Establish refolding conditions to assemble the complete AMO complex in membrane mimics including lipid bilayer nanodiscs, bicelles, etc.

My major scientific area(s) of expertise is/are: Bioinorganic chemistry, inorganic chemistry, inorganic spectroscopy, electronic structure calculations.

To take my project to the next level, my ideal collaborator would have expertise in: Cryo-electron microscopy, membrane mimics.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years]:
None.

Oral Presentations

Sept. 15, 2020

Session VI

*Metallocluster Biosynthesis and
“Insanely Great” Plant Biology*

[Click to Enter Title of Grant/FWP]
(total length of abstract should not exceed 2 pages)

Eric Boyd, Principal Investigator

Eric Shepard, Joan Broderick, Brian Bothner, Jennifer DuBois, Co-PI(s)

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Overall research goals:

Many methanogens can capture, convert, or store energy in the form of interconversions of CO₂ to CH₄, the reduction of N₂ to NH₃, and the reversible oxidation of H₂ to H⁺, with key electron transfer reactions occurring at metal-sulfide clusters and heme centers. However, the mechanisms of sulfur (S) and/or iron (Fe) acquisition and trafficking for use in metal sulfide cluster and heme biosynthesis in these cells remains enigmatic. Our data indicate that several model methanogens can be grown with Earth abundant and low-cost iron disulfide or pyrite (FeS₂) as the sole source of Fe and S. Evidence indicates that FeS₂ is reductively dissolved extracellularly to yield H₂S and soluble iron sulfide (FeS_{aq}), the latter of which is likely to be directly assimilated. The objectives of our project are to define the enzymatic mechanisms and pathways used to acquire and traffic S and Fe from pyrite (FeS₂) for biosynthesis of metal-sulfide metalloclusters and other cellular components (e.g., heme) that are involved in electron transfer and catalysis. Our strategy is to use a suite of genomic, geochemical, physiological, transcriptomic, and proteomic tools to identify a subset of proteins that function in the reduction of FeS₂, to characterize the form(s) of soluble FeS_{aq} that is generated through FeS₂ reduction, and to identify the proteins involved in assimilation/trafficking of this soluble Fe/S substrate. The structural and biochemical properties of proteins involved in reduction of FeS₂ and Fe/S assimilation/trafficking will be investigated using a combination of biophysical and mass spectrometry approaches. Our work is guided by the central hypothesis that *soluble FeS_{aq} species released by enzymatic FeS₂ reduction are directly assimilated and trafficked to meet Fe and S demands in methanogens.*

Our studies are focused on two model methanogen strains that we have shown can use FeS₂ to meet Fe and S demands: *Methanococcus voltae* A3 and *Methanosarcina barkeri* Fusaro. Each of these strains are representative of one the two primary phylogenetic lineages (Class I and Class II, respectively) of characterized methanogens. Class I methanogens tend to use sulfide as a S source, whereas Class II tend to be dependent on cysteine as their S source, providing an opportunity to examine how trafficking of S differs in these two classes of cells.

Significant achievements: 2019-2020

- **Genomics.** We have initiated a comparative genomics analysis of 363 archaeal methanogens and alkanotrophs to determine the distribution of protein homologs involved in *i*) the uptake of Fe and S, *ii*) the biosynthesis of [Fe-S] clusters and heme, and *iii*) coordinating [Fe-S] and heme as destinations for these co-factors. Results indicate that all analyzed organisms synthesize [Fe-S] clusters via the SUF pathway but only select Class II methanogen lineages can synthesize and utilize heme. In particular, class II organisms exhibit a greater apparent usage of heme and a lower usage of [Fe-S] clusters than Class I organisms, presumably as a consequence of higher reduction potentials associated with Class II cells that are in more oxidized settings.
- **Physiology.** Cultivation assays conducted with *M. voltae* and *M. barkeri* indicate that growth is enhanced when provided with mineral Fe/S sources when compared to canonical substrates. Collectively, these results indicate methanogens may prefer mineral sources of Fe/S over soluble forms. To further show that the reduction of FeS₂ is bio-catalyzed, *M. voltae* was grown with FeS₂ that was either free in solution or sequestered in dialysis tubing (3.5 kDa to 100 kDa) with formate as electron donor. Our results indicate that cells cannot utilize FeS₂ when separated from the minerals, indicating

that the cell surface likely has a crucial role in modulating FeS₂ reduction and/or that large extracellular enzymes are involved. This finding is consistent with the observation that cells form extensive biofilms when grown with FeS₂. Additional characterization of the physical interactions between methanogens and FeS₂ using field emission scanning electron microscopy is ongoing.

• **Spectroscopy.** We have conducted spectroscopic analyses of whole cells of *M. voltae* grown with FeS₂ nanoparticles or Fe(II) and sulfide as sole Fe and S sources. AA analyses reveals similar Fe contents per cell when grown with either form of Fe/S. Variable temperature EPR spectroscopy supports the assignment of the major species in samples prepared from both growth conditions as arising from spin 1/2 [4Fe-4S]⁺ cluster centers. These data demonstrate that *M. voltae* whole cells contain similar amounts and common forms of EPR detectable [Fe-S] clusters when grown using FeS₂ nanoparticles or Fe(II)/sulfide as sole Fe and S sources.

• **Transcriptomics and Proteomics.** RNA and protein (intracellular, extracellular) extractions have been optimized with *M. voltae* cells grown with FeS₂ and Fe(II)/sulfide when grown with formate as electron donor. RNA is currently being sequenced. Shotgun proteomics analyses identified 1007 intracellular and 831 extracellular proteins. Quantitative comparison showed that 145 intracellular proteins had statistically significant differences in abundance. Among the proteins that increased in abundance when grown with FeS₂ is an uncharacterized protein (DUF2193) and several proteins involved in Fe transport.

• **Biochemistry.** DUF2193 is highly conserved among methanogens and contains a conserved ensemble of 4 cysteine residues along with other putative [Fe-S] cluster ligands within its C-terminal domain. We have successfully overexpressed and purified the DUF2193 proteins from *M. voltae* and *M. barkeri*. UV-visible spectroscopic analysis of the as-purified proteins reveals low intensity ligand-to-metal charge transfer features typical of protein bound [Fe-S] clusters. We are working towards assignment of the [Fe-S] cluster species via EPR spectroscopy and are attempting to improve [Fe-S] cluster loading via chemical reconstitution techniques. These preliminary observations support the hypothesis that DUF2193 coordinates [Fe-S] cluster species and may have a role in Fe and S trafficking from FeS₂.

Science priorities for the next year (2020-2021):

- Develop voltammetry techniques to further characterize the form of Fe/S that is being released and assimilated during FeS₂ reduction
- Conduct physiology experiments to define the spectrum of Fe and S sources (both soluble and mineral) that support biosynthetic demands and to define the preferred source of Fe and S
- Analyze transcriptomic and proteomic data to identify putative extracellular proteins involved in FeS₂ reduction and intracellular proteins involved in [Fe-S] cluster and heme biosynthesis
- Heterologously express, purify, and characterize proteins involved in FeS₂ reduction and trafficking and subject them to a battery of biochemical and biophysical approaches to define features that underpin function

My major scientific area(s) of expertise is/are: Microbial physiology, bioinformatics, geomicrobiology.

To take my project to the next level, my ideal collaborator would have expertise in: Electrochemistry, structural bioinformatics, archaeal genetics.

Publications supported by this project :

1. C. Greening and E. Boyd. "Editorial: Microbial hydrogen metabolism." *Frontiers in Microbiology*. 11, 56 (2020). 10.3389/fmicb.2020.00056.
2. N.A. Losey, S. Poudel, E.S. Boyd, and M.J. McInerney. "The beta subunit of non-bifurcating NADH-dependent [FeFe]-hydrogenases differ from those of multimeric electron-bifurcating [FeFe]-hydrogenases." *Frontiers in Microbiology*. 11, 1109 (2020). 10.3389/fmicb.2020.01109.

Primary and Secondary Sphere Effects on the Valence Isomerism of Fe–S Clusters

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Overall research goals:

Iron–sulfur (Fe–S) clusters are ubiquitous cofactors in biological energy transduction. They participate in a range of multi-electron redox reactions, serving as electron/hole carriers and/or catalytic cofactors. Their remarkable and diverse reaction chemistry derives from their unique electronic structures, particularly the arrangement and coupling of their valence electrons. The long-term goal of this project is to develop models that predict how the geometric structure of the cluster dictates its electronic structure and how this in turn impacts its biochemical function.

We are focused on elucidating how the primary and secondary coordination spheres of Fe–S clusters dictate the spatial arrangement of their metal valences (each arrangement is a “valence isomer”). Questions we seek to answer include: How does ligation by non-cysteinyl amino acids impact the relative energies of the valence isomers? How do the number, strength, and orientation of hydrogen-bonding interactions impact valence localization? Under what circumstances are excited-state valence isomers thermally accessible, and how (if at all) do they contribute to the observed reactivity? What is the mechanistic significance of valence isomerism in electron transfer and catalysis?

To address these questions, we are systematically studying the phenomenon of valence isomerism in both synthetic and biogenic Fe–S clusters. Our studies of synthetic clusters enable primary sphere effects to be isolated from effects imparted by the polypeptide. Moreover, the tools of chemical synthesis allow for the rational control over molecular structure with exquisite precision. In our work with Fe–S proteins, the energetics of the valence isomers may be tuned by site-directed mutagenesis, and comparisons between the biogenic and synthetic clusters will elucidate the role of the protein in affecting valence isomerism.

Significant achievements (preliminary results pending funding in 2020-2023):

Pending funding for this project, we have built a library of synthetic Fe–S clusters and Fe–S proteins in which we will be able to study valence isomerism.

Science priorities for the next year (2020-2021):

- Preparation of a series of synthetic Fe–S clusters in which valence distribution is systematically tuned via ligand-field effects.
- Comparative spectroscopic analysis of this series (primarily Mössbauer, EPR, and paramagnetic NMR).
- Observation of valence isomerism in an Fe–S protein via Mössbauer spectroscopy.

My major scientific area(s) of expertise is/are: Bioinorganic chemistry; transition-metal chemistry; synthesis; reaction mechanisms; spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: protein structural data analysis, particularly searching for structural trends among large numbers of protein structures.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years]:

None

Atomic Resolution of Lignin-Carbohydrate Interactions in Native Plant Tissues from Solid-State NMR

Tuo Wang, Principal Investigator

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Overall research goals:

The energy-rich and carbon-rich plant cell wall is a sophisticated composite of macromolecules. The interactions between the phenolic polymer lignin and polysaccharides have made the biomass recalcitrant to post-harvest processing. This project aims at developing a biophysical toolbox to enable atomic-level investigations of polysaccharides and lignin using intact stems of maize, *Arabidopsis*, spruce and poplar. We will employ advanced solid-state NMR methods to resolve the roles of electrostatic interactions and covalent linkages in stabilizing lignin-polysaccharide contacts (cellulose, xylan, and glucomannan). We will also determine the domain distribution and hydrophobicity heterogeneity of biopolymers as well as their functional relevance. Comparing wild-type samples with lignin-engineered, transgenic plants will uncover the molecular principles involved in biopolymer interactions and supramolecular assembly. The fundamental knowledge will advance our understanding of energy storage in plants, form the foundation for optimizing the utility of lignocellulose for energy and biomaterial, and inspire the rational design of synthetic polymers and composites with tunable structure and properties. The spectroscopic methods established here are widely applicable to many energy-relevant systems such as plants, algae, microbes, as well as carbon-rich materials and synthetic polymers.

Significant achievements (2020-2025):

We have finished two preliminary studies to understand lignin-carbohydrate interactions in plants. Using *Arabidopsis* and maize stems, we have revealed that lignin self-aggregates to form hydrophobic and dynamically unique nanodomains that accommodate extensive electrostatic contacts with the part of xylan in non-flat conformation. The non-flat xylan further connects to its flat conformers that are coating the surface of cellulose microfibrils, thus bridging the two hydrophobic cores of cell walls, cellulose and lignin, in a conformation-dependent manner. Preliminary data collected on poplar and spruce stems have revealed extensive interactions between lignin and cellulose, which is a feature absent in *Arabidopsis* and maize stems.

Science priorities for the next year (2020-2021):

- Search for covalent lignin-carbohydrate bonds in the stems of poplar, spruce, and maize
- Validate the role of electrostatic interactions in lignin-carbohydrate interactions
- Estimate the dimension of lignin nanodomains in maize and *Arabidopsis*

My major scientific area of expertise is: Solid-State NMR analysis of biomolecules (carbohydrate, lipid, protein, lignin) for understanding their structure, dynamics, and interactions.

To take my project to the next level, my ideal collaborator would have expertise in: plant biology, natural and synthetic polymers, microbiology.

Publications supported by this project: This project just started; there is no publication yet.

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Understanding the mechanism and properties of catalytic Thi4 proteins

Anderew D Hanson, Principal Investigator

Steven D Bruner, Christopher R Reisch, Co-PIs

Jaya Joshi, Jorge Donato Garcia-Garcia, Postdoctoral Research Associates

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Overall research goals:

The Thi4 thiamin synthesis proteins of plants, fungi, and many prokaryotes are suicide enzymes that self-inactivate after a single catalytic cycle because they obtain the sulfur needed to make their thiazole product by destroying an essential cysteine residue in their active site. The resulting rapid turnover of these suicidal Thi4 proteins makes them exceptionally energetically expensive to operate. Intriguingly, non-suicidal – i.e. truly catalytic – Thi4 enzymes have been reported from hyperthermophilic, anaerobic archaea from high-sulfide (HS^-) environments; these Thi4s have no active-site cysteine and instead use HS^- as sulfur donor. Our pilot data indicated that similar catalytic Thi4s having no active-site cysteine ('non-Cys' Thi4s) also occur in mesophilic prokaryotes from low- HS^- environments containing O_2 . Our overall long-term goal is therefore to define the features that allow non-Cys Thi4s to operate as efficient catalysts in air and at low HS^- levels like those in plant cells. If better understood, these non-Cys Thi4s could enable design of energy-conserving replacements for plant Thi4. Specific research goals are to:

1. Screen diverse bacterial non-Cys Thi4s for O_2 sensitivity and operation at low HS^- concentration
2. Apply directed evolution to lower O_2 sensitivity/raise HS^- affinity, and sequence the evolved genes
3. Model structures of Thi4s with contrasting O_2 sensitivities/ HS^- affinities to predict residues involved
4. Determine crystal structures of contrasting Thi4s and of Thi4s before/after directed evolution
5. Use the structure/sequence data to design features needed for catalytic function at high O_2 /low HS^-
6. Implement these features in two *suicidal* Thi4s, analyze outcomes, and refine by directed evolution

Significant achievements (2019-2022):

Goal 1. Screen diverse bacterial non-Cys Thi4s for O_2 sensitivity and operation at low $[\text{HS}^-]$

We surveyed the diversity of non-Cys (i.e. catalytic) Thi4 sequences in prokaryotes, and based on a sequence similarity network constructed with the tools of the Enzyme Function Initiative (**Fig. 1**), we selected 26 representative Thi4 sequences to test for solubility when expressed in *E. coli* and for the capacity to complement an *E. coli* thiazole auxotrophic (ΔthiG) strain in anaerobic and aerobic conditions, with and without cysteine supplementation to increase the intracellular HS^- concentration.

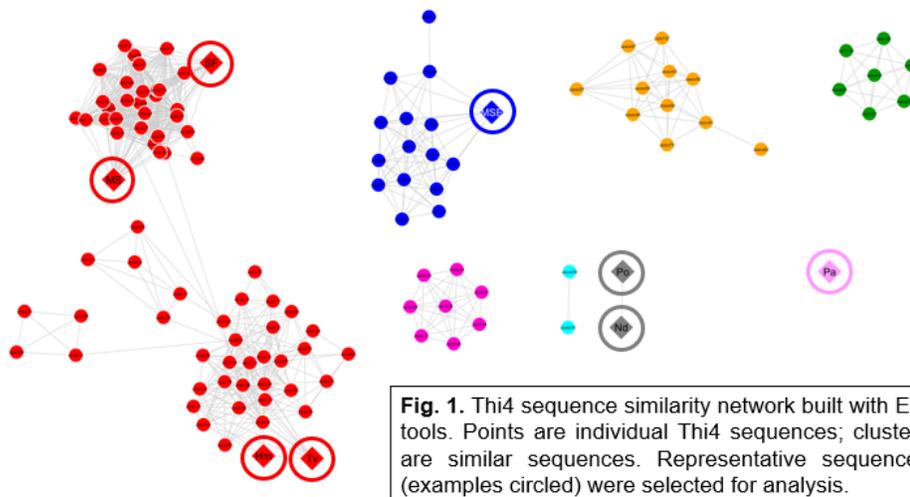


Fig. 1. Thi4 sequence similarity network built with EFI tools. Points are individual Thi4 sequences; clusters are similar sequences. Representative sequences (examples circled) were selected for analysis.

Of the 26 sequences, 21 proved to be soluble. So far 16 of these have been tested for complementation and found to include a good balance between active and inactive proteins. One Thi4 protein that showed complementing activity under aerobic and anaerobic conditions when HS⁻ was supplied was advanced to crystallization trials (see Goal 4 below).

Goal 2. Apply directed evolution to lower O₂ sensitivity/raise HS⁻ affinity

Tests of the *E. coli* EvolvR system for continuous directed evolution showed that this two-plasmid system, developed for use in rich media (<https://doi.org/10.1038/s41586-018-0384-8>), is unsuitable for use in minimal media (required in this project) due to the burden of the plasmid carrying the EvolvR machinery. We are therefore now exploring solutions. In parallel, we have also set up the yeast Ortho-Rep directed evolution system (<https://doi.org/10.1016/j.cell.2018.10.021>), which was developed for use in defined media compatible with this project's requirements. In the set-up work, we designed, built, and validated a multipurpose cloning vector for high-throughput analysis of Thi4 sequences.

Goal 3. Model structures of Thi4s with contrasting O₂ sensitivities/HS⁻ affinities

Modeling work on Thi4 proteins (see Goal 1) with contrasting complementation activities has begun.

Goal 4. Determine crystal structures of contrasting Thi4s

Diffraction quality crystals were obtained from the Thi4 protein from *Thermovibrio ammonificans*. Diffraction data have been collected on a beamline of the Life Sciences Collaborative Access Team facility, Argonne National Laboratory Advanced Photon Source (APS-ANL).

Science priorities for the next year (2020-2021):

- Complete testing of representative Thi4 sequences for solubility and complementing activity
- Complete analysis of the *T. ammonificans* Thi4 crystal structure
- Continue modeling of contrasting Thi4s to predict residues governing O₂ sensitivities/HS⁻ affinities
- Apply continuous directed evolution to *T. ammonificans* and other Thi4s with complementing activity

My major scientific areas of expertise are: Plant and microbial metabolic biochemistry, metabolic engineering, and synthetic biology.

To take my project to the next level, my ideal collaborator would have expertise in: Chemistry of enzyme reactions and side reactions and how these can inflict chemical damage on the enzyme protein. This is an underexplored aspect of mechanistic enzymology with major implications for how long enzymes last in vivo and hence for the energetic cost of replacing them and ultimately bioproductivity.

Publications supported by this project (2019-2022):

1. J.D. García-García, J. Joshi, J.A. Patterson, L. Trujillo-Rodriguez, C.R. Reisch, A.A. Javanpour, C.C. Liu, A.D. Hanson, "Potential and pitfalls of applying continuous directed evolution to plant enzymes." MDPI Life (invited paper for a Special Issue on Synthetic Biology, *to be submitted July 2020*)
2. J. Joshi, Q. Li, S.D. Bruner, A.D. Hanson, "Catalytic THI4s are ecologically diverse but mechanistically unified". Nature Chemical Biology (*to be submitted Fall 2020*)

*Established Project
Abstracts*

Alphabetically by PI last name

Hyperthermophilic Multiprotein Complexes for Energy Conservation and Catalysis

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Overall research goals:

We are investigating the mechanisms of assembly of energy transducing systems, the processes that regulate energy-relevant chemical reactions, the architecture of biopolymers, and the active site protein chemistry leading to efficient bio-inspired catalysts. The novel protein complexes to be studied have the remarkable property of being synthesized (self-assembling) at temperatures near 100°C in a so-called hyperthermophilic microorganism. Some of the novel complexes are involved in the conversion of low potential reducing equivalents into gaseous end products (hydrogen, H₂, and hydrogen sulfide, H₂S) with the concomitant conservation of energy in the form of ion gradients, while others utilize electron bifurcation to conserve energy by the generation of low potential reductant.

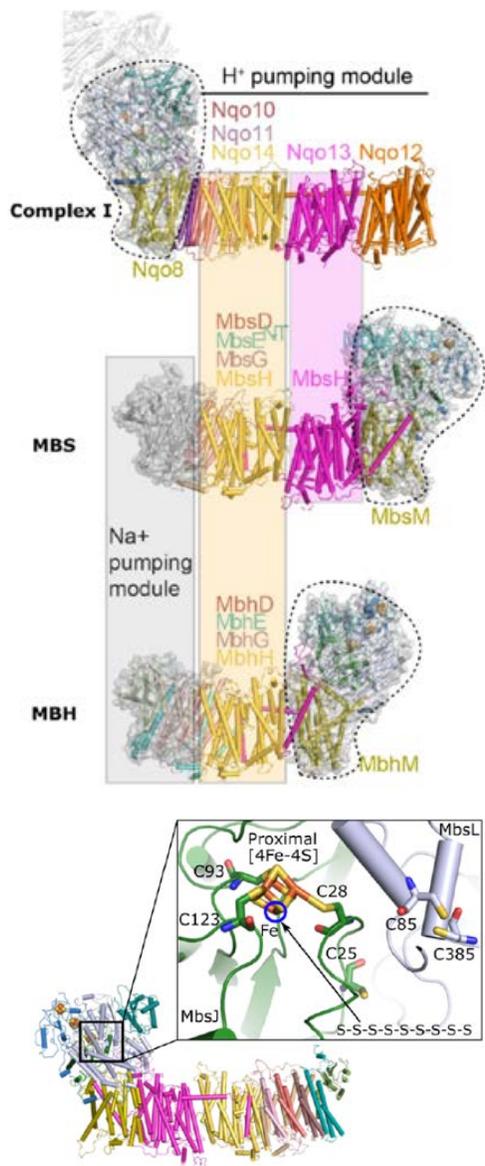
Significant achievements (2018-2021):

Our model microorganism is *Pyrococcus furiosus* (*Pfu*), which grows optimally at 100°C and is thought to represent an ancestral life form. *Pfu* obtains carbon and energy for growth by fermenting carbohydrates and producing H₂ gas or by reducing elemental sulfur (S⁰) to H₂S gas. It also has a respiratory metabolism in which it couples H₂ production by a ferredoxin-dependent, 14-subunit membrane-bound NiFe-hydrogenase (MBH) to sodium ion translocation and formation of a membrane potential that the organism utilizes to synthesize ATP. MBH is highly homologous to modern day NADH-oxidizing, quinone-reducing Complex I of the aerobic respiratory chain. Addition of S⁰ to *Pfu* prevents the synthesis of MBH and induces the synthesis of a homologous 13-subunit membrane-bound complex termed MBS, a sulfane sulfur reductase. This respiratory enzyme catalyzes the reduction of internal S-S bonds in polysulfides (S_n²⁻) but does not itself generate H₂S directly.

The structures of both MBH and MBS have now been determined by cryo-EM in collaboration with Dr. Huilin Li (Van Andel Research Institute). MBH contains a membrane-anchored hydrogenase module that is highly similar structurally to the quinone-binding Q-module of Complex I while its membrane-embedded ion translocation module can be divided into a H⁺- and a Na⁺-translocating unit. The H⁺-translocating unit is rotated 180° in-membrane with respect to its counterpart in complex I, leading to distinctive architectures for the two respiratory systems despite their largely conserved proton-pumping mechanisms (Figure 1). The Na⁺-translocating unit, absent in Complex I, resembles that found in the Mrp H⁺/Na⁺ antiporter and enables H₂ production by MBH to establish a Na⁺ gradient for ATP synthesis. MBS retains all of the structural features thought to be involved in energy transduction in MBH, but contains an additional ion pumping module not present in MBH (Fig. 1). This is thought to enable polysulfide reduction by MBS to conserve more energy than proton reduction by MBH.

MBS represents the so-called “missing link” in the evolution of ubiquitous Complex I. Our structural analysis of MBS now enables a comparison of the structural evolution of a catalytic site that evolved from reducing protons to reducing polysulfide and ultimately quinones. Using spectroscopic and site-directed mutational analyses, we showed that polysulfide reduction occurs at a site-differentiated iron-sulfur cluster (Figure 1). This involves the simplest mechanism yet proposed for reduction of an inorganic or organic disulfide and the first without participation of either protons or an amino acid residue, a reaction of fundamental significance in iron and sulfur-rich volcanic environments of early earth and possibly in the evolution of early life. MBS provides a new perspective on the evolution of modern-day respiratory complexes and provides the simplest known example of catalysis by a biological iron-sulfur cluster. This same iron-sulfur cluster in Complex I is involved in quinone

reduction while in MBH it donates electrons to the H₂-producing NiFe site. This research also provides new insights into how energy conservation by Complex I evolved from a H₂ gas-producing ancestor. **Figure 1.** *Top:* Structural comparison of Complex I, MBS and MBH. *Bottom:* Proposed binding of polysulfide to the unique Fe atom of the proximal [4Fe-4S] cluster of MBS.



Science priorities for the next year (2020-2021):

The priorities for next year include 1) mechanistic and spectroscopic analyses of the reduction of polysulfide by *Pfu* MBS at its unique [4Fe-4S] cluster, 2) characterization of a novel bifurcating NiFe-hydrogenase isolated from a thermophilic bacterium, and 3) investigations of H₂ catalysis by the cytoplasmic hydrogenase (SHI) of *Pfu* using time-resolved IR analyses of site-directed mutants (in collaboration with Dr. Brian Dyer, Emory U.).

My major scientific area(s) of expertise is/are: anaerobic biochemistry and metalloenzymes.

My ideal collaborator has expertise in: biochemistry of Complex I and its mechanism of energy transduction.

Publications supported by this project [2018-2020]:

1. Yu, H., Wu, C.-H., Schut, G. J., Haja, D. K., Zhao, G., Peters, J. W., Adams, M. W. W. and Li, H. (2018) "Structure of an ancient respiratory system" *Cell* **173**, 1636-169 (doi: 10.1016/j.cell.2018.03.071)
2. Wu, C.-H., Schut, G. J., Poole, F. L., Haja, D. K. and Adams, M. W. W. (2018) "Characterization of membrane-bound sulfane reductase - a missing link in the evolution of modern day respiratory complexes" *J. Biol. Chem.* **293**, 16687-16696 (doi: 10.1074/jbc.RA118.005092)
3. Wu, C.-H., Ponir, C. A., Haja, D. K., Adams, M. W. W. (2018) "Improved production of the NiFe-hydrogenase from *Pyrococcus furiosus* by increased expression of maturation genes" *Protein Engin. Design & Selection* **31**, 337-344 (doi: 10.1093/protein/gzy025)
4. Wu, C.-H., Haja, D. K. and Adams, M. W. W. (2018) "Cytoplasmic and membrane-bound hydrogenases from *Pyrococcus furiosus*" *Meths. Enzymol.* **613**, 153-168 (doi: 10.1016/bs.mie.2018.10.009)
5. Sanchez, M. L. K., Wu, C.-H., Adams, M. W. W. and Dyer, R. B. (2019) "Optimizing electron transfer from CdSe QDs to hydrogenase for photocatalytic H₂ production" *Chem. Comm.* **55**, 5579-5582 (doi: 10.1039/c9cc01150a)
6. Crosby, J. R., Laemthong, T., Lewis, A. M., Straub, C. T., Adams, M. W. W. and Kelly, R. M. (2019) "Extreme thermophiles as emerging metabolic engineering platforms" *Curr. Opin. Biotechnol.* **59**, 55-64 (doi: 10.1016/j.copbio.2019.02.006)
7. Yang, W., Vansuch, G. E., Liu, Y., Jin, T., Liu, Q., Ge, A., Sanchez, M., Haja, D. K., Adams, M. W. W., Dyer, R. B., Tianquan, L. (2020) "Surface ligand "liquid" to "crystalline" phase transition modulates the solar H₂ production quantum efficiency of CdS nanorod/mediator/hydrogenases assemblies" *ACS Appl. Mater. Interfaces.* (doi: 10.1021/acsami.0c07820)
8. Haja, D. K., Wu, C.-H., Poole, F. L., Williams, S. D., Jones, A. K. and Adams, M. W. W. (2020) "Characterization of thiosulfate reductase from *Pyrobaculum aerophilum* heterologously expressed in *Pyrococcus furiosus*" *Extremophiles* (doi: 10.1007/s00792-019-01112-9)
9. Vansuch, G. E., Wu, C.-H., Haja, D. K., Blair, S. A., Chica, B., Johnson, M. K., Adams, M. W. W. and Dyer, R. B. (2020) "Metal-ligand cooperativity in the hydrogenase-1 from *Pyrococcus furiosus*" *Chem. Sci.* (accepted)

10. Haja, D. K., Wu, C.-H., Poole, F. L., Ponomarenko, O., George, G. N., and Adams, M. W. W. (2020)
“Improving arsenic tolerance of *Pyrococcus furiosus* by the heterologous expression of a respiratory arsenate reductase” *Appl. Environ. Microbiol.* (accepted)

Allosteric control of electron transfer in nitrogenase and nitrogenase-like enzymes

Edwin Antony, Principal Investigator

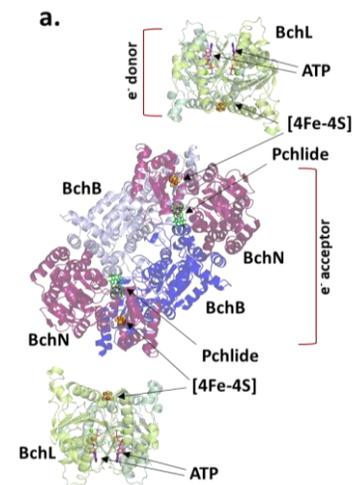
Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine,
Doisy Research Center, St. Louis, MO 63104
Email: edwin.antony@health.slu.edu; Website: www.antonylab.org

Overall research goals:

Many enzymes that catalyze multi-step electron transfer reactions function as higher order complexes with two or more identical active sites. In nitrogenase, the enzyme that reduces dinitrogen to ammonia, we showed that the two halves function in an asymmetric manner with electron transfer transpiring in one half followed by the other. We seek to understand the functional underpinnings of such long-range allosteric control of electron transfer in enzymatic complexes. The structural architecture found in nitrogenase is also conserved in the nitrogenase-like enzymes that function in the biosynthesis of bacteriochlorophyll and coenzyme F430, and likely other related enzyme systems. In addition, these enzyme systems are made of an electron donor and electron acceptor component proteins. ATP-binding driven assembly of the two component proteins trigger electron transfer. Multiple rounds of such assembly and disassembly is required for substrate reduction. We seek to elucidate the structural and mechanistic aspects of such complex enzymatic electron transfer reactions.

Significant achievements (2017-2018, 2018-2019, 2020-2021):

During the current funding period, our efforts have been focused on understanding the allosteric principles underlying the reduction of protochlorophyllide (Pchl_{id}) to chlorophyllide (Chl_{id}) by the enzyme DPOR (dark-operative protochlorophyllide oxidoreductase). DPOR catalyzes a key reductive step in the biosynthesis of bacteriochlorophyll. The BchL homodimer is the electron donor component and the BchN-BchB (BchNB) heterodimer is the electron acceptor component. Two rounds of electron transfer from BchL to BchNB is required for Pchl_{id} reduction. We made the following discoveries:



1) We discovered that a flexible disordered N-terminal region in BchL is autoinhibitive and binds to the [4Fe-4S] cluster and across the interface that binds to BchNB. ATP binding promotes conformational changes within the BchL dimer and relieves autoinhibition.

2) We showed that BchNB functions asymmetrically (similar to nitrogenase) with electron transfer transpiring in one half followed by the other.

3) We showed that BchL can bind to BchNB in the absence of substrate the transfer the first electron from the [4Fe-4S] cluster of BchL to the [4Fe-4S] cluster of BchNB. This preloaded electron can be donated to Pchl_{id}, similar to the ‘deficit spending’ mechanism in nitrogenase.

4) Pchl_{id} is structurally similar to Chl_{id} (except for the location of a double bond) and thus we propose that the transfer of the first electron from BchNB to Pchl_{id} and the asymmetry is ‘substrate-sensing’ mechanism. We showed this by developing a fluorescence assay where a 700-fold increase in Pchl_{id} fluorescence is observed when Pchl_{id} is bound to BchNB and the first electron transfer quenches this reaction.

5) To enable stable purification of [4Fe-4S] containing enzymes we generated a *E. coli* cell line where we corrected a commonly occurring mutation in the *Suf* operon.

Science priorities for the next year (2020-2021):

- Single molecule studies of DPOR. Over the past year we have been developing and optimizing slides that support DPOR function in an anaerobic environment. We will be utilizing this tool to capture the steps in DPOR enzymology.
- Cryo-EM studies of DPOR. We have been working on scaling up our cryo-EM efforts to freeze DPOR samples onto grids in an anaerobic environment. Our focus for the next three years will be to capture the transition states in the DPOR mechanism of action.
- Mechanistic exploration of substrate specificity. We are seeking to understand how DPOR is specific for reducing only the C17=C18 double bond and how orientation of the substrate is accomplished within the active site.

My major scientific area(s) of expertise is/are: Biochemistry, Structural Biology, Enzyme Kinetics, Single Molecule Fluorescence.

To take my project to the next level, my ideal collaborator would have expertise in: Genetics of algal systems, electrochemistry, and/or QQ-MM structural calculations.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years] :

1. Corless E.I., Saad Imran S.M., Watkins, M.B., Origanti S., Bacik J.P., Kitelinger R., Soffe M., Danyal K., Seefeldt L.C., Bennett B., Ando N., and Antony E. "The flexible N-terminus of BchL protects its [4Fe-4S] cluster in oxygenic environments and autoinhibits activity". *BioRxiv*. 811059. doi: 10.1101/840439.
2. Corless E.I., Bennett B., and Antony E. "Substrate recognition induces sequential electron transfer across subunits in the nitrogenase-like DPOR complex". *J. Biol. Chem.* In Press. 2020. doi:10.1074/jbc.RA120.015151.
3. Corless E.I., Mettert E.L., Kiley P.J., and Antony E. "Elevated expression of a functional Suf pathway in *Escherichia coli* BL21(DE3) enhances recombinant production of an iron-sulfur containing protein". *J. Bacteriol.* 2020. 202(3)e00496-19. doi:10.1128/JB.00496-19.
4. Venkiteshwaran K., Pokhrel N., Hussein F., Antony E., and Mayer B.K. "Phosphate removal and recovery using immobilized phosphate binding proteins". *Water Res.* X. 2018. 1:1000003. doi:10.1016/j.wroa.2018.09.003.
5. Seefeldt L.C., Hoffman B.M., Peters J.W., Raugei S., Beratan D.N., Antony E., and Dean D.R. "Energy transduction in nitrogenase". *Acc. Chem. Res.* 2018. 51(9):2179-2186. doi:10.1021/acs.accounts.8b00112.

Cellulose and the control of growth anisotropy

Tobias I Baskin, Principal Investigator

Eri Kamon, Postdoctoral Research Associate (Dec 2017 – Feb. 2020)

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Overall research goals:

For land plants, the cell wall is a sink for fixed carbon that not only has high-capacity but also grows. At the heart of the cell wall are cellulose microfibrils—long crystalline polymers of glucose, around which the rest of the cell wall organizes. Microfibril strength, alignment, and interactions with the surrounding matrix all work to control growth, a control that is exerted not only on rate but also on direction. Thanks to cellulose, expansion is usually anisotropic, and plants control this anisotropy precisely to build organs with specific and functional shapes. Overall, the PI aims to understand how aligned microfibrils dictate the anisotropy of growth.

The project investigates cellulose and the control of growth anisotropy at several levels. On a biochemical level, the project studies the catalytic enzymes (CESAs) responsible for cellulose synthesis. On a cellular level, the project tests the hypothesis that growth anisotropy is controlled by the organization of microfibrils across tens of microns. As part of this effort, the PI is collaborating with scientists at Marine Biological Lab and at Brookhaven National Lab to develop methods where cellulose orientation can be quantified by using advanced polarized light microscopy and by scattering from X-rays. Though diverse, the aims are united by the goal of learning how cellulose synthesis and structure work together to shape the cells and organs of vascular plants.

The project relies on partly roots, which are not only excellent for experimentation but are also a largely untapped reservoir of traits for crop improvement. The project will advance various methods in structural biology, including polarized light microscopy and X-ray scattering, providing opportunities for training biologists in these under-represented skills. Overall, the project will improve understanding of the genetics of cellulose synthesis and the relationship between microfibril organization and growth, allowing plant morphogenesis to be manipulated optimally for humankind.

Significant achievements (2017 - 2021):

Isoform specificity of CESA subunits CESA proteins work in a complex of three isoforms, but it is not known to what extent the role of each isoform differs. Two accomplishments bear on this problem:

- Found that arabidopsis lines, mutant for a given CESA and expressing that CESA's putative ortholog from *B. distachyon*, have wild-type morphology and levels of cellulose. This implies that the diverged genes are in fact orthologous. However, only some of the *B. distachyon* isoforms confer isoxaben resistance on arabidopsis, implying isoforms differ functionally.

- In collaboration with the Center for Lignocellulose Structure and Formation at Penn State, found that cellulose synthase complexes comprising two wild-type isoforms and a catalytically inactive third isoform do make cellulose, but the quantity and organization of the cellulose differs depending on which isoform is inactive. A paper describing these results is being written now.

Mechanism of cellulose organization Although not proposed specifically, two accomplishments reflect collaborations with teams interested in problems related intimately to grant objectives:

- In collaboration with Adam Saffer (Yale University), found that in mutants of arabidopsis making less pectin, cellulose forms thick bundles. As imaged with SEM, birefringence, and polarized fluorescence,

the bundles form helices of a fixed chirality. The chirality accounts for the chiral twisted growth phenotype of the mutants. A paper describing these results is being written now.

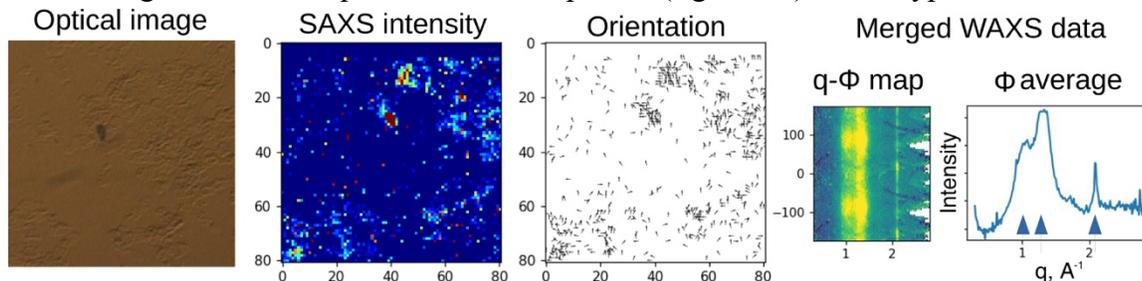
•In collaboration with Lori Goldner (UMass Physics), found that the cellulose synthase complex moves in steps whose distribution suggests that glucan synthesis is limiting compared to crystallization. A paper describing these results is being written now.

A single cell system A major objective of the grant is to develop tobacco BY2 cells as a productive system for studying cellulose synthesis and growth anisotropy. Single cells are ideal for experiments and imaging; also, they simplify the biomechanics. We have three major accomplishments:

•CESA tracking in BY-2 cells. We have made a BY2 line expressing a GFP-tagged homologous CESA and obtained preliminary results characterizing CESA complex movement by using TIRF microscopy.

•Cellulose synthesis and growth anisotropy. By using quantitative polarized-light microscopy, we find that, when cortical microtubule arrays are disrupted, the cell wall cellulose becomes less well organized *before* growth anisotropy changes.

•X-ray scattering analysis. In collaboration with Dr Lin Yang of Brookhaven National Laboratory, at NSLS-II, we have obtained usable WAXS spectra from single BY2 cells and cell wall ghosts. To my knowledge, this is the first report of X-ray analysis of cellulose in the primary cell wall with single cell resolution. In the figure below, cellulose ghosts on mica generate SAXS spectra whose asymmetry is used to align the WAXS spectra, which are pooled (rightmost). Peaks typical of cellulose are present.



Science priorities for the next year (2020-2021):

- Publish the three “in preparation” papers mentioned above.
- Experiments in maize roots to compare kinetics of cellulose disorganization and root swelling.
- Finish phenotyping arabidopsis lines harboring *B. distachyon* CESA genes.
- Experiments in BY2 cells exploiting high resolution tracking of CESA motility.
- Continued X-Ray analysis of single cell walls, including those made in the absence of microtubules.

My major scientific areas of expertise are: Cellulose synthesis; plant genetics; polarized light microscopy; scanning electron microscopy; microtubules; science writing.

To take my project to the next level, my ideal collaborator would have expertise in: Molecular biology.

Publications supported by this project :

Liu D, Zehfroosh N, Hancock BL, Hines K, Fang W, Kilfiol M, Learned-Miller E, Sanguinet KA, Goldner LS, Baskin TI (2017) Imaging cellulose synthase motility during primary cell wall synthesis in the grass *Brachypodium distachyon*. **Scientific Reports** 7, 15111.

[BL Hancock and K Hines were undergraduate students; KA Sanguinet was a Post-Doctoral Fellow who is now an Assistant Professor at Washington State University, Pullman]

Role of HydF in Hydrogenase Maturation

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Overall research goals:

The overall goal of this project is to advance our understanding of the mechanism of active site metal cluster assembly in [FeFe]-hydrogenase, nature's preferred biohydrogen catalyst. We use physical biochemical approaches to elucidate the reactions catalyzed by the three specific hydrogenase maturase enzymes common to all organisms that express [FeFe]-hydrogenase. This research promotes our knowledge of structure/function relationships in complex biological pathways involving metalloenzymes, while additionally contributing to understanding how microbes build the protein cofactors that enable them to capture and convert energy. Moreover, as the steps associated with complex metal cofactor biosynthesis are better understood, the expectation is that the knowledge will inspire and influence the design of new biomimetic catalysts with applications in biohydrogen technologies.

The goals of this project are to develop a molecular-level understanding of the reactions catalyzed by HydE, HydF, and HydG to clearly delineate the steps and [Fe-S] cluster intermediates that are associated with H-cluster biosynthesis. *Specifically, the current project objectives are 1) to probe the molecular details of HydG catalysis, 2) to provide functional and mechanistic insight into HydE, and 3) to define the mechanism of [2Fe]_F assembly on HydF and transfer to HydA.* This work stands to reveal fundamentally unique biochemical transformations and help define valuable paradigms for complex metal cluster assembly in biology.

Significant achievements (2019-2022):

We have shown that HydG proteins containing either trace or no dangler iron species all form multiple equivalents of free (non-Fe bound) CO, while dangler loaded HydG that contains the [5Fe-4S] moiety forms modestly greater amounts of free CO during turnover; these enzyme preparations also produce excess free equivalents of CN⁻. EPR spectroscopy demonstrates that the dangler iron remains intact during turnover. We have shown that regardless of whether HydG contains or lacks the dangler iron species, it activates HydA during *in vitro* hydrogenase maturation. Together, these results are not consistent with the dangler Fe serving as a site for CO and CN⁻ binding to form a synthon. Overall, the *in vitro* activation results mirror the results based on free CO assays, providing support for the idea that free CO and CN⁻ production are relevant to hydrogenase maturation.

We have demonstrated that blue light photolysis of SAM bound to HydG produces a methyl radical trapped in the active site, providing the first evidence of where a radical SAM enzyme reductively cleaves the S-CH₃ bond. We subsequently showed that a methyl radical was also generated via photolysis of reduced HydE-SAM complexes from both the *Clostridium acetobutylicum* (*C.a*) and *Thermotoga maritima* organisms. Our photolysis work using HydE and HydG has provided, for the first time, evidence for a freely tumbling methyl radical in the active site of an enzyme. This work helps define the molecular mechanisms of radical initiation in Radical SAM enzymes, while also making connections to nitrogenase metal cofactor assembly. By defining the mechanisms of radical generation and control in HydE and HydG, an understanding of productive active site chemistry is put forth, which will promote our understanding of the step wise assembly of [2Fe]_F.

Science priorities for the next year (2020-2021):

- Define the role for the dangler iron during HydG catalysis using spectroscopic and functional assays
- Develop a fully defined in vitro hydrogenase maturation assay using purified components
- Further characterize HydE function and interactions with other proteins

My major scientific area(s) of expertise is/are: bioinorganic chemistry, iron-sulfur clusters, radical SAM enzymes, metal cluster assembly, radical reactions in biology.

To take my project to the next level, my ideal collaborator would have expertise in: protein-protein interactions; HYSCORE spectroscopy.

Publications supported by this project 2015 - 2020 :

1. A. S. Byer, E. M. Shepard, J. W. Peters, J. B. Broderick, "Radical S-Adenosyl-L-methionine Chemistry in the Synthesis of Hydrogenase and Nitrogenase Metal Cofactors." *J. Biol. Chem.*, 290, 3987 (2015).
2. J. W. Peters, G. J. Schut, E. S. Boyd, D. W. Mulder, E. M. Shepard, J. B. Broderick, P. W. King, M. W. W. Adams, "[FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation." *Biochim. Biophys. Acta – Mol. Cell Res.*, 1853, 1350 (2015).
3. K. D. Swanson, M. W. Ratzloff, D. W. Mulder, J. H. Artz, S. Ghose, A. Hoffman, S. White, O. A. Zadornyy, J. B. Broderick, B. Bothner, P. W. King, J. W. Peters, "[FeFe]-Hydrogenase oxygen inactivation is initiated at the H-cluster 2Fe subcluster." *J. Am. Chem. Soc.* 137, 1809 (2015).
4. J. N. Betz, N. W. Boswell, C. J. Fugate, G. L. Holliday, E. Akiva, A. G. Scott, P. C. Babbitt, J. W. Peters, E. M. Shepard, J. B. Broderick, "[FeFe]-Hydrogenase Maturation: Insights into the role HydE plays in dithiomethylamine biosynthesis." *Biochemistry* 54, 1807 (2015).
5. E. M. Shepard, A. S. Byer, J. N. Betz, J. W. Peters, J. B. Broderick, "A Redox Active [2Fe-2S] Cluster on the Hydrogenase Maturase HydF." *Biochemistry* 55, 3514 (2016).
6. E. M. Shepard, A. S. Byer, P. Aggarwal, J. N. Betz, A. G. Scott, K. A. Shisler, R. J. Usselman, G. R. Eaton, S. S. Eaton, J. B. Broderick, "Electron spin relaxation and biochemical characterization of the hydrogenase maturase HydF: Insights into [2Fe-2S] and [4Fe-4S] cluster communication and hydrogenase activation," *Biochemistry* 56, 3234 (2017).
7. E. M. Shepard, A. S. Byer, J. B. Broderick, "Iron-Sulfur Cluster States of the Hydrogenase Maturase HydF," *Biochemistry*, 56, 4733 (2017).
8. E. S. Boyd, G. J. Schut, E. M. Shepard, J. B. Broderick, M. W. W. Adams, J. W. Peters, "Origin and evolution of Fe-S proteins and enzymes," in *Iron-Sulfur Clusters in Chemistry and Biology*, edited by Tracey Roualt, de Gruyter, (2017).
9. A. G. Scott, R. K. Szilagy, D. W. Mulder, M. W. Ratzloff, A. S. Byer, P. W. King, W. E. Broderick, E. M. Shepard, J. B. Broderick, "Compositional and Structural Insights into the Nature of the H-Cluster Precursor on HydF," *Dalton Trans.*, 47, 9521 (2018).
10. A. S. Byer, H. Yang, E. C. McDaniel, V. Kathiresan, S. Impano, A. Pagnier, H. Watts, C. Denler, A. Vagstad, J. Piel, K. S. Duschene, E. M. Shepard, T. P. Shields, L. G. Scott, E. A. Lilla, K. Yokoyama, W. E. Broderick, B. M. Hoffman, J. B. Broderick, "Paradigm shift for radical SAM reactions: The organometallic intermediate Ω is central to catalysis," *J. Am. Chem. Soc.*, 140, 8634 (2018).
11. W. E. Broderick, B. M. Hoffman, and J. B. Broderick, "Mechanism of radical initiation in the radical S-adenosyl-L-methionine Superfamily," *Acc. Chem. Res.*, 51, 2611 (2018).
12. A. S. Byer, E. M. Shepard, M. W. Ratzloff, J. Betz, P. W. King, W. E. Broderick, J. B. Broderick, "H-Cluster Assembly Intermediates Built on HydF by the Radical SAM Enzymes HydE and HydG," *J. Biol. Inorg. Chem.*, 24, 783 (2019).
13. H. Yang, S. Impano, E. M. Shepard, C. D. James, W. E. Broderick, J. B. Broderick, and B. M. Hoffman, "Photoinduced Electron Transfer in a Radical SAM Enzyme Generates an S-Adenosylmethionine Derived Methyl Radical," *J. Am. Chem. Soc.*, 141, 16117 (2019).

Elucidating the Cellular Machinery for Lipid Storage in Plants

Kent D. Chapman, Principal Investigator

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Overall Research Goals:

In the last few years, our group and others have identified several new players involved in plant lipid droplet (LD) biogenesis, but the information on how these proteins function to efficiently package lipids into LDs remains to be elucidated. Perhaps as important, the inventory of proteins that participate in the compartmentalization of storage lipids in any tissue in plants is far from complete. Closing gaps in this information is relevant not only in terms of oilseeds and oleaginous fruit tissues that can accumulate large amounts of storage lipids, but also is likely to be important for developing strategies for the biosynthesis of storage lipids in vegetative plant tissues, a concept that is gaining traction for the production of energy-dense biomass. **Our overarching goal is to understand the fundamental biochemical and cellular processes important for compartmentalization of storage lipids in plant tissues, ultimately to enable dramatic increases in the energy storage capacity of plants.** Lipids represent a fully reduced form of carbon, and these hydrophobic molecules are a major store of electrons and energy in plant systems. Understanding the mechanisms of their packaging remains an important question in cell biology research. We are addressing our goal through the following three specific research objectives: **1) Perform a detailed assessment of the mechanisms and functional interactions of SEIPIN, LDIP, and LDAP proteins in plants, 2) Identify and characterize new protein players involved in compartmentalization of neutral lipids in plants, and 3) Characterize variants of known LD biogenesis-related proteins in the packaging of energy-dense storage lipids.**

Significant achievements (2019-2022):

We are currently in the first year of our renewed project funding that began September 1, 2019. Results to date show that a recently identified, hydrophobic protein termed LDIP (LD-Associated Protein [LDAP]-Interacting Protein) works together with both endoplasmic reticulum (ER)-localized SEIPIN and the LD-coat protein LDAP to facilitate the process of LD formation in plants. These data, combined with the analogous function of LDIP to a recently identified protein in mammalian cells called LDAF1 (LD Assembly Factor 1), provide for a new model of LD biogenesis in plant cells with evolutionary connections to LD biogenesis in other eukaryotes. Elsewhere, we recently identified an interaction between SEIPIN and an isoform of the organelle-tethering protein VAP27 (vesicle-associated membrane protein [VAMP]-associated protein 27), and this interaction is required for the formation of normal LDs. These results recently appeared in *Plant Cell* (Greer et al., 2020). Progress in understanding the packaging of other types of neutral lipids into LDs comes from our recent “omics” analysis of jojoba, which is a desert shrub that accumulates wax esters instead of TAG in seeds. A reference-quality genome sequence, along with transcriptomes and LD proteomes, helped to identify relevant LD packaging machinery in jojoba seeds. Data suggest that LDAPs may be key factors for efficient wax-ester packaging into LDs in

jojoba seeds, an unexpected finding since LDAPs are normally low in abundance in proteomes of other oilseed LDs. A paper describing these “-omics” resources was published in *Science Advances* (Sturtevant et al., 2020). This year, our group also contributed several invited reviews.

Science objectives for 2020-2021:

Attention in the coming year will continue to focus research efforts on all three aims. One area that has received less attention, due to lack of personnel, has been the development of an *in-vitro* system to explore the potential role for GTP-binding proteins in LD biogenesis (a sub aim of Objective 2). This remains a future goal, and a post-doctoral fellow at UNT is working to reconstitute LD formation in isolated safflower microsomes in part to lay a foundation for this work in the future. Most importantly, elucidating how cells package and store energy-rich lipid molecules remains our top research priority.

My scientific area(s) of expertise is/are: Lipid Biochemistry, Cell Biology, Protein Biochemistry

To take my project to the next level my ideal collaborator would have expertise in:

Structural Biology/ Cryo-Electron Microscopy, Reconstitution of Cellular Systems *in Vitro*

Publications supported by this project 2019-present:

1. **MS Greer, Y Cai, SK Gidda, N Esnay, FK Kretzschmar, D Seay, E McClinchie, T Ischebeck, RT Mullen, JM Dyer, KD Chapman** (2020) SEIPIN isoforms interact with the membrane-tethering protein VAP27-1 for lipid droplet formation. *The Plant Cell*, in press. <http://www.plantcell.org/content/plantcell/early/2020/07/20/tpc.19.00771.full.pdf>
2. **T Ischebeck, HE Krawczyk, RT Mullen, JM Dyer, KD Chapman** (2020) Lipid droplets in plants and algae: Distribution, formation, turnover and function. *Seminars in Cell & Developmental Biology*. In press, corrected proof is available online <https://doi.org/10.1016/j.semcd.2020.02.014>
3. **N Esnay, JM Dyer, RT Mullen, KD Chapman** (2020) Lipid droplet–peroxisome connections in plants. *Contact*, **3**, 2515256420908765 <https://doi.org/10.1177/2515256420908765>
4. **D Sturtevant, S Lu, Z-W Zhou, Y Shen, S Wang, J-M Song, J Zhong, DJ Burks, Z-Q Yang, Q-Y Yang, AE Cannon, C Herrfurth, I Feussner, L Borisjuk, E Munz, GF Verbeck, X Wang, RK Azad, B Singleton, JM Dyer, L-L Chen*, KD Chapman*, L Guo*** (2020) The genome of jojoba (*Simmondsia chinensis*): a taxonomically-isolated species that directs wax-ester accumulation in its seeds. *Science Advances*, **6** (11), eaay3240. DOI: 10.1126/sciadv.aay3240. *joint correspondence.
5. **S Lu, M Aziz, D Sturtevant, KD Chapman*, L Guo*** (2020) Heterogeneous distribution of erucic acid in *Brassica napus* seeds. *Frontiers in Plant Science*, **10**, 1744. <https://doi.org/10.3389/fpls.2019.01744>
6. **FK Kretzschmar*, NM Doner*, HE Krawczyk, P Scholz, K Schmitt, O Valerius, GH Braus, RT Mullen, T Ischebeck** (2020). Identification of low-abundance lipid droplet proteins in seeds and seedlings. *Plant Physiology*, **182**, 1326. <http://www.plantphysiol.org/content/182/3/1326.long>. *joint first authors.
7. **T Romsdahl, A Shirani, RE Minto, C Zhang, EB Cahoon, KD Chapman*, D Berman*** (2019) Nature-guided synthesis of advanced bio-lubricants. *Scientific Reports*, **9**, 11711. doi:10.1038/s41598-019-48165-6. * joint correspondence.
8. **KD Chapman, M Aziz JM Dyer, RT Mullen** (2019) Mechanisms of lipid droplet biogenesis. *Biochemical Journal*, **476** (13), 1929-1942. <https://doi.org/10.1042/BCJ20180021>
9. **AM Price, NM Doner, SK Gidda, S Jambunathan, CN James, A Schami, O Yurchenko, RT Mullen, JM Dyer, V Puri, KD Chapman** (2020) Mouse Fat-Specific Protein 27 (FSP27) expressed in plant cells localizes to lipid droplets and promotes lipid droplet accumulation and fusion. *Biochimie*, **169**, 43-50. <https://doi.org/10.1016/j.biochi.2019.08.002> Special Issue.

Molecular Mechanisms of Plant Cell Wall Loosening: Expansin Action

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Overall research goals:

Cell enlargement is an essential and limiting process for expansion of the photosynthetic leaf canopy (the plant's collector of solar energy and CO₂) and for determining cell size, which puts an upper limit on the amount of energy-rich, carbon-rich secondary cell wall mass that can be accumulated by a cell. In this project we focus on the wall-loosening action of expansin proteins. Expansin action underpins plant growth, yet its molecular mechanism is enigmatic. Expansins have the remarkable and unique ability to induce wall stress relaxation, polymer creep, and cell wall enlargement, but without traces of enzymatic activity. In contrast, wall-modifying enzymes lack these key loosening activities. Our goal is to elucidate how expansins loosen cell walls at the molecular scale. This is likely to reveal novel aspects of cell wall structure. In recent years we have learned that expansins are more diverse and their actions more complex than previously recognized.

Three classes of proteins make up the expansin superfamily: (1) **α -expansins (EXPAs)** are plant proteins that mediate acid-induced extension of plant cell walls. (2) **β -expansins (EXPBs)** are also plant proteins associated with growth, but their specific biological roles are not well established. We studied ZmEXPB1 that is expressed abundantly in maize pollen and that selectively loosens cell walls from grasses (compared with dicots). (3) **Microbial expansins (EXLXs)** are a polyphyletic group of proteins that facilitate synergistic and pathogenic plant-microbe interactions. We have leveraged the facile expression of bacterial expansins in *E. coli* for study of these proteins by crystallography, site-directed mutagenesis and advanced solid-state NMR (ssNMR) but their biophysical actions, targets, and biological roles likely differ in various ways from plant expansins.

Significant achievements (2018-2020):

- β -Expansin action alters the surface texture of maize cell wall at the nanoscale, by loosening the attachment of glucuronoarabinoxylan (GAX) to the wall. We used atomic force microscopy (AFM) to assess maize cell wall surface texture before and after ZmEXPB1 treatment. In untreated cell walls, cellulose microfibrils appeared to be coated with matrix polysaccharide; after ZmEXPB1 treatment the matrix coating appeared to be swollen, softer and in the process of detaching from microfibril surfaces. We also carried out FESEM imaging with labeling by nanogold tagged CBM3 which binds the crystalline surface of cellulose microfibrils. The extent of CBM3-nanogold labeling increased after treatment with ZmEXPB1, which we interpret to mean GAX was removed from cellulose surfaces. These results further substantiate the unusual loosening action of β -expansin.
- Attempts at expansin expression in Human Embryonic Kidney (HEK) cells for structure-function studies: With our help, professor Zheng-Hua Ye at the University of Georgia attempted to express plant expansins in HEK cells, which we tested in our wall creep assay. Most constructs did not produce appreciable protein. Recombinant ZmEXPB1 was produced in small amounts. We developed a hyper-sensitive cell wall creep assay, which demonstrated detectable activity with the recombinant proteins, but the activity was very low, estimated to be ~1% of the specific activity of native ZmEXPB1. With low protein yields and low specific activity, we deemed this approach to be futile as a platform for future structure-function studies.
- Expression of EXPAs in Arabidopsis root hair cells: We obtained promising results with an alternative approach for potential structure-function studies of α -expansins (EXPAs) *in vivo*. The concept: we generated an expansin knock-out line with a clear phenotype and then complemented it

with wild type or mutant expansin constructs to test for complementation. Using a combination of T-DNA and CRISPR mutagenesis, we created homozygous double mutant lines that lacked EXPA7 and EXPA18 expression. The double mutants failed to form normal root hairs, whose growth (elongation) stalled shortly after initiation. This result is interesting in its own right because so-called 'tip growth' of root hairs is often thought to be based on a mechanism of cell wall growth different from cells that grow diffusely. These results may modify that long-held concept.

- Because of technical obstacles in obtaining an experimental structure (lack of effective heterologous expression), we are developing a computational model of α -expansin and using it to explore various possible mechanisms that EXPAs might use to loosen plant cell walls.

Science objectives for 2020-2021:

- Complete the nanoscale characterization of the wall loosening action of ZmEXPB1.
- Characterize the phenotype of the root-hair deficient *expa8/expa17* double mutant.
- Test for the ability of orthologous and non-orthologous α -expansins to complement the root hair phenotype of the *expa8/expa17* double mutant.
- Develop computational methods to model the loosening of plant cell walls by α -expansin.

My scientific area(s) of expertise is/are: cell wall biophysics and structure; biomechanics; plant molecular biology and evolution; recombinant protein expression; structural biology; modeling; plant cell growth; imaging and nanomechanics by atomic force microscopy.

To take my project to the next level, my ideal collaborator would have expertise in: recombinant expression of 'difficult' proteins in novel expression systems; novel methods for assessing conformation and interactions of complex polysaccharides; polymer biophysics;

Publications supported by this project 2018-2020:

- Cosgrove, D.J. (2018) Diffuse growth of plant cell walls. *Plant Physiology*, 176, 16-27. doi: 10.1104/pp.17.01541 (review)
- Cosgrove, D.J. (2018) Nanoscale structure, mechanics and growth of epidermal cell walls. *Current Opinion in Plant Biology*, 46, 77-86. doi: 10.1016/j.pbi.2018.07.016 (review)
- Chase, W.R. and Cosgrove, D.J. (2018) Carbohydrate Binding Module Family 63. In *Cazylopedia*. <http://www.cazy.org/CBM63.html> (review)
- Hepler, N.K., Bowman, A., Carey, R.E. and Cosgrove, D.J. (2019) Expansin gene loss is a common occurrence during adaptation to an aquatic environment. *Plant J*, 101, 666-680. doi: 10.1111/tpj.14572
- Hepler, N.K. and Cosgrove, D.J. (2019) Directed in vitro evolution of bacterial expansin BsEXLX1 for higher cellulose binding and its consequences for plant cell wall-loosening activities. *FEBS Lett*, 593, 2545-2555. doi: 10.1002/1873-3468.13528
- Chase, W.R., Zhaxybayeva, O., Rocha, J., Cosgrove, D.J. and Shapiro, L.R. (2020) Global cellulose biomass, horizontal gene transfers and domain fusions drive microbial expansin evolution. *New Phytologist*, 226, 921-938. doi: 10.1111/nph.16428
- Cosgrove, D.J. (2020) Theory and Practice in Measuring In-Vitro Extensibility of Growing Plant Cell Walls. *Methods in Molecular Biology*, 2149, 57-72.
- Cosgrove, D.J. (2020) Non-enzymatic action of expansins. *J Biol Chem*, 295, 6782. doi: 10.1074/jbc.L120.013410 (Commentary)
- Cosgrove, D.J. and Anderson, C.T. (2020) Plant Cell Growth: Do Pectins Drive Lobe Formation in Arabidopsis Pavement Cells? *Current Biology*, 30, R660-R662. doi: <https://doi.org/10.1016/j.cub.2020.04.007> (Invited Commentary)

Versatility of electron donors to heterodisulfide reductase in hydrogenotrophic methanogens

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Overall research goals:

We are characterizing the metabolism of hydrogenotrophic methanogens. These organisms generate CH₄ as a product of metabolism and catalyze the terminal steps in the degradation of organic matter in anoxic environments. Specifically, we are investigating electron flow and energy conservation in methanogens from the order Methanomicrobiales, a group that is ubiquitous in anoxic environments including freshwater and marine sediments, wastewater reactors, and the rumen.

Hydrogenotrophic methanogens are thought to prefer H₂ as an electron donor for CO₂ reduction to CH₄. However, in addition to H₂, many can also utilize electron donors such as formate, ethanol, secondary alcohols, or carbon monoxide. We seek to understand how these alternative electron donors integrate into the core methanogenic pathway. The terminal step of methanogenesis is carried out by heterodisulfide reductase (Hdr) and catalyzes the exergonic reduction of a heterodisulfide of two methanogenic cofactors: CoM-SH and CoB-SH. This exergonic reaction is coupled to the reduction of ferredoxin through flavin-based electron bifurcation (FBEB) (Fig. 1A). In most hydrogenotrophic methanogens, electrons for these coupled reactions are derived from H₂ oxidation by an associated hydrogenase (Mvh). However, several members of the order Methanomicrobiales lack genes encoding Mvh. The source of electrons for Hdr in these organisms is unknown. Additionally, most methanogens from the order Methanomicrobiales utilize one or more alternative electron donors and it is unknown how electrons derived from substrates other than H₂ feed into the Hdr reaction. The main goal of this project is to characterize the FBEB reaction catalyzed by Hdr in methanogens from the Methanomicrobiales. Results will expand our understanding of this ubiquitous group and will provide insight into pathways of methane production in natural and industrial settings.

Significant achievements (2018-2020):

We have discovered that *Methanoculleus thermophilus* is naturally competent, providing a mechanism to generate mutants, express proteins with affinity tags, and express genes from other organisms. This advance will enable future studies to understand the physiology and metabolism of methanogens from the Methanomicrobiales. As a proof of concept, we have generated mutants that lack components of the type IV-like pilus and shown that pili are essential to DNA uptake. These experiments are described in Fonseca *et. al.* (publication 1).

Leveraging the ability to transform *M. thermophilus*, we tagged and affinity purified Hdr to determine which electron donors participate in the FBEB reaction. *M. thermophilus* was grown with H₂ as an electron donor, harvested, and lysed. Cell lysate was subjected to immobilized metal affinity chromatography to purify Hdr and interacting proteins were identified through mass spectrometry. Interestingly, despite cultures having been grown with H₂, only a formate dehydrogenase was found associated with Hdr. A biochemical assay for Hdr activity showed that robust activity was only observed when purified protein complex was provided formate as an electron donor (Fig. 1B and 1C). These data suggest that formate is the preferred electron donor for methanogenesis in *M. thermophilus*. These results raised the question of the source of formate in cultures where H₂ was the only supplied electron donor. We found that whole cells of *M. thermophilus* catalyze a reversible formate hydrogenlyase activity; this activity produces the formate required for Hdr activity. Many members of the Methanomicrobiales lack genes encoding Hdr associated hydrogenases, but encode several copies of formate dehydrogenase. We hypothesize that these organisms likely catalyze formate-dependent heterodisulfide reduction.

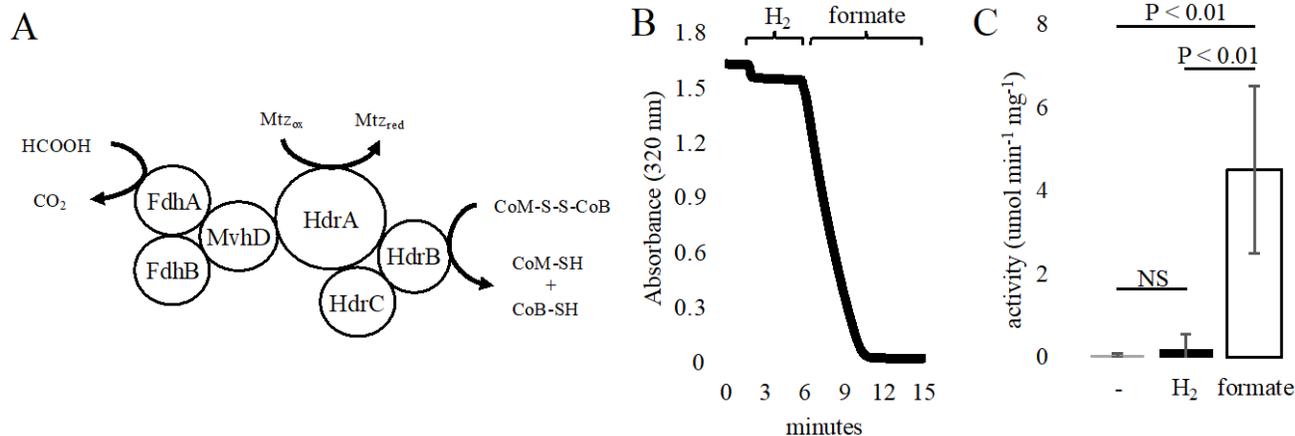


Figure 1. Formate dependent Hdr activity in *M. thermophilus*. **A**. Diagram of the reaction catalyzed by Hdr. **B and C**. Formate dependent reduction of both CoM-S-S-CoB and ferredoxin by the Hdr protein complex. Robust activity was only observed in the presence of formate. Activity was monitored by observing reduction of metronidazole (mtz). Panel B shows example data from one experiment. Panel C shows average activities (\pm standard deviation) from four independent experiments.

Science priorities for the next year (2020-2021):

- Determine the mechanism whereby electrons from alcohols catalyze heterodisulfide reduction in methanogens that utilize secondary alcohols as an electron donor.
- Fdh catalyzes two reactions: reduction of heterodisulfide and reduction of coenzyme F₄₂₀. To gain insight in the mechanism regulating these activities, we will test whether Fdh catalyzes both reactions when bound to Hdr.
- Determine the subunit interactions between Fdh, MvhD, and Hdr enzymes.

My major scientific area(s) of expertise is/are: Microbial physiology and metabolism. Anaerobic microbiology. Genetics and molecular biology.

To take my project to the next level, my ideal collaborator would have expertise in: Structural biology and biochemistry of oxygen sensitive enzymes. Enzymology.

Publications supported by this project:

1. D. Fonseca, M.F. Abdul Halim, M. Holten, K. Costa "Type IV-like pili facilitate transformation in naturally competent archaea." *J Bacteriol*, (2020) *In press*.
2. M.F. Abdul Halim, L. Day, K. Costa "Formate dependent heterodisulfide reduction in a Methanomicrobiales archaeon." *In preparation*.

The DOE Center for Plant and Microbial Complex Carbohydrates at the University of Georgia

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Overall research goals:

The overall goal of our research is to enhance our understanding of rhamnogalacturonan I (RGI), an important and extremely complex pectin polysaccharide present in most plants. RGI is a major component of the pectic polysaccharides in plants, and thus knowledge of the structure and biosynthesis of this polysaccharide is essential to the understanding of the plant cell wall. We are approaching the goal of this research on two separate but interconnected tracks: (1) through the development of analytical methods to determine the structure of RGI and (2) through elucidating the biosynthesis of RGI. The structural studies focus on using permethylation as a means to solubilize insoluble RGI fractions in organic solvents for composition and structure analysis by mass spectrometry and NMR. In addition, we aim to understand the structural variety of RGI from different plant species and tissues, as well as waste industrial plant biomass. A component of our studies includes RGI that is covalently attached to arabinogalactan-protein (AGP). Another part of our efforts is to develop a suite of linkage specific enzymes that can be used to selectively break down RGI, allowing us to gain understanding of how structure contributes to biological function. In addition, these enzymes will be used to generate a library of RGI polymers and oligosaccharides for use as acceptors in studies of glycosyl transferases involved in RGI synthesis. The long-term goals of the RGI biosynthesis studies are to identify the enzymes that add the glycosyl and non-glycosyl substituents during the synthesis of the RGI backbone and side chains. A secondary goal is the application of the methods being developed to the structural characterization of other insoluble or sparingly soluble plant and microbial polysaccharides through our many collaborative efforts of the DOE Center. In addition, we will also disseminate our methods and protocols through number of hands-on training courses/workshops.

Significant achievements

We have developed and published a method for the glycosyl composition analysis of whole cell wall samples by permethylation and GC-MS.¹ We also applied permethylation to solubilize plant cell walls for NMR structure analysis. An NMR chemical shift database generated from a number of permethylated polysaccharide standards has enabled structure determination of both the major and minor polysaccharides.² We have authored a tutorial on methylation analysis as a book chapter³ and have published one and submitted 4 manuscripts detailing the structural analysis of non-RGI carbohydrates.⁴

We have discovered that duckweed RGI and apiogalacturonan containing pectic AGPs can form borate diesters with each other and with the pectin RGII, offering an explanation for plant resistance to breakdown in high pH water. We further found that the major RGI released from both citrus peels and sugar beet residues with endopolygalacturonase/pectin methyl esterase after oxalate and carbonate extraction are covalently attached RGI-AGP complexes, and some of the RGIs have bound boron in the form of boric acid monoesters.

We have established a method for mass production of duckweed biomass for pectin isolation from monocots. A library of more than 30 plasmids for the expression of recombinant enzymes specifically involved in RGI deconstruction was assembled and screened for heterologous expression. In addition, we have completed constructs for the heterologous expression of several RGI transferases including putative arabinosyltransferases, β -1,4-galactosyltransferases, and two putative pectin synthesis enzymes from a new glycosyltransferase family 47 subfamily in liverwort. Using a subset of these enzymes, we demonstrate that *Arabidopsis thaliana* galactosyltransferase 1 (AtGALS1) enzymes can transfer azido-functionalized sugar nucleotide donors to selected synthetic plant cell wall oligosaccharides on a glycan array, and determined that AtGALS1 is able to utilize more sugar donors than was currently known, generating unnatural xylogalactan oligosaccharides that are potential new biomaterials.⁵

Prior to this project, a family of RGI:rhamnosyltransferases (RRT) had been identified, but the identity of the galacturonosyltransferases that incorporate GalA (galacturonic acid) into the RGI backbone was unknown. The identity of the first RGI:galacturonosyltransferase has now been confirmed. We expressed this enzyme in HEK293 cells and completed assays to demonstrate that it is an RGI backbone specific GalA transferase (GalAT).

Combining the GalAT and RhaT activities, we demonstrated elongation of RGI backbone oligosaccharides *in vitro*. We established preparatory-scale methods to purify UDP-Rha and RGI oligosaccharide acceptors necessary to complete these studies.

In order to make our tools and methodologies available to investigators outside of the DOE Center, we are offering virtual training courses in glycoscience which have been well received by the scientific community with over 300 registered course participants.

Science priorities for the next year (2020-2021):

1) The permethylation methodology will be optimized to obtain NMR sample solutions from whole cell wall preparations for quantitation and molecular weight determination of cell wall polysaccharides. Furthermore, a rigorous study of the impact of permethylation on uronic acids will be undertaken to establish an efficient permethylation protocol for solubilizing insoluble RGI fractions for composition and structure analysis by GC-MS and NMR. 2) Detailed structural analysis of the RGI-AGPs purified from different plant materials, especially the RGI-AGPs from citrus peels and sugar beet residues. We propose to locate the attachment site on sugar residues on the RGI for boron/boric acid monoesters and will determine if all RGI molecules have boron covalently attached. 3) We will scale up tissue production to further build our substrate library for structural elucidation of RGI using NMR and enzyme techniques and evaluate their robustness. A key goal of our gene toolbox project is to optimize enzymes that are able to cleave the RGI backbone in the presence of full side-chain structures, which is necessary for determining fine structure of RGI and implementation of high throughput (HTP)-polysaccharide fingerprinting. 4) Further priorities for year 2 are to complete a biochemical characterization of the RGI:GalAT enzyme and to expand our assays to study RGI backbone elongation when this enzyme is combined with individual purified RhaT enzymes.

My major scientific area(s) of expertise is/are:

Carbohydrate structural characterization of plant and microbial polysaccharides by MS, HPLC, and NMR. Carbohydrate chemistry; plant cell wall pectins and glycoproteins. Pectin and hemicellulose biosynthesis and structure. Characterization of carbohydrate active enzymes. Pectin biosynthesis, structure, and function. Purification of glycosyltransferases and polysaccharides. Biochemical characterization of glycosyltransferase enzyme function.

To take my project to the next level, my ideal collaborator would have expertise in:

Mutagenesis of biomass, targeted enzymatic degradation of plant polysaccharides. Atomic force microscopy, biomechanics and protein crystallography, biological function of carbohydrates.

A subset of publications out of 42 total supported by this project:

¹ Black, I., Heiss, C., & Azadi, P. (2019). Comprehensive monosaccharide composition analysis of insoluble polysaccharides by permethylation to produce methyl alditol derivatives for gas chromatography/mass spectrometry. *Analytical chemistry*, 91(21), 13787-13793.

² Ikenna E. Ndukwe, Ian Black, Christian Heiss and Parastoo Azadi (2020) Evaluating the Utility of Permethyated Polysaccharide Solution NMR Data for Characterization of Insoluble Plant Cell Wall Polysaccharides. *Anal. Chem.*, *under review*.

³ Ian Black, Artur Muszinski, Kathryn Lockwood, Christian Heiss, Parastoo Azadi (2020) Linkage Analysis of Oligo- and Polysaccharides – A Tutorial, *in Methods in Molecular Biology / Mass Spectrometry of Glycoproteins*, Arnaud Delobel, Ed., Springer.

⁴ a) Christian Heiss, Ian Black, Mayumi Ishihara, Mehmet Tatli, In-Cheol Yeo, Timothy P. Devarenne, and Parastoo Azadi (2020) Structure of the Polysaccharide Sheath from the B Race of the Green Microalga *Botryococcus braunii*. *Algal Res.*, *under review*; b) Mehreen Zeb, Linda E. Tackaberry, Hugues B. Massicotte, Keith N. Egger, Kerry Reimer, Grace Lu, Christian Heiss, Parastoo Azadi, and Chow H. Lee (2020) Structural elucidation and immuno-stimulatory activity of a novel polysaccharide containing glucuronic acid from the fungus *Echinodontium tinctorium*. *Carbohydr. Polymers*. *Under review*; c) Maitte Docampo-Palacios¹, Anislay Alvarez¹, Olubu Adiji¹, Daylin Gamio-tea-Turro², Alexander B. Valerino-Diaz², Luis P. Viegas³, Angelo de Fatima^{1,4}, Giulio Pasinetti⁵ and Richard A. Dixon¹ (2020) Glucuronidation of Methylated Quercetin Derivatives: Chemical and Biochemical Approaches. *J. Agric. Food Chem.* *Under review*; Sapeck Srivastava, Christian Heiss, David Zuckerman, Jeffery So, Koen Semeijn, Radnaa Naran, Parastoo_Azadi, and Egbert Hoiczky. (2020) Structure and function of a novel osmoregulated periplasmic fiber-forming high-molecular-weight carbohydrate of *Myxococcus xanthus*. *mBio*, *under review*; e) Wong, J.E., Gysel, K., Birkefeldt, T.G., Vinther, M., Muszyński, A., Azadi, P., Laursen, N.S., Sullivan, J.T., Ronson, C.W., Stougaard, J. and Andersen, K.R. (2020) Structural signatures in EPR3 define a unique class of plant carbohydrate receptors. *Nat. Commun.* **11**, 3797.

⁵ Colin Ruprecht, Max P. Bartetzko, Deborah Senf, Anna Lakhina, Peter J. Smith, Maria Soto, Hyunil Oh, Daniel Varon Silva, Mads H. Clausen, Kelley W. Moremen, Michael G. Hahn, Breeanna R. Urbanowicz, and Fabian Pfrengle (2020) A Glycan Array-Based Assay for the Identification and Characterization of Plant Glycosyltransferases *Angewandte Chemie*. 2020 May 12. doi: 10.1002/anie.202003105.

Resolving protein-semiquinone interactions by advanced EPR spectroscopy.

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Antony R. Crofts, Robert B. Gennis, Co-PIs

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Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we propose to study, - the Q_A and Q_B sites of the bacterial reaction center (RC), the Q site of the *cyt bo*₃ quinol oxidase, and the Q_i site of the *bc*₁ complex, - all operate using ubiquinone, but have different electron transfer partners, and different operating potentials. EPR probes interactions between the electron spin of SQ and local magnetic nuclei, which provide direct information about *spatial* and *electronic* structure of the SQ and the immediate protein and solvent environment. The main question to be addressed is that of how the protein environment modifies the spatial and electronic structure of the SQ in different sites to fit the physiological function.

Significant achievements 2018-2020:

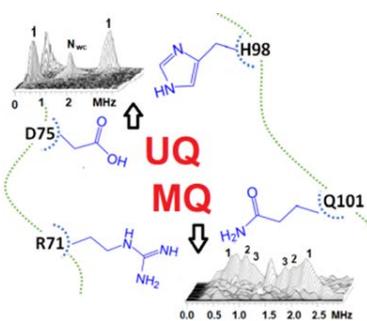
The ubiquinol binding site of *cyt bo*₃ from *E. coli* accommodates menaquinone and stabilizes a functional menasemiquinone. *Cyt bo*₃, one of three terminal oxygen reductases in the aerobic respiratory chain of *E. coli*, has been well characterized as a ubiquinol oxidase. Its ability to catalyze the two-electron oxidation of ubiquinol-8 requires the enzyme to stabilize the one-electron oxidized

ubisemiquinone (USQ) species that is a transient intermediate in the reaction. This USQ was detected and studied in detail using EPR techniques. *Cyt bo*₃ has been shown recently to utilize also demethylmenaquinol-8 as a substrate which, along with menaquinol-8, replaces ubiquinol-8 when *E. coli* is grown under micro-aerobic or anaerobic conditions. In the current work, we show that steady-state turnover with 1,3-dimethyl-2,4-naphthoquinone (DMN) a water-soluble menaquinol analogue, is just as efficient as with ubiquinol-1. These findings imply that the menasemiquinone (MSQ) must also be stabilized

by interacting with the active site residues of *cyt bo*₃. The suggestion was supported by previous studies on the *cyt aa*₃-600 menaquinol oxidase from *Bacillus subtilis*, a close homologue of *cyt bo*₃ that utilizes menaquinol-7 as its native substrate and stabilizes a MSQ species. Based on the similarity of the amino acid sequences four polar residues have been implicated in binding to the quinol at the Q-site in *cyt bo*₃ and *cyt aa*₃-600: R71(R70), D75(D74), H98(H94), and Q101(E97) (*B. subtilis* numbering).

Using pulsed EPR spectroscopy and ¹⁵N uniform labeling of the protein, we demonstrate that the same residues in *cyt bo*₃ that stabilize the USQ state also stabilize the MSQ state of menaquinone-4, with the hydrogen bond strengths and the distribution of unpaired spin density accommodated to the different substrate. Catalytic function with menaquinol is more tolerant of mutations at the active site than with ubiquinol. A mutation of one of the stabilizing residues (R71H in subunit I) that eliminates ubiquinol oxidase activity of *cyt bo*₃ does not abolish activity with soluble menaquinol analogues. **Ref. 4.**

Influence of hyperfine coupling strain on two-dimensional ESEEM spectra from I=1/2 nuclei. Hydrogen bonding between semiquinone (SQ) intermediates and sidechain or backbone nitrogens in protein quinone processing sites (Q-sites) is a common motif. Our systematic studies of H-bonds with SQs in ¹⁵N labeled proteins have previously provided us with an extensive collection of ¹⁵N HYSCORE spectra produced by N-H...O nitrogen donors. Examination of these spectra has indicated in several cases lineshape distortions not described by available theoretical models. They manifest in the form of the “boomerang” lineshape and low intensity lines in the opposite quadrant. We were able to explain all observed artificial phenomena in the ¹⁵N spectra of SQs considering a single mechanism – strain of the isotropic hyperfine interaction. Their appearance is regulated by the relative values of the strain width Δa and parameter $\delta =$



$|2a+T|-4v_{15N}$. Clearly resolved effects from the α -strain are only expected to appear when part of the strain broadened cross-ridge approaches or satisfies the condition $(2(a\pm\Delta a)+T) \sim 4v_{15N}$. The intensity and shape of the cross-features in the opposite quadrant depend on the strain distribution function and the Δa and δ values. We found similar lineshape distortions in previously published spectra from ^{15}N , ^{57}Fe ([Fe-Fe]-hydrogenase), ^{29}Si (Ti^{3+} in $\text{SiO}_2\text{-TiO}_2$), and ^{31}P (VO^{2+} in bones and model systems) nuclei that can be explained using the approach applied for the analysis of the ^{15}N spectra of SQs. These examples clearly show that hyperfine strain produces new spectral features in 2D spectra, and ignorance of their true nature may lead to errors in spectral interpretations and resulting structural models. **Refs. 1,6.**

Collaborative work. Our experience in pulsed EPR and available state-of-the-art equipment have attracted the attention of researches from the UIUC and other laboratories. The spectroscopic approach and isotope labeling methodology developed under this project was proven completely applicable to more complex systems as paramagnetic metals and clusters in proteins and other systems including frozen solutions and catalysts. Currently we collaborate with several laboratories of PIs supported by the Physical Biosciences program or involved in the projects funded by the BES. Most successful is the collaborative work with the laboratory of Prof. Yi Lu (UIUC) devoted to the studies of an engineered binuclear Cu_A (Cu_2S_2) site in azurin and cytochrome *c* peroxidase with the aim to characterize peculiarities of an electronic structure. **Refs. 2,5.**

Science objectives for 2020-2021:

- We will study the influence of mutations on SQ_A in bacterial reaction center from *Rhodobacter sphaeroides*. We will focus on the M265IS and M265IT mutations induced 80-120 mV negative shifts in the E_m of Q_A , with little perturbation of any other properties, including binding affinity.
- Previous studies showed that the 2-methoxy group is essential for simultaneous function of Q_A and Q_B , i.e. electron transfer from Q_A to Q_B works for 2- $\text{CH}_3\text{O-Q}$ and does not work for 3- $\text{CH}_3\text{O-Q}$. We will characterize the interactions of the reconstituted 2- and 3-monomethoxyQs with protein in the Q_A^- state by performing 2D ESEEM and ENDOR in native and ^{15}N labeled RC, and H_2O or D_2O solvent.

My scientific area(s) of expertise is/are: Continuous-wave and pulsed EPR; magnetic resonance as a structural tool and its application in inorganic biochemistry, photosynthesis, structure-function relations in metalloproteins and quinone processing sites studies.

To take my project to the next level, my ideal collaborator would have expertise in: We are open for any collaborative work requiring EPR approaches. Pulsed EPR spectroscopy and methods of selective isotope labeling employed in this work will be beneficial for structural studies of metal cofactors and radical species in any other areas of bioenergetics, photosynthesis, and bioinorganic chemistry.

References to work supported by this project 2018-2020:

1. Dikanov, S.A., Taguchi, A.T. (2018) Two-dimensional pulsed EPR resolves hyperfine coupling strain in nitrogen hydrogen bond donors of semiquinone intermediates. *J. Phys. Chem. B* **122**, 5205–5211.
2. Dikanov, S. A., Berry, S. M., Lu, Y. (2019) HYSCORE insights into the distribution of the unpaired spin density in an engineered Cu_A site in azurin and its His120Gly variant. *Inorg. Chem.* **58**, 4437–4445.
3. Taguchi, A.T., Evans, E.D., Dikanov, S.A., Griffin, R.G. (2019) Convolutional neural network analysis of two-dimensional Hyperfine Sublevel Correlation Electron Paramagnetic Resonance spectra. *J. Phys. Chem. Lett.* **10**, 1115–1119.
4. Ding, Z., Sun, C., Yi, S.M., Gennis, R.B., Dikanov, S.A. (2019) The ubiquinol binding site of cytochrome *bo*₃ from *Escherichia coli* accommodates menaquinone and stabilizes a functional menasemiquinone. *Biochemistry* **58**, 4559–4569.
5. Mirts, E.N., Dikanov, S.A., Jose, A., Solomon, E.I., Lu, Y. (2020) A binuclear Cu_A center designed in an α -helical protein scaffold. *J. Am. Chem. Soc.* **142**, 32, 13779–13794.
6. Dikanov, S.A., Taguchi, A.T. Influence of hyperfine coupling strain on two-dimensional ESEEM spectra from $I=1/2$ nuclei. *Appl. Magn. Reson.* (accepted, DOI: 10.1007/s00723-020-01246-6).

Activation of recombinant methyl-coenzyme M reductase in the methanogenic archaeon *Methanococcus maripaludis* and the role of post-translational modifications

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Overall research goals:

At present, we have expressed recombinant holoenzyme of methyl-coenzyme M reductase or Mcr in the methanogen *Methanococcus maripaludis*. While the recombinant Mcr contains the correct subunit composition, posttranslational modifications (or PTMs) and coenzyme F₄₃₀, the activity is still low. We propose that the low activity is due to incomplete activation. The proposed research will address the possibilities that 1) the activating enzymes present in *M. maripaludis* do not recognize the recombinant Mcr from even genetically closely related species, 2) the recombinant activation proteins expressed in *E. coli* do not form the correct conformations or Fe-S clusters, and 3) the tagging of the recombinant proteins inhibits their activation. It will also address the possibility that components necessary for full activation are missing from the core activation complex.

In parallel to our studies of expression of recombinant Mcr, an in-frame deletion mutant was constructed for the gene encoding the methanogen marker protein 10 (Mmp10). The gene encoding Mmp10 in *M. maripaludis* was deleted with a new genetic tool, resulting in the complete loss of the 5-C-(S)-methylarginine PTM in the McrA subunit and a 40-60 % reduction of methane formation by whole cells. Site-directed mutagenesis of *mmp10* resulted in a differential loss of the arginine methylation among the mutants *in vivo*.

Building upon these preliminary studies, **the proposed research has three specific aims:**

- 1) **Characterization of the activation proteins from *M. maripaludis* and other methanogens.** This aim will address the possibilities that the recombinant activation proteins expressed in *E. coli* do not form the correct conformation, tagging of the recombinant proteins inhibits their activity, and additional components of the activation complex are yet to be identified.
- 2) ***In vitro* activation of the recombinant Mcr.** Further experiments will be performed to optimize the activation to obtain basic insights into the process. A reliable assay will be established for the activation process *in vitro* to enable a detailed characterization of the function of the individual activation components. The studies will include comparison of activation of the native and recombinant Mcr with different peptide tags. In addition, effects of the Me-Arg and thio-Gly PTMs of Mcr on activation will be examined. The Mcr substrates for the activation assay will be generated in *Methanococcus* mutants unable to perform PTMs.
- 3) **Characterization of the Mmp10 protein and its role in Mcr PTM.** The Me-Arg PTM of Mcr is catalyzed by the Mmp10 protein. While the recombinant Mmp10 from *Methanosarcina* has been characterized *in vitro*, the protein was expressed in *E. coli* and missing the cobamide cofactor [Radler et al. (2019) *J Biol Chem* **294**, 11712-11725). Activity was detected when cobalamin was present in the assay. In our experience, archaeal proteins expressed in bacteria seldom contain the native cofactors. For that reason, the recombinant Mmp10 protein expressed in *Methanococcus* will be characterized. Because the

Methanococcus and *Methanosarcina* proteins are not closely related, these experiments will not duplicate efforts in other laboratories.

Significant achievements ([Current Funding, 2020-2022]):

Although regulated heterologous expression systems are well developed in bacterial and yeast systems, they are still relatively primitive in archaea. Recently, we developed the phosphate promoter *Ppst* to decouple expression from growth. During growth on high phosphate, expression is reduced to about one-fifth of that of other strong promoters. Under this condition, we expect that the presence of heterologous genes will have little effect on growth, and the selection for downregulated spontaneous mutants would be reduced. In preliminary experiments, expression of the TAP-FLAG tagged Mcr was about 5 % of the total protein following growth with low phosphate, and the coenzyme F430 content was near 100 %.

The activating complex, component A3a, contains multiple subunits. Five were proposed to be essential: component A2, iron-sulfur flavin protein (Isf), Mmp7, MrcC, and an ATP-binding protein. The expression of all five proteins in *E. coli* was optimized, and iron-sulfur clusters were reconstituted. The Isf protein contained 50% [4Fe-4S] clusters and no flavin, MrcC contained 20% [4Fe-4S] clusters. With this set of protein about 0.5% of the Mcr activity was restored. Since the recombinant A2 and Mmp7 are highly unstable, they and other proteins were expressed in *M. maripaludis* as well. While MrcC and the ATP-binding protein were successfully expressed, A2 and Mmp7 were not. Since pull down experiments showed a high affinity between A2 and Mmp7, a plasmid was designed that contains both genes to determine if they will form a stable complex when coexpressed.

The composition of the activation complex A3a is expected to vary depending on the electron donor during growth on different substrates. Searching for different forms, another complex was detected that contained Mmp3, 5, 6, 7, 15, and 17. In some methanogens the genes for these Mmps are clustered on the genome, possibly forming an operon. In a collaborative experiment with the group of Dr. Steven Mansoorabadi at Auburn University, it was shown that the *M. acetivorans* gene cluster encoding A2, Mmp2, 3, 5, 6, 7, 15, 17, indeed formed a complex when coexpressed in *E. coli* from a pETDuet vector. The connection between the A3a and this complex is not clear. In addition to activation, the Mmp cluster might be involved in the assembly and folding of Mcr.

Science priorities for the next year (2020-2021):

- In vitro activation of the Mcr is our highest priority.

My major scientific area(s) of expertise is/are: Whitman: Microbial physiology of methanogenesis; Duin: Anaerobic enzymology, bioinorganic chemistry, EPR spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: Bioinformatics.

Publications supported by this project [over the past 3 years]:

The first grant period, which began 09/01/2017, resulted in two publications:

1. Lyu, Z, C-W Chou, H Shi, L Wang, R Ghebream, D Phillips, Y Yan, EC Duin, WB Whitman (2018) Ordered assembly of methyl-coenzyme M reductase in the methanogenic archaeon *Methanococcus maripaludis*. J. Bacteriol. 200: e00746-17; doi: 10.1128/JB.00746-17
2. Lyu, Z, N Shao, C-W Chou, S Hao, R Patel, EC Duin, and WB Whitman (2020) Post-translational methylation of arginine in methyl-coenzyme M reductase has a profound impact on both methanogenesis and growth of *Methanococcus maripaludis*. J. Bacteriol. 202: e00654-19, doi: 10.1128/JB.00654-19.

Tuning Directionality for CO₂ Reduction in the Oxo-acid:ferredoxin oxidoreductase superfamily

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Overall research goals:

The oxo-acid:ferredoxin oxidoreductase (OFOR) enzyme superfamily represents one of the best examples to study the reversible transformation of CO₂. Members of the family are responsible for both oxidizing oxo-acids such as pyruvate, as in a PFOR, to produce electrons that are taken up in a ferredoxin (Fd) pool, and yield acetyl-CoA. However, OFORs must also operate in the reductive direction, such as the OGOR enzyme that produces oxo-glutarate from CO₂ and succinyl-coA, taking up electrons from Fd proteins. How nature biases oxidation *versus* reduction is not well understood for the OFOR superfamily. We have hypothesized that the rate-determining steps of catalysis involving electron transfer (ET) may be responsible, at the level of specific unimolecular rate constants or the macroscopic redox potential. Our research goals are to (a) determine the impact of redox potential of internal “wiring” of an OFOR enzyme upon catalytic rate constants and (b) monitor the impact of redox partners (Fds) of diverse potential upon the same. As our project has continued, increasing focus has fallen on the roles of specific (or non-specific) Fd proteins, which have tunable redox potentials that themselves act as the determinants of the ‘bias’ or directionality of catalytic chemistry.

Significant achievements ([2016-2020]):

- We have recently published the complete characterization of the first reverse TCA cycle OFOR member, the oxo-glutarate:ferredoxin oxido-reductase (OGOR) from *Magnetococcus marinus* MC-1, including solving the structure of that enzyme in its resting state, and with substrate bound, at 1.84 Å and 2.8 Å resolution, respectively. The OGOR enzyme, *Mm* KorAB has a single [4Fe-4S] cluster, in comparison with other structurally characterized PFOR enzymes. We have current and long-range interests in attempting to understand why nature has chosen to oblate the typical additional 2 x [4Fe-4S] cluster-bearing Ferredoxin (Fd) domain that are found in most OFOR family members.
- We examined the Fd-dependent properties associated with catalysis. In the context of both a synthetic library of single, double and triple mutants of a given set of homologous Fd proteins, we have examined the impact of Fd mediator potential on the ability to support oxidative versus reductive catalysis. Our first paper, now in submission, describes these relationships for a ‘canonical’ oxidative PFOR and its ortholog associated with CO₂ reduction. Ongoing efforts that too early for publication include comparative effects when examining different Fd scaffolds (i.e., redox mediators that have multiple FeS clusters, instead of a single [4Fe-4S] cluster).
- We continue to make exciting measurements of the direct electrochemistry of OFOR family members giving us access to the direct redox potentials of the 3 x [4Fe-4S] cluster bearing OFORs for the first time. We have achieved this previously (last PB contractors meeting) with the enzymes from *Chlorobium tepidum* and *Moorella thermoacetica*. Recently, in collaboration with Prof. William Metcalf (UIUC) we have also address a methanogenic PFOR, and its Fd-bearing subunit independently. These data are critical, as they illustrate the impact of binding of a Fd subunit to the entirety of a multi-polypeptide enzyme. Intriguingly, there are shifts in the 2x[4Fe-4S] redox potentials in the “PFOR-free” Fd sub-unit, but also a greater sense of electrochemical reversibility, indicating that ordering between sub-units leads to enhance ET rate.

- We have recently reported with Prof. Gordana Dukovic at University of Colorado, Boulder, that *Mm* KorAB can be docked with a variety of nano-crystals to develop a light-induced process for capture of CO₂ and the generation of products. Intriguingly one of our major findings (Publication 5 below) shows that photo-induced ET can be very fast, but the enzyme itself controls the rate of electronic input. As KorAB and other PFORs are now known to undergo significant conformational changes presumed to be linked to Fd binding, ET, and Fd release, we have learned important mechanistic information about how to better design such photo-bio hybrid systems in the future.
- Finally, we have worked with Prof. Russ Hille at UC-Riverside, to examine the redox chemistry of the FeS clusters of the soluble Formate Dehydrogenase of *C. necator*, allowing us to assign redox potentials for several sub-states of the iron-sulfur clusters and Mo-based cofactor.

Science priorities for the next year (2020-2021):

- Now that we have the ability to pre-reduce the enzyme in hand, pre-steady-state kinetic analyses of the KorAB enzyme electron transfer reactions, in the presence of oxidative and reductive substrates.
- Continued mapping of the impact of residues in proximity to the [4Fe-4S] clusters of OFOR family members, in order to elucidate the impact upon redox potential, ET kinetics and catalysis.
- Leveraging our success on wild-type *Mm*KorAB integration with semi-conductor nanorods with different nano-structured light collectors.
- Deploying the mutated Fds into a synthetic biology format to examine the impact upon a model system (such as *E. coli*)

My major scientific area(s) of expertise is/are: Metalloenzymology, and Electrochemistry/electrocatalysis.

To take my project to the next level, my ideal collaborator would have expertise in: Anaerobic synthetic biology; ability to make site-directed mutants in Wood-Ljungdahl Pathway or rTCA-cycle bearing micro-organisms.

Publications supported by this project [2016-2020] :

1. Li B and Elliott SJ. (2016) The catalytic bias of 2-oxoacid:ferredoxin oxidoreductase in CO₂ evolution and reduction through a ferredoxin-mediated electrochemical assay, *Electrochimica Acta*, 199, 349-356. DOI: [10.1016/j.electacta.2016.02.119](https://doi.org/10.1016/j.electacta.2016.02.119)
2. Chen PYT, Li B, Drennan CL and Elliott SJ. (2019) Identification and characterization of a CO₂-fixing 2-oxoacid:ferredoxin oxidoreductase and its physiological redox partner. *Joule*, 3, 1-17. 10.1016/j.joule.2018.12.006
3. Walker LM, Li B, Niks D, Hille R, Elliott SJ. (2019) Deconvoluting the redox potentials of formate dehydrogenase, *J. Biol. Inorganic Chemistry*, 24(6):889-898.
4. Atkinson JT, Campbell IJ, Thomas EE, Bonitatibus SC, Elliott SJ, Bennett GN, Silberg JJ. (2019) Metalloprotein switches that display chemical-dependent electron transfer in cells, *Nature - Chemical Biology*, 15, 189-195.
5. Hamby H, Li B, Shinopoulos KE, Keller HR, Elliott SJ, Dukovic G. (2020) Light-driven carbon-carbon bond formation of CO₂ reduction catalyzed by complexes of nanorods and a 2-oxoacid oxidoreductase, *Proc. Natl. Acad. Sci.*, 117(1): 135-140

Extracellular Charge Transport in Microbial Redox Chains: Linking the Living and Non-Living Worlds

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Overall research goals:

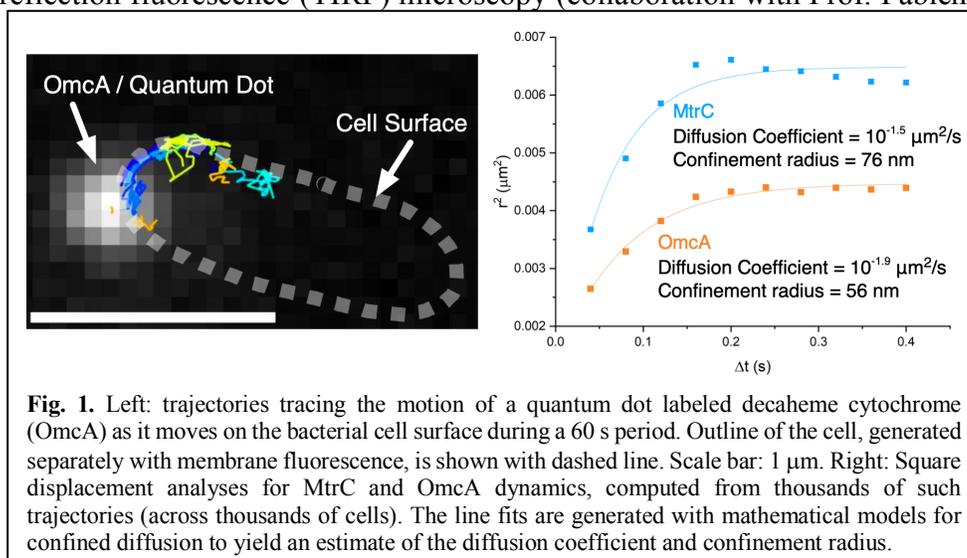
The overarching goal of our work is to understand the fundamentals, limits, and prevalence of extracellular electron transport (EET) conduits that link microbial metabolism to solid-state electrodes. A biophysical understanding will enable energy conversion at hybrid biomolecular/synthetic interfaces. The objectives of the current grant (2019-2022) are:

- (1) Quantify the diffusive dynamics of bacterial outer-membrane multiheme cytochromes through *in vivo* single molecule tracking on cell surfaces and membrane nanowires. The results will directly test the hypothesis that protein dynamics facilitate a collision-exchange mechanism of inter-protein electron transport over micrometer length scales.
- (2) Electrochemically characterize the outer-membrane porin-cytochrome Cyc2 from chemolithotrophic Fe(II)-oxidizers, as a model conduit of inward EET. The overarching goal is to shed light on cathode-microbe EET, which remains poorly understood relative to its microbe-anode counterpart.
- (3) Develop solid-state physics and electrochemical techniques to measure inter-species electron transport in anaerobic oxidation of methane consortia where multiheme cytochromes are hypothesized to link methanotrophic archaea and sulfate-reducing bacteria.

Significant achievements (2019-2022):

Goal 1. We successfully implemented and quantified quantum dot (QD) labeled single-molecule trajectories of the bacterial electron conduits MtrC and OmcA on the cell surface of the model extracellular electron transfer organism *Shewanella oneidensis* MR-1. To accomplish these measurements, we engineered the decaheme cytochromes with an acceptor peptide fusion tag, allowing us to label the biotinylated cytochromes with streptavidin-functionalized QDs. These constructs were cloned into deletion backgrounds and expression/labeling was confirmed prior to single molecule tracking with total internal reflection fluorescence (TIRF) microscopy (collaboration with Prof. Fabien Pinaud, USC).

By analyzing thousands of cells and QD trajectories, we have built significant statistics for quantitative analyses of the diffusive dynamics. The mean square displacement analysis revealed confined diffusion behavior with a confinement radius of ~56-76 nm, representing the lateral range of the molecule in between collisions. This confinement is in agreement with our



electron cryo-tomography measurements of cytochrome density on the bacterial membrane nanowires of *Shewanella*. Preliminary analyses reveal confined diffusion coefficients in the $10^{-1.5}$ - 10^{-2} $\mu\text{m}^2/\text{s}$ range (pending corrections for curvature effects). Taking these dynamics into account, and using realistic electron hopping timescales and cytochrome densities, collision-exchange simulations reveal significant electron transport rates (up to 10^4 s^{-1}) along membrane nanowires and the cellular surface.

Goal 2. We have demonstrated a heterologous expression approach for analyzing the electrochemical activity of Cyc2 from two Fe(II)-oxidizing bacteria (*Mariprofundus ferrooxydans* PV-1 and *Acidithiobacillus ferrooxidans*) in cytochrome-deficient mutants of the model EET organism *Shewanella*. This approach overcomes challenges posed by the difficulty of cultivating Fe(II)-oxidizing bacteria, obtaining sufficient protein quantities for functional assays, and distinguishing biotic from abiotic Fe-oxidation. Preliminary electrochemical measurements show enhanced electron uptake from cathodes by Cyc2-expressing *Shewanella*, in the presence of fumarate as electron acceptor. In addition to engineering *Shewanella* for inward EET, this preliminary data represents the first direct confirmation of EET via this electron conduit. The stage is now set for more detailed characterization of this conduit.

Goal 3. Recent activity measurements, genomic/transcriptomic studies, and modeling suggest that the syntrophy within anaerobic oxidation of methane (AOM) consortia is based on direct electron transport via large cytochrome complexes expressed by both the anaerobic methanotrophic archaea (ANME) and sulfate-reducing bacteria (SRB). To test this hypothesis of direct inter-species electron conduction, we developed a new experimental apparatus for solid-state conduction, electrochemical measurements, and microscopy of AOM aggregates in an anaerobic setup, in collaboration with Victoria Orphan's group. Using this setup, we measured the current-voltage characteristics of AOM aggregates crossing interdigitated gold electrode arrays, and found the aggregates to be conductive under both dry and hydrated conditions when measured anaerobically (conductance decayed quickly upon air exposure). Preliminary electrochemical (voltammetric) measurements are also showing the first hints of redox signatures from ANME-1/SRB incubations applied to electrodes.

Science priorities for the next year (2020-2021):

- Complete and publish the study describing the diffusive dynamics of electron transfer proteins, and the impact of these dynamics on micrometer scale extracellular electron transport.
- Confirm the redox characteristics observed in anaerobic oxidation of methane consortia, and their contribution to inter-species electron transport.

My major scientific area(s) of expertise is/are: Biological electron transport, *in vivo* microscopy, scanning probe measurements, bioelectrochemistry, nanoscience..

To take my project to the next level, my ideal collaborator would have expertise in: Time-resolved measurements, synthetic biology.

Publications supported by this project 2018-2020:

1. A. Karbelkar, A.R. Rowe, M.Y. El-Naggar, *An Electrochemical Investigation of Interfacial Electron Uptake by the Sulfur Oxidizing Bacterium Thioclava electrotropha ElOx9*, *Electrochimica Acta*, 324, 134838 2019
2. A.R. Rowe, S. Xu, E. Gardel, A. Bose, P. Girguis, J.P. Amend, M.Y. El-Naggar, *Methane-linked mechanisms of electron uptake from cathodes by Methanosarcina barkeri*, *mBio*, e02448-18, 2019
3. S. Xu, A. Barrozo, L.M. Tender, A.I. Krylov, M.Y. El-Naggar, *Multiheme Cytochrome Mediated Redox Conduction Through Shewanella oneidensis MR-1 Cells*, *Journal of the American Chemical Society*, 140, 32, 10085-10089, 2018
4. N.L. Ing, M.Y. El-Naggar, A.I. Hochbaum, *Going the distance: long-range conductivity in protein and peptide bioelectronic materials*, *The Journal of Physical Chemistry*, 122, 10403-10423. ACS Editors Choice.
5. G.W. Chong, A.A. Karbelkar, M.Y. El-Naggar, *Nature's Conductors: What can microbial multi-heme cytochromes teach us about electron transport and biological energy conversion?* *Current Opinion in Chemical Biology*, 47, 7-17, 2018
6. M.D. Yates, S.M. Strycharz-Glaven, J.P. Golden, J. Roy, S. Tsoi, J.S. Erickson, M.Y. El-Naggar, S. Calabrese Barton, L.M. Tender, *Characterizing Electron Transport through Living Biofilms*, *Journal of Visualized Experiments*, 136, 2018
7. P. Subramanian, S. Pirbadian, M.Y. El-Naggar, G.J. Jensen, *The ultrastructure of Shewanella oneidensis MR-1 nanowires revealed by electron cryo-tomography*, *PNAS*, 115, E3246-E3255, 2018.
8. A.R. Rowe, P. Rajeev, A. Jain, S. Pirbadian, A. Okamoto, J.A. Gralnick, M.Y. El-Naggar, K.H. Nealson, *Tracking electron uptake from a cathode into Shewanella cells: implications for generating maintenance energy from solid substrates*, *mBio*, 9, e02203-17, 2018

Electron Transport in Acetotrophic Methanogens

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Overall research goals:

The long-term goal is a more complete biochemical understanding of the conversion of acetate to methane that guide improvements in the rate and reliability of biomethanation of renewable biomass, an alternative to fossil fuels. The metabolism of acetate by methane-producing anaerobes (acetotrophic methanogens) accounts for at least two-thirds of the methane produced by anaerobic microbial food chains transforming renewable biomass to methane (biomethanation) as a carbon-neutral biofuel. Furthermore, acetotrophic methanogenesis is the rate-limiting step in the biomethanation of biomass. Only two genera of acetotrophic methanogens (*Methanosarcina* and *Methanosaeta*) have been described for which much is known of one-carbon pathways leading from the methyl group of acetate to methane. Much less is known concerning electron transport pathways that control the distribution of electrons to methane coupled to energy conservation.

The overall research goal of this grant period is a more detailed understanding of redox reactions and the enzymes and proteins that direct and regulate the flow of electrons in acetotrophic methanogens.

Significant achievements (2017-2020):

- Biochemical and physiological characterization of the first archaeal flavodoxin to be investigated.
- Original discovery of respiratory growth in methanogens.
- Original discovery of ferredoxin- and $F_{420}H_2$ -dependent, electron-bifurcating, heterodisulfide reductases from the domains *Bacteria* and *Archaea*, and characterization of the archetype.
- Elucidation and characterization of key electron transport reactions driving $Fe(III)$ -dependent reverse methanogenesis.

Science priorities for the next year (2020-2021):

- Atomic resolution structure of the acetyl-CoA decarbonylase/synthase multienzyme complex.
- Mechanistic understanding of the coenzyme F_{420} electron bifurcating complex HdrA2B2C2.
- Mechanistic understanding of ferredoxin disulfide reductases from the domain *Archaea*.
- Biochemical and physiological characterization of flavodoxins from acetotrophic methanogens.

My major scientific area(s) of expertise is: Biochemistry and physiology of anaerobic microbes.

To take my project to the next level, my ideal collaborator would have expertise in: Advanced spectroscopic methods, cryo-electron microscopy, x-ray crystallography.

Publications supported by this project:

1. Ferry JG (2020) *Methanosarcina acetivorans*: A model for mechanistic understanding of acetoclastic and reverse methanogenesis. *Front. Microbiol.* 11(1806). 10.3389/fmicb.2020.01806.
2. Prakash D, et al. (2019) Structure and function of an unusual flavodoxin from the domain *Archaea*. *Proc. Natl. Acad. Sci. U. S. A.* 116(51):25917-25922.
3. Prakash D, Chauhan SS, & Ferry JG (2019) Life on the thermodynamic edge: Respiratory growth of an acetotrophic methanogen. *Sci. Adv.* 5(8):1-6.
4. Guan Y, et al. (2019) Comparative genomics of the genus *Methanohalophilus*, including a newly isolated strain from kebrit deep in the Red Sea. *Front. Microbiol.* 10. 10.3389/fmicb.2019.00839.
5. Yan Z & Ferry JG (2018) Electron bifurcation and confurcation in methanogenesis and reverse methanogenesis. *Front. Microbiol.* 9:1-10. 10.3389/fmicb.2018.01322.
6. Prakash D, et al. (2018) Towards a mechanistic and physiological understanding of a ferredoxin:disulfide reductase from the domains *Archaea* and *Bacteria*. *J. Biol. Chem.* 293:9198-9209.
7. Holmes DE, et al. (2018) Electron and proton flux for carbon dioxide reduction in *Methanosarcina barkeri* during direct interspecies electron transfer. *Front. Microbiol.* 9:1-11.
8. Yan Z, Wang M, & Ferry JG (2017) A Ferredoxin- and $F_{420}H_2$ -dependent, electron-bifurcating, heterodisulfide reductase with homologs in the domains *Bacteria* and *Archaea*. *mBio* 8:e02285-02216. doi: 10.1128/mBio.02285-02216.
9. Jasso-Chavez R, Diaz-Perez C, Rodriguez-Zavala JS, & Ferry JG (2017) Functional role of MrpA in the MrpABCDEF Na⁺/H⁺ antiporter complex from the archaeon *Methanosarcina acetivorans*. *J. Bacteriol.* 199:e00662-00616.
10. Guan Y, Haroon MF, Alam I, Ferry JG, & Stingl U (2017) Single-cell genomics reveals pyrrolysine-encoding potential in members of uncultivated archaeal candidate division MSBL1. *Environ. Microbiol. Rep.* 9:404-410.
11. Nazem-Bokae H, Gopalakrishnan S, Ferry JG, Wood TK, & Maranas CD (2016) Assessing methanotrophy and carbon fixation for biofuel production by *Methanosarcina acetivorans*. *Microb. Cell Fact.* 15:10.
12. Yan Z, Joshi P, Gorski CA, & Ferry JG (2018) A biochemical framework for anaerobic oxidation of methane driven by Fe(III)-dependent respiration. *Nat. Commun.* 9:1642.
13. Vullo D, Kumar RSS, Scozzafava A, Ferry JG, & Supuran CT (2018) Sulphonamide inhibition studies of the beta-carbonic anhydrase from the bacterial pathogen *Clostridium perfringens*. *J. Enzyme Inhib. Med. Chem.* 33:31-36.
14. Nazem-Bokae H, Yan Z, Maranas CD, & Ferry JG (2018) The biochemistry and physiology of respiratory-driven reversed methanogenesis. *Methane Biocatalysis: Paving the Way to Sustainability*, eds Kalyuzhnaya MG & Xing X-H (Springer International Publishing AG, Cham, Switzerland), pp 183-197.
15. Grisewood MJ, Ferry JG, & Maranas CD (2018) Computationally exploring and alleviating the kinetic bottlenecks of anaerobic methane oxidation. *Front. Environ. Sci.* 6:1-18. 10.3389/fenvs.2018.00084.
16. Soo VW, et al. (2016) Reversing methanogenesis to capture methane for liquid biofuel precursors. *Microb. Cell Fact.* 15:11. doi: 10.1186/s12934-016-10397-z.
17. Mueller TJ, et al. (2015) Methane oxidation by anaerobic archaea for conversion to liquid fuels. *J. Ind. Microbiol. Biotechnol.* 42:391-401.
18. Kumar AK, Kumar RS, Yennawar NH, Yennawar H, & Ferry JG (2015) Structural and biochemical characterization of a ferredoxin:thioredoxin reductase-like enzyme from *Methanosarcina acetivorans*. *Biochemistry* 54:3122-3128. [Click here to enter text.](#)

Redox-regulation of electron flow in an anaerobe

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Overall research goals:

Our goal is to understand how anaerobic bacteria regulate and control intracellular electron flow. By understanding how these organisms control electron flow, we hope to uncover new ways to increase the release of energy-rich compounds. We recently realized that we can manipulate the redox state of the cytoplasm in the phototrophic anaerobe *Rhodospseudomonas palustris* (*Rpal*) by growing cells at different light intensities. Under high light, the cytoplasm is relatively reduced, and at low light, it is relatively oxidized. Using thiol-reactive chemical probes and activity-based proteomics, we found that this change in the redox state of the cytoplasm also alters the redox state of key metabolic enzymes, including nitrogenase. Thiol-based modification of enzymes in response to redox shifts is a powerful mechanism to regulate enzyme activity posttranslationally, and this led us to hypothesize that changes in the redox status of proteins is a posttranslational regulatory mechanism that *Rpal* uses to adjust intracellular electron flow. In this project period, we proposed the following goals to test this central hypothesis.

Goal 1. To test the hypothesis that changes in the redox state of *Rpal* proteins happens rapidly in response to changes in light intensity and this is reflected in rapid changes in the activities of redox-sensitive enzymes.

Goal 2. To test the hypothesis that there are specific redox-sensitive cysteine residues in nitrogenase and in proteins associated with nitrogenase that modulate the rate at which it catalyzes the conversion of N_2 to NH_3 and H_2 .

Goal 3. To test the hypothesis that *Rpal* has thioredoxins and glutaredoxins that mediate redox-responsive modifications of proteins in response to low light.

Significant achievements (years of current funding, 2019-2021):

In trying to determine the role of redox regulation in controlling electron flow to different metabolic modules of *Rpal*, we have learned the following in the past year:

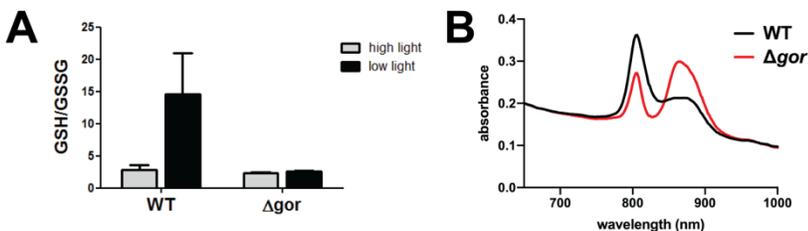


Fig. 1. (A) The ratio of GSH/GSSG increases under low light intensity, and this is dependent on expression of glutathione oxidoreductase (*gor*). (B) Absorbance spectra of intact cells show that a *gor* mutant is unable to synthesize a redox-regulated LH complex (absorbance at 800 nm), and instead only produces LH complexes seen in high light (absorbance at 800 and 860 nm).

transmitting this redox signal under low light intensities.

We found that low light cells had a 3-fold higher ratio of GSH/GSSG than high light cells. The increased

1. Light intensity alters the GSH/GSSG ratio in *Rpal*

We believe that under low light intensities electron flux decreases, leading to more oxidized Trx and Grx. This in turn leads to oxidation of key enzymes involved in electron flow in the cell, a form of posttranslational modification that allows cells to adjust their metabolism to lower light intensities. Mutational analysis suggested that there may be a role for a glutaredoxin and glutathione in

ratio was dependent on the activity of Gor, a glutathione oxidoreductase, because this ratio did not increase in a *gor* mutant exposed to low light (Fig. 1). A *gor* mutant was also unable to synthesize a light harvesting (LH) complex that predominates under low light and is known to be redox regulated (Fixen et al., 2019) (Fig. 1). These findings indicate that an increased ratio of GSH/GSSG is needed for *Rpal* to respond to low light intensity, and this increased ratio in cells exposed to low light intensity is achieved through the activity of Gor. These findings are exciting because glutathione has been shown to affect photosynthetic membrane expression in other purple bacteria. Additionally, a new thiol, N-Me-BSH, was identified in anoxygenic phototrophs belonging to the *Chlorobiaceae*. and increased amounts of reduced N-Me-BSH are observed under low light intensities, suggesting an increase in reduced thiols under low light intensity may be a general feature of anoxygenic photosynthesis. It is still unclear what purpose this response serves. We believe that our work will allow us to gain insight into why anoxygenic phototrophs increase the amount of reduced thiols in response to reduced light intensities.

2. Low light intensity may affect FeS cluster biosynthesis and assembly

We also found that deletion of a gene encoding a monothiol glutaredoxin, *grxD*, resulted in increased synthesis of a redox-regulated LH complex under high light intensity, which suggests that in the absence of GrxD the proteome becomes more oxidized. Consistent with this, we found that expression of *grxD* is repressed under low light intensity in all 17 *Rpal* strains tested so far (Fixen et al., 2019). Monothiol glutaredoxins have been shown to play a role in posttranslational modification of proteins by deglutathionylation of proteins in yeast. In this context, down-regulation of *grxD* under low light intensity could lead to an increase in glutathionylated proteins. To test this, we plan on determining if proteins we identified as altering their redox state under low light intensity are glutathionylated.

Despite this role in deglutathionylation, monothiol glutaredoxins have mostly been studied for their role in FeS biosynthesis. In both eukaryotes and prokaryotes, monothiol glutaredoxins have been implicated in FeS cluster assembly and trafficking and play a role in signaling iron availability to iron regulatory proteins. Since *grxD* is down-regulated in cells exposed to low light intensity, it is possible that FeS cluster trafficking may also be impaired, and some of our initial results suggest this may be true. Roughly 20% of the proteins identified as being more oxidized under low light intensity are predicted to bind FeS clusters or other metals, including Fer1, the main electron donor to nitrogenase, as well as nitrogenase itself. In fact, we see significant overlap in the number of genes down-regulated under low light intensities and down-regulated when we increase the concentration of iron in the medium. We believe this may point to an impairment in metal trafficking in cells grown under low light intensity, which may be a mechanism *Rpal* uses to slow its metabolism in response to fluctuating light conditions.

Science priorities for the next year (2020-2021):

- Determine the role of GSH and potentially glutathionylation in adapting to decreased light intensity.
- Determine the role of GSH in regulating nitrogenase activity and other metabolic units that are redox-sensitive.
- Determine if the monothiol glutaredoxin, GrxD, plays a role in glutathionylation and FeS cluster trafficking.

My major scientific area(s) of expertise is/are: Bacterial physiology, in particular, nitrogen fixation, anoxygenic photosynthesis, redox regulation; molecular biology; bacterial genetics.

To take my project to the next level, my ideal collaborator would have expertise in: Biochemistry, EPR, proteomics.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years]:

Fixen, K. R., Oda, Y., and Harwood, C.S. (2019). Redox regulation of a light-harvesting antenna complex in an anoxygenic phototroph. *mBio* 10(6): e02838-19. DOI: 10.1128/mBio.02838-19

DNRA: Catalyzing Multi-Electron Reductions Using a Pentaheme Scaffold

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Overall research goals:

Pentaheme cytochrome *c* nitrite reductase (NrfA) catalyzes the remarkable six-electron reduction of NO_2^- to NH_4^+ . Currently, there are several unanswered questions concerning the precise molecular mechanism of NrfA and the exact electron flow through this intriguing enzyme. The long-term goals of this project are to (i) ascertain the precise enzymatic mechanism of NrfA, (ii) elucidate the strategy for the storage and flow of electrons, and (iii) determine how the flow of electrons is regulated. The specific objectives for this two-year proposal are to investigate key NrfA protein-protein interactions (including interactions with its physiological donor NrfH), demonstrate stepwise reduction of NrfA, and initiate mechanistic studies. To accomplish these objectives, we will employ a synergistic combination of biochemical, kinetic, spectroscopic, and electrochemical methods to trap and interrogate reaction intermediates. We will focus our studies on the NrfA enzyme of *Geobacter lovleyi*, a DNRA bacterium identified for its environmental relevance and, as time permits, we will integrate the *in vitro* mechanistic studies with *in vivo* studies to interpret the data within the biological context. Successful completion of this project will provide insight into how NrfA stores and regulates the flow of electrons, and it will also lay the foundation for subsequent detailed mechanistic studies to ascertain how this unique pentaheme enzyme orchestrates the challenging multi-electron and multi-proton reduction of NO_2^- to NH_4^+ .

Significant achievements (2019-2021):

- We optimized culturing conditions with acetate and nitrate to maximize growth yields of *G. lovleyi* during DNRA. We also developed a heterologous expression system for *G. lovleyi* NrfA in *Shewanella oneidensis* MR-1, and a purification protocol that involves the use of Strep-tag II affinity and size exclusion chromatography.
- We developed a robust NrfA activity assay based on literature precedent. *G. lovleyi* NrfA expressed and purified from *S. oneidensis* had a calculated rate constant (k_{cat}) and Michaelis constant (K_M) that agree with published values.
- We performed spectroscopic characterizations of the enzyme including UV-Vis, EPR, and dynamic light scattering. While the UV-Vis and EPR data are consistent with the previously characterized NrfA homologs, dynamic light scattering provided evidence that *G. lovleyi* NrfA is a monomer in solution up to concentrations as high as $\sim 300 \mu\text{M}$.
- We succeeded in the quantitative, EPR-monitored redox titration of NrfA using Ti(III) citrate, and we demonstrated that we can reduce NrfA stoichiometrically. We also generated EPR simulations of the WT NrfA in its oxidized resting state with heme 1 in the high-spin state and of the cyanide

bound form where heme 1 is low-spin. Our ongoing simulations have shed light on the exchange interactions between hemes 1, 3, and 4. We were also able to demonstrate that either heme 4 or 5 is reduced first.

- We generated an interface mutant by altering its interface salt bridges to introduce charge-to-charge repulsion and by substituting the hydrophobic interface residues with hydrophilic residues to increase solubility. The rationale for generating these mutants was to prevent productive NrfA-NrfA interactions, thereby inhibiting electron transfer at the dimer interface.
- We solved the crystal structure of the *G. lovleyi* NrfA at 2.55 Å resolution. The structure revealed a dimer fold and heme configuration consistent with other structurally characterized NrfA homologs. Surprisingly, the *G. lovleyi* NrfA features an arginine residue in the active site region that would be otherwise occupied by Ca²⁺ in other structurally characterized NrfA homologs. Site-directed mutagenesis of this arginine to lysine, glutamine, or alanine resulted in variants that are no more than 3% active compared to the WT enzyme, suggesting that this arginine is critical to catalysis.
- We performed amino acid sequence alignment of 445 refined NrfA sequences and identified bacteria with NrfA proteins carrying the critical arginine (Arg) residue found in *G. lovleyi* NrfA. This finding suggests a novel subclass of Arg-containing, Ca²⁺-independent NrfA enzymes. The alignment helped us identify diagnostic sequences specific to both Ca²⁺-dependent and Ca²⁺-independent NrfA proteins. We constructed a phylogenetic tree from the refined alignment and identified 4 separate nodes marking the emergence of Arg-containing NrfA enzymes. The independent appearance of these branches provided evidence for the convergent evolution of this new subclass of enzymes in four groups of bacteria. From the phylogenetic analysis, we inferred that the emergence of Ca²⁺-independent NrfA proteins may allow these bacteria to ammonify nitrate and nitrite in environments where Ca²⁺ is limited or tightly controlled.

Science priorities for the next year (2020-2021):

The specific goals of this 2-year research proposal are to:

Goal 1: Characterize key NrfA protein-protein interactions

- A. Clone, express, and purify NrfH
- B. Characterize NrfA-NrfH interactions and ascertain the impact of these interactions
- C. As appropriate, generate, clone, express, and purify linked NrfA-NrfA and NrfA-NrfA* dimers

Goal 2: Determine the flow of electrons to the active site

- A. Demonstrate systematic stepwise reduction of hemes in NrfA
- B. Determine NrfA heme redox potentials

Goal 3: Elucidate the detailed mechanism of NO₂⁻ reduction

- A. Clarify the role of the conserved Arg residue near the active site in this subclass NrfA enzymes
- B. Characterize intermediates in the reaction cycle

Major scientific areas of expertise are: Hegg: Role of metals in biological systems; mechanistic enzymology. Lehnert: spectroscopy and simulation, quantum-chemical calculations. Reguera: Biological electron transfer, nutrient cycling.

To take my project to the next level, my ideal collaborator would have expertise in: protein film voltammetry to assess the redox potentials of the various hemes in both WT *G. lovleyi* NrfA and amino-acid variants. To address this issue, we recently established a collaboration with Prof. Sean Elliott at Boston University.

Publications supported by this project:

1. J. Campeciño, S. Lagishetty, Z. Wawrzak, V. Sosa Alfaro, N. Lehnert, G. Reguera, J. Hu, E.L. Hegg. "Cytochrome *c* nitrite reductase from the bacterium *Geobacter lovleyi* represents a new NrfA subclass." *J. Biol. Chem.* **2020**, *295*, 11455–11465. [DOI: 10.1074/jbc.RA120.013981]

DE-SC0010666 “Mechanistic Studies of Energy-Relevant Molybdenum Enzymes”

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Overall research goals:

We are building on the results obtained during the previous funding period and further examine the enzyme's reaction mechanism in order to gain a deeper understanding of the chemical mechanism by which formate is reversibly oxidized to CO₂ at the enzyme's molybdenum center. The overarching goal is to understand how the structure (both physical and electronic) of the molybdenum center relates to its reactivity. This will be accomplished through a combination of physicochemical methods, and we anticipate that this work will inspire the development of new catalytic systems that are both selective and efficient in accelerating the interconversion of energy-relevant one-carbon compounds. We will focus on the full-length FdsABG formate dehydrogenase from *Cupriavidus necator* as well as three “simple” formate dehydrogenases that possess only the molybdenum center and a single [4Fe-4S] iron-sulfur cluster: FdhF from *E. coli*, which has a selenocysteine ligand to the molybdenum; the FdhF from *Pectobacterium atrosepticum*, which has a cysteine rather than selenocysteine coordinated to the metal; and the C-terminal fragment of the FdsA subunit of the FdsABG from *C. necator*, which also has a cysteine coordinated to the metal.

We are also pursuing an extremely promising opportunity to collaborate with Dr. Cara Lubner at the National Renewable Energy Laboratory, to study NfnI (a bifurcating NAD⁺-dependent NADPH:ferredoxin oxidoreductase) from *Pyrococcus furiosus*. This enzyme is a member of a newly recognized family of enzymes that utilize flavin-based bifurcation to generate low-potential reducing equivalents for nitrogen fixation and carbon assimilation, an evolutionarily ancient mechanism of energy conservation in biology. The goal of this work is to examine the rapid-reaction kinetics of the reductive and oxidative half-reactions of NfnI. This will involve the reaction of oxidized NfnI_{ox} with NADPH using both stopped-flow spectrophotometry and freeze-quench EPR, and will include a pH dependence and solvent kinetic isotope effect study to ascertain the role of protonation/deprotonation events in the course of the reaction. We will also investigate the oxidative half-reaction, the reaction of reduced enzyme with NAD⁺ in the absence and presence of ferredoxin to ascertain the extent to which the two oxidation events of catalysis are tightly linked. Finally, we perform enzyme-monitored turnover experiments, monitoring enzyme directly in the course of turnover in the course of reaction with NADPH, NAD⁺ and ferredoxin. The results will be integrated with those of the above reductive and oxidative half-reaction studies to obtain a detailed kinetic picture of turnover.

Significant achievements (2019-2020):

- Have demonstrated the ability of FdsABG to reduce CO₂ using NADH as reductant, and shown that the reaction can be driven by an NADH-regenerating system.
- Have collaborated to show that the Mo-containing sulfite oxidase reduces nitrite to NO under physiological conditions.
- Have collaborated to determine the reduction potentials of both the CO dehydrogenase of *O. carboxidovorans* and the FdsABG formate dehydrogenase.
- Have determined the crystal structure of the FdsBG fragment of the *C. necator* formate dehydrogenase and examined the kinetics of its reduction by NADH.

Science priorities for the next year (2020-2021):

- Purify the SeCys-containing *E. coli* and Cys-containing *P. atrosepticum* FdhF formate dehydrogenases and characterize them both crystallographically and kinetically.
- Pursue mechanistic studies of the FdsDABG formate dehydrogenase from *C. necator*.
- Undertake collaborative CryoEM studies of FdsDABG.
- Examine the rapid reaction kinetics of NfnI with its substrates, NADPH, NAD⁺ and ferredoxin.

My major scientific area(s) of expertise is/are: enzyme reaction mechanism; rapid reaction kinetics; protein structure/function; EPR spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: purification and characterization of bifurcating flavoproteins.

Publications supported by this project [Supported by DOE DE-SC0010666]:

Yu, X., Niks, D., Ge, X., Liu H., Hille, R., and Mulchandani, A. (2019) Synthesis of Formate from CO₂ Gas Catalyzed by an O₂-tolerant NAD-Dependent Formate Dehydrogenase and Glucose Dehydrogenase. *Biochemistry* 58, 1861-1868.

Bender, D., Kaczmarek, A.T., Niks, D., Hille, R., Schwarz, G. (2019) Mechanism of nitrite-dependent NO synthesis by human sulfite oxidase. *Biochem. J.*, 476, 1805-1815.

Walker, L.M., Li, B., Niks, D., Hille, R. and Elliott, S.J. (2019) Deconvolution of reduction potentials of formate dehydrogenase from *Cupriavidus necator*, *J. Biol. Inorg. Chem.* 24, 889-898.

Kalimuthu, P., Petitgenet, M., Niks, D., Dingwall, S., Hille, R., and Bernhardt, P.V. (2020) The oxidation-reduction and electrocatalytic properties of CO dehydrogenase from *Oligotropha carboxidovorans*. *Biochim. Biophys. Acta* 1861, #148118.

Young, T., Niks, D., Hakopian, S., Tam, T.K., Yu, X., Hille, R. and Blaha, G. (2020) Crystallographic and kinetic analyses of the FdsBG subcomplex of the cytosolic formate dehydrogenase FdsABG from *Cupriavidus necator*. *J. Biol. Chem.*, in press

Hille, R., Young, T., Hakopian, S., Tran, T.K., Yu, X., Mulchandani, A., and Blaha, G. (2020) Structure: function studies of the cytosolic, Mo- and NAD⁺-dependent formate dehydrogenase from *Cupriavidus necator*, *Inorganics* 8, #41 (doi:10.3390/inorganics8070041).

Towards the Mechanism of N₂ Fixation by Nitrogenase

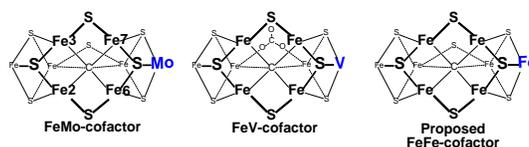
Brian Hoffman, Principal Investigator

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Overall research goals:

[Nitrogenases are the microbial enzymes responsible for the biological reduction of N₂ to two molecules of NH₃. There are three known isozymes of nitrogenase, named for the metal ion *M*, incorporated into their active-site cofactor; Mo-nitrogenase, V-nitrogenase, and Fe-nitrogenase. Mo-nitrogenase is the most phylogenetically distributed and the V- and Fe-nitrogenases are, respectively, expressed under Mo-, or Mo- and V-, depleted growth conditions. Mo-nitrogenase is the best characterized of the three, although V- and Fe-nitrogenases have garnered increased attention in recent years both for their unique features and for comparisons and contrasts with Mo-nitrogenase. An overarching aim of the work of our research team, involving Seefeldt, Dean, and Raugei, is to gain molecular level insights into the mechanism of N₂ reduction by the three nitrogenase variants. A particular focus of the work supported by the DOE is to gain insights into the mechanism of reduction of other, typically energy relevant, small compounds, including C₂H₂, CO, and CO₂.



Structures of the FeMo-cofactor of Mo-nitrogenase, FeV-cofactor of V-nitrogenase, and proposed structure of the FeFe-cofactor of Fe-nitrogenase. View is looking down on the Fe₂, 3, 6, 7 face

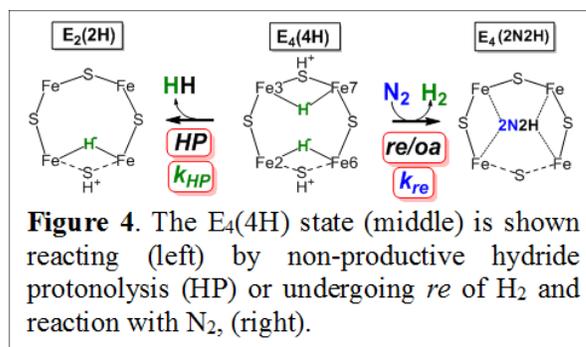
Significant achievements ([2018-2022]):

Mechanism of Fe- and V-Nitrogenases: We have completed the demonstration that all three nitrogenases exhibit the *reductive-elimination/oxidative-addition (re/oa)* mechanism for N₂ binding and activation, which has been established by our team, with the attendant limiting stoichiometry of H₂/N₂ → 1 as P_{N₂} → ∞. The results further provide a comparison of key kinetic parameters for the three nitrogenases, thus providing a complete picture of the differences in reactivity of the catalytically central E₄(4H) intermediate among the three.

The isoform reactivities are well-described by the competition between the productive (rate-constant, k_{re}) and non-productive (k_{HP}) reactions of the E₄(4H) Janus intermediate illustrated in Fig 4. Analysis reveals that k_{HP} for the two alternative nitrogenases are comparable to that for the Mo variant, whereas k_{re} for V-nitrogenase is three-fold smaller than for the Mo form, and that for Fe-nitrogenase is ten-fold smaller.

Electronic Properties of V-Nitrogenase and its Intermediates: Preliminary studies have revised the long-held view that the resting-state cofactor of this isozyme has $S = 3/2$. They have further suggested that vanadium may be in the V(III) oxidation state throughout the catalytic cycle.

CO as Substrate and as Inhibitor of H⁺ Reduction for the Three Nitrogenase: We presented a comparative study of the reactivity of the three isozymes with CO, examining CO both as a substrate and as an inhibitor of H⁺ reduction. For Mo-nitrogenase, there is neither detectable reduction of CO nor inhibition of H⁺ reduction. V-nitrogenase shows CO reduction and inhibition of H⁺ reduction that depends on the CO partial pressure; ethylene (C₂H₄) is the major reduction product with a maximum specific activity of ~7.5 nmol C₂H₄/nmol VFe protein/min at 1 atm CO. Fe-nitrogenase is shown to



reduce CO to methane (CH₄) as the major product with a specific activity of 4.8 nmol CH₄/nmol FeFe protein/min at 0.05 atm CO. At higher P(CO), CH₄ production by Fe-nitrogenase progressively declines down to ~10% of the maximal rate under one atm CO. H⁺ reduction, as well as total electron flow through Fe-nitrogenase, are inhibited as P(CO) increases, both plateauing at ~40% inhibition.

To explore the differences in the cofactor environment among the three isozymes, we carried out a structural alignment of the Mo-, V-, and Fe-nitrogenases. By focusing on residues having variation in at least one isozyme, we identified variations of side chain size, charge, and H bonding ability. Interestingly, much of the variation is near the charged homocitrate portion of the cofactor, with many of the residues sufficiently close for interactions with its oxygen atoms. We also considered how alternative pathways from CO to different products might be favored in the different isozymes.

The Reduction of Substrates by Nitrogenases: This *Chemical Reviews* article recounted recent progress, in particular the work of our team, in addressing how nitrogenase catalyzes the reduction of an array of substrates. New insights into the mechanism of N₂ and proton reduction, derived primarily through our team's efforts, were first considered. This was followed by a summary of recent gains in understanding the reduction of a number of other nitrogenous compounds not considered to be physiological substrates. Progress in understanding the reduction of a wide range of C-based substrates, including CO and CO₂, was also discussed, and remaining challenges in understanding nitrogenase substrate reduction were considered.

Science priorities for the next year (2020-2021):

- We will continue to advance our understanding of the N₂ activation mechanism at the active site of all three nitrogenases. We seek to combine further spectroscopic analysis with theory to answer some key outstanding questions, such as how are electrons accumulated stepwise on FeMo-co in going from the E₀ to E₄ state, what are the differences in reactivity of the hydrides in the E₄ states in the three nitrogenases, and what is the transition state for N₂ binding and H₂ release for all three nitrogenases? Likewise we shall extend our study of the active-site cofactor of V-nitrogenase.
- A particular focus will be to extend the high-flux kinetic approach to other substrates for all three isozymes, beginning with C₂H₂ as a model substrate that can undergo two-electron reduction at multiple E_n stages (n = 2-4) in competition with hydride protonolysis, and does not undergo the *re*⁻ process at all. We will turn this foundational work to gaining mechanistic insights into the mechanism of CO and CO₂ reduction by all three nitrogenases.

My major scientific area(s) of expertise is/are: Advanced paramagnetic resonance spectroscopies: electron transfer and chemical kinetics; inorganic reaction mechanisms.

To take my project to the next level, my ideal collaborator would have expertise in: Fields that complement the expertise of our primary team members and the expert collaborators we sometimes work with.

Publications supported by this project [Enter Publications Supported by This Grant/FWP]:

1. D.F. Harris; D.A. Lukoyanov; H. Kallas; C. Trncik; Z-Y. Yang; P. Compton; N. Kelleher; O. Einsle; D.R. Dean; B.M. Hoffman; L.C. Seefeldt, "Mo-, V-, and Fe-Nitrogenases Use a Universal Eight-Electron Reductive-Elimination Mechanism to Achieve N₂ Reduction", *Biochemistry*, 2019, 58, 3293-3301, DOI: 10.1021/acs.biochem.9b00468
2. L.C. Seefeldt; Z-Y. Yang; D.A. Lukoyanov; D.F. Harris; D.R. Dean; S. Rauegi; B.M. Hoffman, "Reduction of Substrates by Nitrogenases", *Chemical Reviews*, 2020, 120 (12), 5082-5106, DOI: 10.1021/acs.chemrev.9b00556
3. A. Peng; M.C. Kung; M.O. Ross; B.M. Hoffman; H.H. Kung, "The Role of Co-ZSM-5 Catalysts in Aerobic Oxidation of Ethylbenzene", *Topics in Catalysis*, 2020, DOI: 10.1007/s11244-020-01305-z
4. D.F. Harris; E. Jimenez-Vicente; Z-Y. Yang; B.M. Hoffman; D.R. Dean; L.C. Seefeldt, "CO as a Substrate and Inhibitor of H⁺ Reduction for the Mo-, V-, and Fe-Nitrogenase Isozymes", *JACS*, 2020, submitted

MSU-DOE Plant Research Laboratory Subproject B: Integrating energy supply and demand in the biological solar panel

Gregg Howe, Christoph Benning, Federica Brandizzi, Danny Ducat, Sheng Yang He, Jianping Hu, Cheryl Kerfeld, David Kramer, Tom Sharkey, Berkley Walker, Co-PI(s)

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Overall research goals: The conversion and storage of the energy in sunlight into energy-dense organic molecules depends on a set of highly complex cellular processes that operate across multiple temporal and spatial scales. The overarching objective of Subproject B is to understand biological solar panels as a highly integrated, complex photochemical system and to work collaboratively across disciplines to improve photosynthetic efficiency through redesign of component parts. Thematic area **B1** focuses on understanding the biosynthesis and turnover of photosynthetic membranes within the chloroplast, and the functional interactions of this organelle with the endoplasmic reticulum required for photosynthetic processes. Theme **B2** explores the mechanisms underlying the connection between photosynthesis and photorespiratory metabolism, and how these linkages are modulated by environmental factors. **B3** seeks to better understand the role of carbonic anhydrases in carbon sensing and storage. **B4** uses engineered plant and cyanobacterial systems to modify carbon metabolism and partitioning, with the goal of understanding how carbon flux under natural conditions may constrain overall photosynthetic capacity. Finally, **B5** develops and implements new methodologies for measuring photosynthetic metabolism to better understand how primary energy capture reactions are coordinated with the energy demands of the biological solar panel.

Significant achievements (2019-2020):

- We found that three phosphatidic acid (PA) phosphatases in Arabidopsis are localized to the plastid envelope. Biochemical and genetic approaches were used to test the hypothesis that these enzymes are associated with different leaflets of the envelope and affect different pools of PA.
- We showed that a chloroplast inner envelope-associated rhomboid protein (RBL10) is proteolytically active and may mediate PA flipping through the inner envelope membrane. RBL10 is present in a large protein complex that includes acyl-carrier protein 4 (ACP4). Mutants defective in ACP4 have strong lipid phenotypes, suggesting a functional relationship between RBL10 and ACP4.
- We conducted a suppressor screen in the background of a thylakoid lipase (PLIP3)-overexpression line to identify mutations in the chloroplast export machinery for jasmonate (JA) precursors, as proxy for fatty acids. Several mutants were isolated and sequenced to identify the causal mutation. The first mutant identified was affected in a gene proposed to be involved in coordinating ABA and JA responses.
- We used a JA-hypersensitive mutant (*jazD*) of Arabidopsis as a new tool to study the rapid turnover of thylakoid membranes and associated remodeling of chloroplast lipids. Several mutants defective in JA-induced thylakoid turnover were identified in a genetic screen.
- We characterized several Arabidopsis Vap proteins (Vap27-1/27-3/27-4/27-6) that accumulate at the ER-chloroplast interface and found photosynthetic defects. We also identified and are starting to characterize several VAP-interacting proteins.
- We continued to study mechanistic connections between peroxisomes and photosynthetic metabolism. Specifically, we characterized genetic suppressors of the Arabidopsis photorespiratory mutant, *hpr1*. This work is providing insight into how the photorespiratory pathway interacts with plastid-to-nucleus retrograde signaling pathways involved in plant growth.
- Carbonic anhydrase (CA) mutants (*aca1*, *aca2*, *βca1*, *βca5*, *βca6*, *γca2* and *γcal1*) in Arabidopsis were isolated and characterized for growth and photosynthetic phenotypes. CA isoforms are being tested for subcellular localization and encapsulation in proteinaceous shells in collaboration with the Kerfeld lab.
- We discovered the likely source of unlabeled carbon that enters Calvin-Benson cycle metabolites during CO₂ labeling. We showed that glucose-6-phosphate (G6P) dehydrogenase contributes to a G6P shunt

during photosynthesis and that the gene encoding a G6P transporter is induced by high light. Results from flux labeling studies support the G6P shut hypothesis.

- We studied energy metabolism in Arabidopsis mutants in which carbon partitioning between growth and defense processes is systematically tuned through manipulation of JAZ repressors. We characterized suppressor mutants in which growth-defense tradeoffs are uncoupled. Most of these mutants contain null mutations in genes encoding the phyB photoreceptor or subunits of the Mediator complex. This work highlights the importance of signaling in the regulation of energy homeostasis during stress responses.
- Studies in cyanobacteria showed that the combination of heterologous electron (cytochrome P450) and carbon (sucrose) sinks can contribute additively to photosynthetic performance, including increased quantum efficiency of PSII, increased electron transport rates, and reduced PSI P700⁺ under light pulses. Installment of these two heterologous sinks can partially compensate for the loss of other photoprotective mechanisms including the flavodiiron proteins (Flv1/3) and alternative respiratory terminal oxidase activity. These results will inform efforts to engineer metabolic pathways into cyanobacteria without compromising fitness.

Science priorities for the next year (2019-2020):

- Determine the sub-chloroplast location of PA phosphatases and develop a functional model to explain their location. We will also explore the functional relationship between RBL10 and ACP4 to explain lipid phenotypes of RBL10-deficient mutants.
- Further characterize suppressor mutants with the goal of identifying inner envelope transporters for fatty acid-derived compounds. In parallel, we will perform an RNA-seq time course experiment to identify genes involved in lipid remodeling during thylakoid membrane disassembly.
- Photosynthetic characterization of CA-deficient mutants, with emphasize on *βca5* and higher-order CA mutants. *βCA1* and *βCA5* will be prioritized for sub-cellular localization, structural, and kinetic characterization. In addition, a suppressor screen of *βca5* will be conducted.
- We will characterize the *vaps* quadruple mutant, as well mutants defective in various VAP-interacting proteins.
- Further characterization of *hpr1* suppressors and continued investigation of the role of photorespiration in plant interaction with the biotic environment, in collaboration with Project A.
- Determine role of G6P dehydrogenase in providing carbon to the Calvin-Benson cycle, and in releasing CO₂ that occurs during photosynthesis. In parallel, we will determine the role of the G6P transporter in regulating carbon export from the Calvin-Benson cycle. We will assess how photosynthetic electron transport is regulated to cope with triose phosphate use limitation in a stochastic environment.
- Determine the effect of MYC regulators on carbon partitioning to various metabolic sinks in Arabidopsis. We will begin to investigate the mechanism by which CDK8 modulates energy metabolism, as well as the influx of carbon into the shikimate pathway during growth-to-defense transitions.
- Having completed a genetic screen for putative target regulatory genes involved in source/sink energy balance in *S. elongatus* PCC 7942, promising lead candidates with established roles in light-sensing, circadian balancing of metabolism, or unknown function (5 candidate genes) will be characterized for broader roles in energy balancing.
- Determine the long-term role of photorespiration in photoprotection and nitrate assimilation (in collaboration with project A). We will use flux approaches to investigate how photorespiration integrates into central metabolism and how it is adapted to elevated temperatures in extremophiles.

Our major scientific areas of expertise are: biochemistry, genetics, plant physiology, molecular and cell biology. To take my project to the next level, my ideal collaborator would have expertise in: Structural biology and modeling.

Publications supported by this project [2019-2020] :

1. A.A. Lavell, C. Benning, "Cellular organization and regulation of plant glycerolipid metabolism." Plant & Cell Physiol, 60:1176-1183 (2019). doi: 10.1093/pcp/pcz016

2. A. Lavell, J.E. Froehlich, O. Baylis, A. Rotondo, C. Benning, "A predicted plastid rhomboid protease affects phosphatidic acid metabolism in *Arabidopsis thaliana*." *Plant J*, 99:978-987 (2019). doi: 10.1111/tpj.14377
3. B.J. Walker et al., "Flexibility in the energy balancing network of photosynthesis enables safe operation under changing environmental conditions." *Plants*, 9(3): p. 301 (2020).
4. J. Li, S. Tietz, J. Cruz, D. Strand, Y. Xu, J. Chen, D. Kramer, J. Hu, "Photometric screens identified *Arabidopsis* peroxisome proteins that impact photosynthesis under dynamic light conditions." *Plant J*, 97: 460-474, (2019).
5. J. Li, S.M. Weraduwege, A.L. Preiser, S. Tietz-Rhodes, S.E. Weise, D.D. Strand, J.E. Froelich, D.M. Kramer, J. Hu, T.D. Sharkey, "A cytosolic bypass and G6P shunt in plants lacking peroxisomal hydroxypyruvate reductase." *Plant Physiol*, 180: 783-792 (2019).
6. A.M. McClain, T.D. Sharkey, "Triose phosphate utilization and beyond: from photosynthesis to end product synthesis." *J Exp Bot*, 70, 1755-1766 (2019).
7. A.M. McClain, T.D. Sharkey, "Building a better equation for electron transport estimated from chlorophyll fluorescence: Accounting for non-photosynthetic light absorption." *New Phytol*, 225, 604-608 (2020).
8. A.L. Preiser, N. Fisher, A. Banerjee, T.D. Sharkey, "Plastidic glucose-6-phosphate dehydrogenases are regulated to maintain activity in the light." *Biochem J*, 476, 1539-1551 (2019).
9. T. Ren, S.M. Weraduwege, T.D. Sharkey, "Prospects for enhancing leaf photosynthetic capacity by manipulating mesophyll cells morphology." *J Exp Bot*, 70, 1153-1165 (2019).
10. T. Sharkey, "Is triose phosphate utilization important for understanding photosynthesis?" *J Exp Bot*, 70, 5521-5525 (2019a).
11. T.D. Sharkey, "Discovery of the canonical Calvin-Benson cycle." *Photosyn Res*, 140, 235-252 (2019b).
12. T.D. Sharkey, "Emerging research in plant photosynthesis." *Emerging Topics in Life Sciences*, in press (2020).
13. T.D. Sharkey, A.L. Preiser, S.M. Weraduwege, L. Gog, "Source of ^{12}C in Calvin Benson cycle intermediates and isoprene emitted from plant leaves fed with $^{13}\text{CO}_2$." *Biochemical J*, in press (2020).
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Engineering a Functional Equivalent of Nitrogenase for Mechanistic Investigations of Ammonia Synthesis

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Overall research goals:

The overarching research goal of the project is to use NifEN of *Azotobacter vinelandii* as a mutational platform to construct partially defective or fully functional MoFe protein mimics for mechanistic investigations of ammonia synthesis by nitrogenase. Genetic methods (mutagenesis and homologous recombination) will be used to strategically reconstruct defective or functional mimics of MoFe protein, and biochemical (metal and enzymatic assays) and spectroscopic (EPR and XAS/EXAFS analyses) methods will be employed to monitor and analyze the (re)construction process. Success in generating partially defective variants on a NifEN template will facilitate capture of the reaction intermediates of N₂ reduction for mechanistic investigations of nitrogenase; whereas success in generating an active nitrogenase equivalent on a NifEN template will enable identification of all functional determinants for the catalytic activity of nitrogenase and provide a proof-of-concept for minimizing the essential *nif* gene set for future transgenic expression of nitrogenase via synthetic biology. Together, these efforts not only contribute to a better understanding of the mechanism of ammonia synthesis by nitrogenase, but also have the long-term potential in developing energy-efficient strategies for nitrogenase-based ammonia synthesis and generating modified enzymes with improved efficiency in ammonia or hydrogen production.

Significant achievements (2016-2020):

- The enzyme nitrogenase uses a suite of complex metal cofactors to reduce dinitrogen (N₂) to ammonia. Mechanistic details of this reaction remain sparse. During the last grant period, we reported a 1.83 Å crystal structure of the nitrogenase molybdenum-iron (MoFe) protein captured under physiological N₂ turnover conditions (see publication #1 below). This structure reveals asymmetric displacements of the cofactor belt sulfurs (S2B or S3A and S5A) with distinct dinitrogen species in the two αβ dimers of the protein. The sulfur-displaced sites are distinct in the ability of protein ligands to donate protons to the bound dinitrogen species, as well as the elongation of either the Mo-O5 (carboxyl) or Mo-O7 (hydroxyl) distance that switches the Mo-homocitrate ligation from bidentate to monodentate. These results highlight the dynamic nature of the cofactor during catalysis and provide evidence for participation of all belt-sulfur sites in this process. It also provides the guidance for our proposed mutational work of NifEN, a structural and functional homolog of MoFe protein.
- NifEN plays a crucial role in the biosynthesis of nitrogenase, catalyzing the final step of cofactor maturation prior to delivering the cofactor to MoFe protein (or NifDK), the catalytic component of nitrogenase. The difficulty in expressing NifEN, a complex, heteromultimeric metalloprotein sharing structural/functional homology with NifDK, is a major challenge in the heterologous expression of nitrogenase. During the last grant period, we reported the expression and engineering of *Azotobacter vinelandii* NifEN in *Escherichia coli* (see publication #3 below). Biochemical and spectroscopic analyses demonstrate the integrity of

the heterologously expressed NifEN in composition and functionality and, additionally, the ability of an engineered NifEN variant to mimic NifDK in retaining the matured cofactor at an analogous cofactor-binding site. This work represents an important step toward piecing together a viable pathway for the heterologous expression of nitrogenase and identifying variants for the mechanistic investigation of this enzyme.

Science priority for the next year (2020-2021):

- Restoring a catalytically active M-cluster site of NifEN that enables at least some N₂-reducing activity.

My major scientific area(s) of expertise is/are: Molecular biology, structural biology, biochemistry, bioinorganic chemistry.

To take my project to the next level, my ideal collaborator would have expertise in: Mössbauer spectroscopy.

Publications supported by this project:

1. Kang W, Lee CC, Jasniewski AJ, Ribbe MW, Hu Y. (2020) Structural evidence for a dynamic metallocofactor during N₂ reduction by Mo-nitrogenase. **Science** 368(6497):1381-1385. doi: 10.1126/science.aaz6748.
2. Hu Y, Ribbe MW. (2020) Special issue on nitrogenases and homologous systems. **Chembiochem** 21(12):1668-1670. doi: 10.1002/cbic.202000279.
3. Solomon JB, Lee CC, Jasniewski AJ, Rasekh MF, Ribbe MW, Hu Y. (2020) Heterologous expression and engineering of the nitrogenase cofactor biosynthesis scaffold NifEN. **Angew Chem Int Ed Engl** 59(17):6887-6893. doi: 10.1002/anie.201916598.
4. Jasniewski AJ, Lee CC, Ribbe MW, Hu Y. (2020) Reactivity, mechanism, and assembly of the alternative nitrogenases. **Chem Rev** 120(12):5107-5157. doi: 10.1021/acs.chemrev.9b00704.
5. Newcomb MP, Lee CC, Tanifuji K, Jasniewski AJ, Liedtke J, Ribbe MW, Hu Y. (2020) A V-nitrogenase variant containing a citrate-substituted cofactor. **Chembiochem** 21(12):1742-1748. doi: 10.1002/cbic.201900654.
6. Lee CC, Ribbe MW, Hu Y. (2019) Purification of nitrogenase proteins. **Methods Mol Biol** 1876:111-124. doi: 10.1007/978-1-4939-8864-8_7.

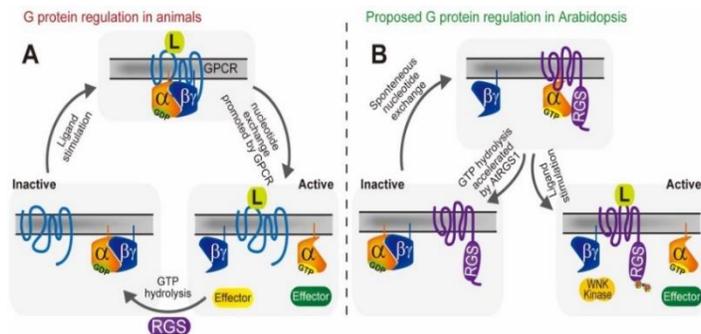
Arabidopsis Regulator of Signaling 1: a sensor for light fluctuation used to control photosynthesis efficiency

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Overall research goals (2018-2021 cycle): Figure 1B shows that the plant G signaling pathway has a different architecture. This different architecture is important in dynamic light environments, consistent with our modeling showing the emergent property of Dose-Duration Reciprocity conferring a shadow detector. We propose that this emergent property is a coping mechanism to



optimize the production of sugar in a light environment that has fluctuations both extended (e.g. predictable diurnal, weather) and transient (unpredictable sun spots, cloud cover). In other words, while energy conversion must tolerate an unstable energy source with regard to intensity and duration, it therefore must be highly regulated to maintain high fitness.

At the crux of this unusual signaling architecture is a chimeric protein, AtRGS1, a 7TM domain protein coupled to an RGS protein. The other feature of his architecture is that the central element, the G protein complex, is self-activating. For years, naturally we assumed that AtRGS1 accelerates the GTPase activity of the

Figure 1 The mechanism for regulation of the active state of plant G proteins is completely opposite to the mechanism in animals. In animals, the ligand (L, chartreuse) stimulates the stimulator (GPCR, blue), whereas in plants, the ligand inhibits the inhibitor (7TM RGS, purple). The plant Gα subunit spontaneously exchanges GDP for GTP when inhibition is relieved thus plants do not need GPCRs. The Gβγ dimer recruits a WNK kinase (gold) which then phosphorylates AtRGS1 causing it to be endocytosed. In both plants and animals, active G proteins interact with effectors (yellow and green) to control their activity (usually catalytic).

G protein until this inhibition is itself inhibited (by glucose). But this makes no sense energetically. Instead of accelerating G cycling as the main mechanism for keeping the G protein in the inactive state, we propose now that AtRGS1 stabilizes the G protein complex at

some point in the cycle, most likely the GDP bound and the GDP+Pi transition states. Specifically, we propose that D-glucose shifts the binding equilibrium from AtGPA1::AGB1 toward AtGPA1::AtRGS1, thus freeing up AGB1 (and its obligate partner AGG) to recruit WNK kinases to phosphorylate AtRGS1 which causes rapid endocytosis. The FRET data support this. This internalization of AtRGS1 physically uncouples AtRGS1 (G inhibitor) from AtGPA1 (the self-activating Gα subunit). We showed that this process is sensitive to dose and duration of glucose thus the predictions are that in the absence of glucose perception, control of photosynthesis efficiency in a *fluctuating* light environment will be lost. The photosynthesis phenomics data support this.

Significant Achievements of 2019-2020: While significant, to our disappointment, we learned that *in vitro* expression of AtRGS1 cannot yield sufficient amounts for the proposed structural studies (e.g. nanodisks in conjunction with NMR) despite extensive optimization over the last 2 years. However, we can get biochemical amounts and this has spurred great progress in a different area. We are thrilled to report that we have preliminary evidence for the long sought-after ligand to AtRGS1 is **UDP glucose** and the mechanism how fixed sugars are utilized to detect light dynamics for optimizing photosynthesis. We have a body of data ready for submission for publication that

supports the following scenario: Carbon fixation from photosynthesis which are sugars, primarily sucrose, are transported in the apoplast where sucrose meets the plasma membrane of distal cells. Then at the plasma membrane this photosynthate is converted to UDP-glucose by sucrose synthase SUS1 and/or SUS4 in a complex that includes AtRGS1, the heterotrimeric G protein, and at least one kinase (probably WNK1, 8, and/or 10). UDP-glucose induced conformational change activates G signaling which results in phosphorylation-dependent de-repression of the G protein complex by AtRGS1. The following rapid AtRGS1-dependent events are: 1) monomerization of AtRGS1 (seconds), 2) an increase in calcium (30 s), activation of MAPK cascade (4 min), and gene expression (30 minutes).

Science priorities for 2020-2021: The highest priority is to wrap up this UDP-glucose story in a high impact peer-reviewed publication.

My major scientific areas of expertise are: genetics, mathematical modeling, cell biology, plant physiology, signal transduction

To take my project to the next level, my ideal collaborator would have expertise in: expression of membrane proteins using *Pichia pastoris*.

Publications supported by BES:

- Yan, C, et al (2020) An intact heterotrimeric G-protein complex is required for the N-acyl ethanolamine-induced, transcriptionally-mediated chloroplast responses in developing Arabidopsis. *Plant Physiology (in press)*
- Ross-Elliott, TJ, Watkins J, Shan X, Lou F, Dreyer B, Tunc-Ozdemire M, Jia H, Yang J, Wu L, Trusov Y, Krysan P, Jones AM (2020) Biased signaling: Distinct ligand-directed plasma membrane signalosomes using a common RGS/ G protein core. *BioRxiv doi.org/10.1101/666578*
- Lou, F, Abramyan, TM, Jia, H, Tropsha, A, and Alan M. Jones (2019) An atypical heterotrimeric G α protein has substantially reduced nucleotide binding but retains nucleotide-independent interactions with its cognate RGS protein and G $\beta\gamma$ dimer. *J. Biological and Structural Dynamics Dec 23;1-15*
- Jia, H, Song, G, Werth, EG, Walley, JW, Hicks, LM and Jones, AM 2019 Receptor-like kinase phosphorylation of Arabidopsis heterotrimeric G-Protein G α -Subunit AtGPA1 *Proteomics* DOI: 10.1002/pmic.201900265
- Urano D, Leong R, Wu TY, Miura K, Jones AM Quantitative morphological phenomics of rice G protein mutants portend autoimmunity disease. *Developmental Biology* **457**: 83-90
- Escudero, V. et al (2018) A mitogen-activated protein kinase phosphatase 1 (MKP1) negatively regulates the production of reactive oxygen species during Arabidopsis immune responses. *The Plant Journal* **32**(4):464-478
- Liang Y, Zhao X, Jones AM, Gao Y G (2018) proteins sculpt root architecture in response to nitrogen in rice and Arabidopsis *Plant Science* **274**: 129-136
- Li, B, Urano, D, Mowrey, DD, Dokholyan, NV, Torres, MP, Jones, AM (2017) Tyrosine phosphorylation switching of a G protein substrate. *J. Biol Chem.* **293**(13):4752-4766.
- Tunc-Ozdemir, M., Liao, K-L, Jones, AM (2018) Long-distance communication in Arabidopsis involving a self-activating G protein. Feb 26 DOI: 10.1002/pld3.37
- Liao K-L, Melvin, CE, Sozzani R, Jones RD, Elston TC, Jones AM (2017) Dose-Duration reciprocity for G protein activation: Modulation of kinase to substrate ratio alters cell signaling. *PLoS One* **12**, e0190000
- Escudero, V et al (2017) Alteration of cell wall xylan acetylation triggers defensive responses that counterbalance the immune deficiencies of plants impaired in the β subunit of the heterotrimeric G protein. *Plant J* **92**(3):386-399
- Tunc-Ozdemir, M, Li, B, Jaiswal, DK, Urano, D, Jones, AM, Torres, MP (2017) Predicted functional implications of phosphorylation of regulator of G protein signaling protein in plants. *Front Plant Sci.* **8**:1456.
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- Tunc-Ozdemir, M, Jones, AM (2017) Ligand-induced dynamics of a heterotrimeric G protein-coupled receptor kinase complex *PLoS ONE* **12**: e0171854
- Liao, K-L, Jones, RD, McCarter, P, Tunc-Ozdemir, M, Draper, JA, Elston TC, Kramer, D, Jones, AM (2017) A shadow detector for photosynthesis efficiency. *J. Theoret. Biol.* **414**: 231-244

Electron Bifurcation and Pyrophosphate-Mediated Energy Conservation in *Syntrophus aciditrophicus*

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Overall research goals:

Our overall research goals are to understand how microorganisms conserve energy and grow using catabolic reactions that operate close to thermodynamic equilibrium and to elucidate the biochemical basis of the critically important microbial mutualism called syntrophy. Syntrophy is a near-equilibrium process that is the rate-limiting step in the conversion of organic matter to methane and carbon dioxide. A more in-depth understanding of syntrophy is essential to develop efficient biomethanation processes to convert organic wastes and crop residues into the energy-rich fuel, methane. We discovered that the syntrophic, fatty and aromatic acid degrader, *Syntrophus aciditrophicus*, uses a novel approach, an acetyl-CoA synthetase (Acs1), to synthesize ATP from acetyl-CoA. However, we know very little about how syntrophic bacteria reoxidize their reduced cofactors (NADH and reduced electron transfer flavoprotein (Etf_{red})) even though the reoxidation of reduced electron carriers is the critical process in syntrophy. The objectives of this project are to determine whether: (1) the reoxidation of NADH occurs by NADH-linked hydrogenases and formate dehydrogenases, (2) a sodium gradient is used to drive pyrophosphate synthesis, which can then be used by Acs1 to make ATP, and (3) the

Acs1 of *S. aciditrophicus* has an active site that prefers functioning in the ATP-forming direction.

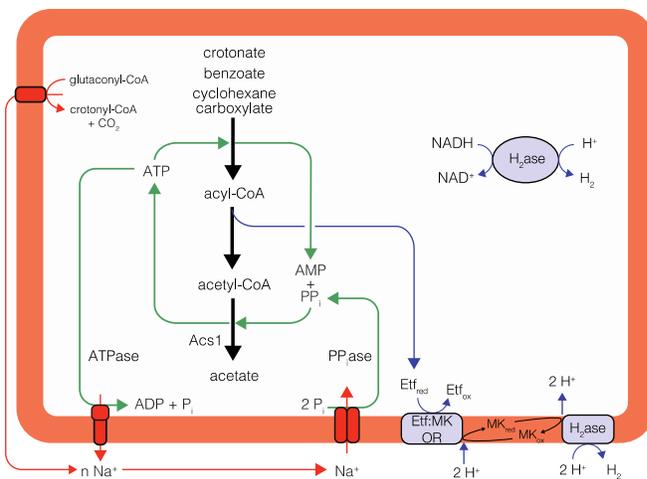


Figure 1. Bioenergetic model for pyrophosphate cycling, ATP synthesis by Acs1, and electron flow in *S. aciditrophicus*. Acs1 uses pyrophosphate made during substrate activation or by membrane-bound pyrophosphatases. The chemiosmotic energy needed for pyrophosphate synthesis and hydrogen production from Etf_{red} is generated by ATP hydrolysis by ATP synthase or glutacetyl-CoA decarboxylation. NADH is reoxidized by an NADH-linked hydrogenase.

Significant achievements (2018-2021):

- We have elucidated the biochemical basis for the mutualism called syntrophy.

Syntrophic bacteria generate NADH and

Etf_{red} during fatty and aromatic acid degradation. We have shown that both *S. aciditrophicus* and the syntrophic, fatty acid degrader, *Syntrophomonas wolfei*, use ferredoxin-independent hydrogenases (non-electron-bifurcating) to reoxidize NADH coupled to hydrogen production. Hydrogen production from hydrogenases that use only NADH is limited by thermodynamic constraints to a maximum of about 60 Pa. Thus, continual reoxidation of NADH requires continual hydrogen use, which explains, in part, the obligate requirement for a hydrogen-using partner such as a hydrogenotrophic methanogen. The second key protein is an Etf:menaquinone oxidoreductase that serves as the membrane input complex for electrons derived from the oxidation of acyl-coenzyme A (CoA) intermediates. We identified a protein conduit of electron flow from acyl-CoA dehydrogenase to the Etf:menaquinone oxidoreductase in *S. aciditrophicus* and *S. wolfei*. The Etf:menaquinone oxidoreductase is predicted to reduce menaquinone. Reduced menaquinone can then transfer electrons to membrane-bound hydrogenases or formate dehydrogenases. Hydrogen production from electrons derived from acyl-CoA oxidation also requires low hydrogen partial pressures. Thus, continual oxidation of acyl-CoA intermediates requires continual hydrogen use and is the second biochemical process that explains the obligate requirement for a hydrogen-using partner.

- The flavin-containing beta subunits of the non-bifurcating NADH-dependent hydrogenases from *S. aciditrophicus* and *S. wolfei* share several features with the flavin-containing beta subunits from non-bifurcating NADH-dependent enzymes such as NADH:quinone oxidoreductases and formate dehydrogenases. The beta

subunits of these non-bifurcating NADH-dependent enzymes differ from electron-bifurcating hydrogenases and formate dehydrogenases in the number of [Fe-S] centers and in conserved residues near predicted cofactor binding sites. These differences can be used to distinguish members of these two groups of enzymes and may be relevant to the differences in ferredoxin-dependence and ability to mediate electron-bifurcation.

- *Syntrophus aciditrophicus* uses the same enzymes in a reversible manner to degrade but also to make aromatic and alicyclic compounds. Energy is conserved by the unique synthesis of ATP from AMP and pyrophosphate by an AMP-forming, acetyl-CoA synthetase, *SaAcs1*. Reversibility means that the core reactions operate at near-equilibrium conditions, including those involved in ATP formation as expected from catabolic reactions that are close to thermodynamic equilibrium. Reversibility also means that the metabolic pools of syntrophic metabolizers are susceptible to environmental perturbations, which is consistent with the observed inhibition of syntrophic metabolism by the accumulation of their end products of metabolism, acetate, hydrogen, and formate. This work provides a mechanistic understanding of how microorganisms grow using catabolic reactions that operate close to thermodynamic equilibrium.

- In collaboration with Dr. Derek Lovley's group, *S. aciditrophicus* has been shown to produce electrically conductive pili and grow via direct interspecies electron transfer. The *S. aciditrophicus* type IV pilin had the same diameter and conductance as the electrically conductive pilin in *Geobacter sulfurreducens*. Thus, interspecies electron transfer during syntrophy can involve pilin-mediated direct electron transfer in addition to hydrogen and formate transfer.

- Alanine substitutions were introduced at six active-site residues of *SaAcs1* that are hypothesized to play a key role in stabilization of ATP. The *SaAcs1* variants were initially tested for activity in the AMP-forming directions where the substitutions at Ser²⁷³ and Arg⁵³⁶ resulted in no AMP-forming activity. Substitutions at Gly³⁹⁵, Thr⁴²⁰, and Trp⁴²¹ resulted in lower AMP-forming activity compared to wildtype *SaAcs1*.

Science objectives for 2020-2021:

- We will use homology modeling/docking and X-ray crystallography to determine the position of amino acid side chains in the *SaAcs1* active site and to identify the conformation of *SaAcs1* in the presence and absence of ATP and/or acetate. Furthermore, we will continue to characterize the effects of amino acid substitutions hypothesized to be important for interactions with ATP in the active site of *SaAcs1*.
- We will show that ATP hydrolysis by the membrane-bound ATP synthase generates a sodium gradient that can be used to drive pyrophosphate synthesis. Purified *Acs1* and acetyl-CoA will be added to the membrane system to show that sodium ion gradient-driven pyrophosphate synthesis can be used for ATP production from AMP and acetyl-CoA by *Acs1*, verifying our bioenergetics model (Fig.1).

Our scientific area(s) of expertise are: microbial physiology, anaerobic protein crystallization, x-ray crystallography, transcription regulation, molecular biology

To take our project to the next level, our ideal collaborator would have expertise in: protein computational modeling and docking, systems biology, membrane protein crystallography.

Publications supported by this project 2018-2020:

1. K. L. James, J. W. Kung, B. R. Crable, H. Mouttaki, J. R. Sieber, H. H. Nguyen, Y. Yang, Y. Xie, J. Erde, N.Q. Wofford, E.A. Karr, J.A. Loo, R. R. Ogorzalek Loo, R. P. Gunsalus, and M. J. McInerney. 2019. *Syntrophus aciditrophicus* uses the same enzymes in a reversible manner to degrade and synthesize aromatic and alicyclic acids. *Environ. Microbiol.* 21: 1833-1846. DOI: 10.1111/1462-2920.14601
2. D. J. F. Walker, K. P. Nevin, D. E. Holmes, A.-E. Rotaru, J. E. Ward, T. L. Woodard, J. Zhu, T. Ueki, S. S. Nonnenmann, M. J. McInerney, and D. R. Lovley. 2020. *Syntrophus* conductive pili demonstrate that common hydrogen-donating syntrophs can have a direct electron transfer option. *ISME J* 14: 837–846. <https://doi.org/10.1038/s41396-019-0575-9>.
3. N. A. Losey, S. Poudel, E. S. Boyd, and M. J. McInerney. 2020. The beta subunit of non-bifurcating NADH-dependent [FeFe]-hydrogenases differs from those of multimeric electron-bifurcating [FeFe]-hydrogenases. *Front. Microbiol.* 2020: 11: 1109. doi: [10.3389/fmicb.2020.01109](https://doi.org/10.3389/fmicb.2020.01109).
4. M. K. Nobu, T. Narihira, R. Mei, Y. Kamagata, P. KH Lee, P-H. Lee, M. J. McInerney, and W.-T. Liu. Catabolism and interactions of uncultured organisms shaped by eco-thermodynamics in methanogenic bioprocesses. *Microbiome* 8, 111 (2020). <https://doi.org/10.1186/s40168-020-00885-y>

MSU-DOE Plant Research Laboratory Subproject C: Characterizing and Engineering Subcellular and Cellular Modules for Photosynthetic Productivity

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Overall research goals: Our overarching goal is to gain a fundamental mechanistic understanding of cyanobacterial photosynthesis--the single cell natural solar panel--that can be applied to guide strategies for engineering improvements in primary productivity. We focus on the structure, function & interconnectivity of two cyanobacterial modules: light harvesting & the carboxysome. These modules are prominent components of cyanobacterial photosynthesis with features broadly useful for bioengineering applications.

Significant achievements (partial list, please see publication list):

- Development of a semi-wet cyanobacterial disc method for analyzing carbon assimilation in *Fremyella diplosiphon*. This method allows measuring the dynamic nature of carbon assimilation and the impact of environmental regulation of the carbon concentrating mechanism (CCM) on carbon response curves.
- Continued development of a bacterial microcompartment shell protein toolbox for modular construction of novel intracellular architectures and compartments with desirable functions. In this funding period, three projects related to this goal have been published. Briefly, the work described in these manuscripts have extended the range of potential electron transfer reactions and the capacity to visualize *in vivo* assembly of BMC-based nanoscaffolds, utilizing advanced imaging tools while identifying the likely requirement for polymerization ‘terminators’
- Additional tools were developed enabling experimenter control over the stability of endogenous target proteins in cyanobacteria. Using this system, we have demonstrated rapid downregulation of essential proteins and components of the carboxysome.
- Ongoing research has identified a link between the engagement of heterologous metabolic pathways (*i.e.*, an inducible “carbon sink” in the form of a sucrose secretion pathway) and restructuring of the cyanobacterial CCM.

Science priorities for the next year (2020-2021):

- Develop methods for the direct measurements of gas permeability across carboxysome shells
- Structurally and functionally characterize the mechanism of electron transfer between the shell and the lumen in a model system.
- Evaluate potential mechanisms of carbon balancing on cyanobacterial CCM, including identification of specific, two-component signaling mechanisms that may mediate the restructuring of carboxysomes following the induction of a heterologous sink or after sucrose feeding.
- Utilize components of the McdAB system to explore the role of carboxysome positioning on photosynthetic efficiency.
- Pursue structural and activity assays and compartmentalization of plant carbonic anhydrases (collaboration with PRL Project B)

Our major scientific area(s) of expertise is/are: The Project C Team has expertise in structural, molecular and synthetic biology and biochemistry.

To take our project to the next level, our ideal collaborator would have expertise in: Advanced imaging of live bacterial cells, including extended time lapse imaging and algorithmic analysis of phenotypic datasets.

Computational approaches for predictive modeling of organic and inorganic carbon in cyanobacteria and development of methods for integrating imaging across scales of organization.

Publications supported by this project (2019-2020):

1. Sommer, M. Sutter, M., Gupta, S., Turmo, A., Burton, R.L., Saechao, C., Chan, L-J.G., Petzold, C.J., Ralston, C.Y., Kerfeld, C.A. Heterohexamers Formed by CcmK3 and CcmK4 Increase the Complexity of Beta-Carboxysome Shells. *Plant Physiology* 179: 156, 2019.
2. Sutter, M., Laughlin, T.G., Sloan, N.B., Serwas, D., Davies, K.M., Kerfeld, C. A. Structure of a Synthetic beta-carboxysome shell. *Plant Physiology* 181: 1050, 2019.
3. Greber, B.J., Sutter, M., Kerfeld, C.A. The plasticity of molecular interactions governs bacterial microcompartment shell assembly. *Structure* 7:749, 2019.
4. Ferlez, B., Sutter, M., Kerfeld, C.A. Glycyl radical enzyme-associated microcompartments: redox replete bacterial organelles. *mBio* 10: e02327, 2019.
5. Dominguez-Martin, M.A., Polivka, T., Sutter, M., Ferlez, B., Lechno-Yossef, S., Montgomery, B.L., Kerfeld, C.A. Structural and spectroscopic characterization of HCP2. *Biochimica Biophysica Acta* 1860:414, 2019.
6. Dominguez-Martin, M.A. Kerfeld, C.A. Engineering the Orange Carotenoid Protein for Applications in Synthetic Biology. *Current Opinion in Structural Biology* 57:110, 2019.
7. Gupta, S., Dominguez-Martin, M.A., Bao, H., Sutter, M., Feng, Pawlowski, E.G., Feng, J., Chan, L-J.G., Petzold, C.J., Ralston, C.Y., Kerfeld, C.A. Molecular mechanism of structural rearrangements during photoregulation of OCP and binding of FRP. *Journal of Biological Chemistry*, 294:8848, 2019.
8. Sutter, M., McGuire, S., Ferlez, B., Kerfeld, C.A. Structural Characterization of a Synthetic Tandem-domain Bacterial Microcompartment Shell Protein Capable of Forming Icosahedral Shell Assemblies. *ACS Synthetic Biology* 8:668, 2019.
9. Young, E. J., Sakkos, J.K., Huang, J., Wright, J.K., Kachel, B., Fuentes-Cabrera, M., Kerfeld, C.A. & Ducat, D. Visualizing in Vivo Dynamics of Designer Nanoscaffolds. *NanoLetters* 20, 208, 2019.
10. Santos-Merino, M., Singh, A.K., Ducat, D.C. New applications of synthetic biology tools for cyanobacterial metabolic engineering. *Frontiers in Bioengineering and Biotechnology* 7: 33, 2019.
11. Rohnke, BR, Rodríguez Pérez, KJ, Montgomery, BL. Linking the Dynamic Response of the Carbon Dioxide-Concentrating Mechanism to Carbon Assimilation Behavior in *Fremyella diplosiphon*. *mBio*, 11: e01052, 2019.
12. Rohnke BA, Kerfeld CA, Montgomery BL Binding Options for the Small Subunit-Like Domain of Cyanobacteria to Rubisco. *Frontiers in Microbiology*, 11:187, 2020.
13. Lechno-Yossef, S, Rohnke, BA, Belza, ACO, Melnicki, MR, Montgomery, BL, Kerfeld, CA. Cyanobacterial Carboxysomes Contain an Unique Rubisco-Activase-Like Protein. *New Phytologist*, 225: 793, 2020.
14. Plegaria, J.S., Yates, M.D., Glaven, S.M., Kerfeld, C.A. Redox Characterization of electrode-immobilized Bacterial Microcompartment Shell Proteins Engineered to Bind Metal Centers. *ACS Applied Biomaterials* 3: 685, 2020.
15. Huang, J., Ferlez, B.H., Young, E.J., Kerfeld, C.A., Kramer, D.M., Ducat, D.C. Functionalization of Bacterial Microcompartment Shell Proteins With Covalently Attached Heme. *Frontiers in Bioengineering and Biotechnology* 7: 432, 2020.
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Mechanism of Photochemical N₂ Reduction

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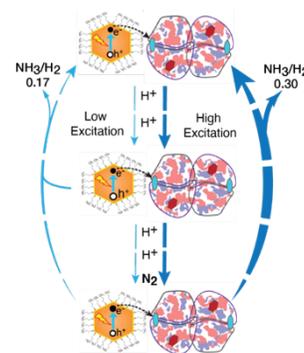
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Overall research goals:

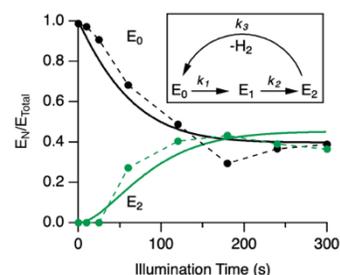
The proposed work focuses on defining the photochemical reaction mechanism of one of the most energy demanding and difficult chemical reactions: the activation and reduction of dinitrogen (N₂) to ammonia (NH₃) by nitrogenase. The enzyme nitrogenase catalyzes this chemical transformation by coupling ATP hydrolysis by the Fe protein to drive the sequential delivery of electrons to the MoFe protein for N₂ activation and reduction to NH₃. In this work, nanocrystalline materials are used to photochemically activate electron transfer into MoFe protein, in lieu of ATP-dependent electron transfer by the nitrogenase Fe protein. The tunable light-harvesting properties of nanocrystals will enable comprehensive analysis of the energetic and kinetic requirements for injection of electrons into MoFe protein, transfer of electrons through the protein, and N₂ reduction chemistry at the catalytic site FeMo-cofactor. Integration of nanocrystal photochemistry with nitrogenase will further enable the development and use of light-driven biophysical techniques to resolve key structural and functional questions of the MoFe protein catalytic mechanism. The ability to synchronize electron injection with tuning of photochemical potential will enable profound new insights into the mechanisms that control electron transfer and catalysis and will provide a deeper understanding of the energetic and kinetic requirements for achieving difficult, energy demanding, multi-electron chemical transformations.

Significant achievements (2018-2021):

- Photochemical reactions of MoFe protein and CdS quantum dot (CdS) complexes were studied to determine how light intensity affects the selectivity of H₂ and NH₃ production. Using ¹⁵N₂ as a substrate, light-driven N₂ reduction by CdS:MoFe protein was demonstrated to produce increasing levels of ¹⁵NH₃ relative to H₂ as the light intensity, and the rate of CdS excitation and electron transfer to MoFe protein, increased. Thus a threshold photoexcitation rate is required to shift the reaction products from H₂ towards ¹⁵NH₃ (Scheme on the right). Overall the analysis of the effect of excitation rate on MoFe protein catalysis provides a deeper understanding of how electron flux controls product selectivity and informs on the requirements for enabling MoFe protein to function in the solar-driven reduction of N₂ to ammonia.



- Light-controlled reduction of CdS:MoFe protein has enabled biophysical studies to define the early intermediates in the photochemical N₂ reduction reaction cycle. By use of low-photoexcitation rates to control MoFe protein reactivity with cryo-trapping, we were able to explore the stepwise formation of early intermediates of the N₂ reduction reaction. Electron paramagnetic resonance (EPR) spectroscopy was used to identify time-dependent changes in the MoFe protein resting state, E₀, and the accumulation of a paramagnetic, two-electron reduced intermediate, E₂. Modeling the interconversion of E₀ and E₂ (figure on the right) revealed how controlled photoexcitation can provide access to certain reaction intermediates that otherwise can be difficult to resolve.



- In molecular systems composed of a light capture molecule and a catalytic enzyme, the ultrafast processes of light conversion and electron transfer must be coupled to the slow processes of catalysis to enable substrate activation and product formation. In multi-electron reactions, for example N₂ reduction to ammonia by nitrogenase, rates and efficiencies of electron transfer have a significant effects on the overall efficiency of the conversion. In nanocrystal-MoFe protein complexes, the electron transfer rates and efficiencies are key to understanding what controls MoFe protein reactivity and selectivity in the photochemical reaction. Transient absorption spectroscopy is being used to measure these properties for a CdS nanorod-MoFe protein system. This approach is being used to determine how small nanocrystal changes impact the outcome of multi-electron photochemical reactions catalyzed by redox enzymes.

Science priorities for the next year (2020-2021):

- Identify MoFe protein E-state progression by photochemical and chemical freeze-quench EPR
- Perform *in situ* controlled illumination of CdS-MoFe protein complexes for cryo-trapping and EPR analysis of photochemical reaction intermediates, and use multi-dimensional EPR for structural characterization to determine the ligands (e.g., H₂, hydride, and N₂) of intermediates.
- Develop a complete reaction model of the photochemical N₂ reduction reaction by MoFe protein.

My major scientific area(s) of expertise is/are: Photo-driven EPR, FTIR and photochemical based techniques for mechanistic studies of enzymes that catalyze reduction-oxidation reactions.

To take my project to the next level, my ideal collaborator would have expertise in: Photoelectrochemistry.

Publications supported by this project:

1. J.K. Utterback, J.L. Ruzicka, H.R. Keller, L.M. Pellows, G. Dukovic. "Electron Transfer from Semiconductor Nanocrystals to Redox Enzymes." *Ann. Rev. Phys. Chem.* *71*, 335 (2020). DOI:10.1146/annurev-physchem-050317-014232.
2. J.K. Utterback, J.L. Ruzicka, H. Hamby, J.D. Eaves, G. Dukovic. "[Temperature-Dependent Transient Absorption Spectroscopy Elucidates Trapped-Hole Dynamics in CdS and CdSe Nanorods.](#)" *J. Phys. Chem. Letts.* *10*, 2782 (2019). DOI:10.1021/acs.jpcclett.9b00764.
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MSU-DOE Plant Research Laboratory Subproject A: Robust Photosynthesis in Dynamic Environments

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Overall research goals: The project focuses on one of the grand challenges in basic energy science, understanding how the components of natural photosynthesis are integrated into living organisms. Over past funding periods, we have developed enabling technologies that enhance our ability to probe and understand energy capture and storage *in vivo* under environmental conditions relevant to the field. The data obtained using these tools has revealed new processes that support efficient photosynthesis in dynamic environments, and which can be synthesized into a generalizable model for regulation of the light reactions of photosynthesis in the green lineage. In this period, we expand our work to study how robust this model is, and whether it applies to diverse organisms, including cyanobacteria. We are most interested in cases where the paradigm fails, potentially revealing new mechanisms and fundamental limitations to photosynthetic efficiency. We have three highly interactive components: 1) Development of enabling technologies to enhance our ability to probe energy capture and storage *in vivo* under environmental conditions; 2) Understanding processes that support efficient photosynthesis; and 3) Disseminating knowledge, methods and technology to empower a broader community.

Significant achievements (2019-2020):

The power and limits of the “pmf paradigm” for regulation of photosynthesis. The development of underlying concepts of this project were presented in two publications (1, 2), emphasizing co-regulation of early events in photosynthesis with downstream metabolic processes, and focusing on the diversity of mechanisms that allow photosynthesis to balance efficiency with the avoidance of photodamage. Experimental work over this period is built on these concepts, highlighting processes that limit plants’ abilities to respond to rapidly changing conditions, the extent of genetic (species or variety) variations and fundamental biophysical mechanisms that limit these capabilities. Space limitations allow us to highlight only a few of these projects and collaborations, but include areas such as development of biophysical and synthetic biology platforms for understanding fundamental processes of long range electron transfer, the co-evolution of the chloroplasts and nuclear genomes in response to changes in photosynthetic capacity, etc. (3-7).

The light potentials of photosynthesis. We have identified two types of photosynthetic limitations under dynamic lighting, what we term “light potential” (LPs): 1) the ability to use light energy productively following a rapid increase in illumination (productive light potential, PLP); and 2) the ability to responsively activate/relax photoprotective processes (quenching light potential, QLP). We developed protocols and instruments to probe the extents and mechanistic bases for limitations to PLP and QLP under real world conditions and established a global, open-science project to test these hypotheses across diverse species and environments (photosynq.org/projects/productive-and-photoprotective-light-potentials-1). We now have evidence that PLP and/or QLP are “tuned” in different genotypes to function more rapidly, and that the increased responsiveness is associated with higher sustained rates of photosynthesis and photosynthetic productivity. Using our high throughput phenotyping platforms, we identified mutant lines with altered LP associated with changes in photorespiratory capacity, leading to the discovery of new components of the photorespiratory system (8) as well as a bypass pathway to overcome limitations in peroxisomal hydroxypyruvate reductase (9).

The role of the thylakoid proton motive force (*pmf*) and ATP synthase in regulating photosynthesis. We showed that short-term changes to CO₂ availability can result in limitations in carbon metabolism at the level of triose-phosphate utilization, leading to co-regulation of the light and dark reactions by governing proton flux through the ATP synthase (10, 11). It has been suggested that the chloroplast ATP synthase is particularly inefficient because of its high ATP/H⁺ stoichiometry and that engineering its c-subunit could improve the productivity photosynthesis. However, our detailed kinetic simulations (12) suggest this high ratio is required to prevent formation of excess transthylakoid $\Delta\psi$, over-acidification of the lumen, and resultant photodamage.

New regulatory mechanisms revealed by exploring the responses of photosynthesis to combinations of environmental fluctuations. Biophysical and biochemical processes are required for phototrophs to withstand “real world” fluctuations in multiple environmental conditions simultaneously, especially light and temperature dynamics, which most immediately impact energy capture reactions. The lipid composition of photosynthetic membranes is unique and varies across strains, though specific roles of these lipid species are not well understood. Efforts in the Kramer lab using high throughput phenotyping of genomic diversity panels, and the Benning lab using genetics and biochemical approaches, independently converged to identify PG16:1t (phosphatidyl glycerol with a 16:1 trans fatty acid attached in its *sn2* position) as playing a key role in responses to low temperatures. We found this thylakoid-specific lipid requires fatty acid desaturase (FAD4), as well as a redox active peroxiredoxin (PRXQ) that link PG16:1t synthesis to the redox state of the chloroplast (13). A resulting hypothesis is that PG16:1t modulates the function of photosynthetic complexes in the thylakoid membrane or acts as a part of a signaling cascade. At the other extreme, we studied combinations of high temperature and high light. In some species, e.g. tobacco or Arabidopsis, we found no evidence that photorespiration does not provide a photoprotective role, at least under short term exposures to these conditions (2). By contrast, in heat tolerant (but not sensitive) genotypes of cowpea both photorespiration and alternative electron acceptors provide substantial photoprotection at high temperatures (14). These findings suggest that there are distinct genetic/species specific differences in the mechanistic bases for protecting against environmental challenges.

Diversity in photosynthetic regulation. Cyanobacteria have distinct mechanisms for regulating the light reactions that may not directly involve the canonical “pmf paradigm” (1), and thus we evaluated the tradeoff between heterologous metabolic sinks and “wasteful” photoprotective mechanisms in the model cyanobacterium *Synechococcus elongatus* PCC 7942. Briefly, in work that bisects goals in Subprojects A and B, we have found that combining two distinct heterologous metabolic sinks (cytochrome P450 and sucrose export) can contribute to additive effects on photosynthetic performance (15), with important implications for energetic balance in cyanobacterial photosynthetic metabolism and for maximizing photosynthetic efficiency by decreasing dissipative oxygen reduction (DOR) (15, 16).

The elusive signal to induce pyrenoid formation in green algae and its importance in responses of photosynthesis to hyperoxia. To gain insights into how algae respond to hyperoxia, which serves as a major feedback limitation in growth, we compared responses to high O₂ in natural isolates of *Chlamydomonas reinhardtii*. We found that, in addition to the classical induction of a pyrenoid by low CO₂, the pyrenoid is also induced by high O₂ (hyperoxia), even at high levels of inorganic C (CO₂ or bicarbonate). This led us to the discovery that H₂O₂ is the elusive signal that controls pyrenoid formation. We also show that differences in the activation process may play a role in intraspecies variations in pyrenoid morphology, which have been reported for over 100 years, and may be related to the observed genotypic-dependencies in tolerances to hyperoxia (17).

Science priorities for the next year (2019-2020):

- In addition to the projects described above, we will follow up on several new discoveries. Results on delayed fluorescence and electroluminescence, which may have identified new states of photosystems I and II that are important for preventing recombination reactions that can lead to the production of singlet oxygen. We will test this in vivo and, eventually, under real world conditions.
- Results from high throughput phenotyping, both using imaging in simulated environments (using the DEPI platform) and in the real world (through the global PhotosynQ project) suggest that there are previously-unknown photosynthetic regulatory mechanisms that contribute to the avoidance of photodamage in different genetic variants in a species. We are developing the bioinformatics and statical tools to identify their specific genetic bases.
- We will further elucidate the redox signaling pathway uncovered by the work on 16:1t, towards understanding how changes in lipid content affect specific photosynthetic processes.

Our major scientific areas of expertise are: Biophysics, Biochemistry, Physiology, Genetics and Genomics, instrumentation, data analysis and modeling related to photosynthesis.

To take my project to the next level, my ideal collaborator would have expertise in: Experts in photochemistry, structural biology, kinetics and modeling who are also interested in how their systems have been optimized by evolution.

Publications supported by this project [2019-2020]:

1. Kanazawa, A., Davis, G. A., N., F., Neofotis, P., and Kramer, D. M. (2020) Diversity in photoprotection and energy balancing in terrestrial and aquatic phototrophs, in *Photosynthesis in Algae* (Larkum, A. W. D., Grossman, A., and Raven, J. A., Eds.).
2. Walker, B. J., Kramer, D. M., Fisher, N., and Fu, X. (2020) Flexibility in the Energy Balancing Network of Photosynthesis Enables Safe Operation under Changing Environmental Conditions, *Plants* 9, 301.
3. Major, I. T., Guo, Q., Zhai, J., Kapali, G., Kramer, D. M., and Howe, G. A. (2020) A Phytochrome B-independent Pathway Restricts Growth at High Levels of Jasmonate Defense, *Plant physiology In Press*.
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5. Flood, P. J., Theeuwens, T., Schneeberger, K., Keizer, P., Kruijjer, W., Severing, E., Kouklas, E., Hageman, J. A., Wijffjes, R., Calvo-Baltanas, V., Becker, F. F. M., Schnabel, S. K., Willems, L. A. J., Ligterink, W., van Arkel, J., Mumm, R., Gualberto, J. M., Savage, L., Kramer, D. M., Keurentjes, J. J. B., van Eeuwijk, F., Koornneef, M., Harbinson, J., Aarts, M. G. M., and Wijnker, E. (2020) Reciprocal cybrids reveal how organellar genomes affect plant phenotypes, *Nat Plants* 6, 13-21.
6. Huang, J., Zarzycki, J., Gunner, M. R., Parson, W. W., Kern, J. F., Yano, J., Ducat, D. C., and Kramer, D. M. (2020) Mesoscopic to Macroscopic Electron Transfer by Hopping in a Crystal Network of Cytochromes, *J Am Chem Soc* 142, 10459-10467.
7. Huang, J., Ferlez, B. H., Young, E. J., Kerfeld, C. A., Kramer, D. M., and Ducat, D. C. (2020) Functionalization of Bacterial Microcompartment Shell Proteins With Covalently Attached Heme, *Frontiers in Bioengineering and Biotechnology* 7, 43.
8. Li, J. Y., Tietz, S., Cruz, J. A., Strand, D. D., Xu, Y., Chen, J., Kramer, D. M., and Hu, J. P. (2019) Photometric screens identified Arabidopsis peroxisome proteins that impact photosynthesis under dynamic light conditions, *Plant Journal* 97, 460-474.
9. Li, J., Weraduwage, S. M., Preiser, A. L., Tietz, S., Weise, S. E., Strand, D. D., Froehlich, J. E., Kramer, D. M., Hu, J., and Sharkey, T. D. (2019) A Cytosolic Bypass and G6P Shunt in Plants Lacking Peroxisomal Hydroxypyruvate Reductase, *Plant physiology* 180, 783-792.
10. McClain, A. M., and Sharkey, T. D. (2019) Triose phosphate utilization and beyond: from photosynthesis to end product synthesis. , *Journal of Experimental Botany* 70, 1755-1766.
11. McClain, A. M., and Sharkey, T. D. (2020) Building a better equation for electron transport estimated from chlorophyll fluorescence: Accounting for non-photosynthetic light absorption. *New Phytologist* 225, 604 - 608., *New Phytologist* 225, 604-608.
12. Davis, G. A., and Kramer, D. M. (2020) Optimization of atp synthase c-rings for oxygenic photosynthesis, *Frontiers in Plant Science* 10, 1778.
13. Horn, P. J., Smith, M. D., Clark, T. R., Froehlich, J. E., and Benning, C. (2020) PEROXIREDOXIN Q stimulates the activity of the chloroplast 16:1(Delta3trans) FATTY ACID DESATURASE4, *Plant J* 102, 718-729.
14. Osei-Bonsu, I., Hoh, D., Chattopadhyay, A., Tessmer, O., Roberts, P. A., Huynh, B. L., Kuhlert, S., Chen, J., Kanazawa, A., and Kramer, D. M. (2020) On the genetic and mechanistic bases of natural variations in photosynthesis responses to abiotic stresses, *Plant Cell and Environment submitted*.
15. Santos-Merino, M., Torrado, A., Davis, G. A., Röttig, A., Bibby, T. S., Kramer, D. M., and C., D. D. (2020) Testing the capacity of heterologous metabolic sinks to replace photoprotective mechanisms in cyanobacteria. Under Review. , *Under review*.
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17. Neofotis, P., Temple, J., Tessmer, O. L., Bibik, J., Norris, N., Poliner, E., Lucker, B., Wijetilleke, S., Withrow, A., Sears, B., Mogos, G., Frame, M., Hall, D., Weissman, J., and Kramer, D. M. (2020) The induction of pyrenoid synthesis by hydrogen peroxide and its implications for the natural diversity of tolerance to hyperoxia in Chlamydomonas, *eLife submitted*.

Mechanism of Ethylene Production from the Common Metabolite, 2-Oxoglutarate, by the Ethylene-Forming Enzyme (EFE)

Carsten Krebs, Principal Investigator

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Overall research goals:

Ethylene-forming enzyme (EFE) from *Pseudomonas syringae* is an ambifunctional iron(II)- and 2-(oxo)glutarate-dependent (Fe/2OG) oxygenase. In its major reaction, EFE fragments 2OG to ethylene and three equivalents of CO₂, a four-electron oxidation that differs radically from the outcomes of other members of this large enzyme family. Its secondary reaction conforms to the usual *modus operandi*: oxidative decarboxylation of 2OG to succinate is coupled to hydroxylation of C5 of L-arginine, which is a required activator of the major pathway. We aim to define the structural and mechanistic bases for the complex reactivity of EFE. In particular, we seek to identify the branch point between the two pathways and elucidate the mechanism of the unusual multiple-electron and multiple-bond fragmentation reaction leading to ethylene.

Significant achievements (2016-2020):

We have shown that, consistent with precedent, L-Arg hydroxylation proceeds via an iron(IV)-oxo (ferryl) intermediate. Owing to the large kinetic isotope effect (²H-KIE) on hydrogen-atom transfer ($k_H/k_D \sim 20$), the presence of deuterium at C5 of L-Arg slows decay of the ferryl intermediate, allowing it to accumulate to > 11% of the total iron. The observed insensitivities of (i) the relative yields of the two products and (ii) more than half the reaction flux of a single turnover (as monitored by stopped-flow absorption spectrophotometry) to C5 deuteration imply that the detected ferryl complex is not on the major, ethylene-producing pathway. Thus, the branch point must be earlier in the reaction sequence.

We solved x-ray crystal structure of the anoxically prepared EFE•Fe(II)•2OG•L-Arg reactant complex and the EFE•Fe(II)•NHA•L-Arg complex, wherein NHA (*N*-hydroxysuccinamate) is a mimic of the persuccinate intermediate. The persuccinate complex is a good candidate for the branchpoint of the ethylene-generating and arginine-oxidizing pathways. Thus, we consider the geometric and electronic structure of this complex as potentially insightful for rationalizing the reactivity.

The Asp191 → Glu substitution, amounting to simple insertion of a methylene unit into an iron-coordinating amino acid, markedly shifts the partition ratio toward the hydroxylation pathway and permits greater ferryl accumulation. The nearly complete abolition of ethylene production by this nearly conservative substitution, as well as its much less pronounced effect on ferryl-mediated L-Arg oxidation, imply that the unusual primary reaction has more rigid stereoelectronic requirements than the canonical pathway leading to formation of the ferryl complex and L-Arg oxidation.

We used 3,3,4,4-*d*₄-2OG (*d*₄-2OG) to test for a change in the partition ratio of the ethylene-producing and Arg-oxidizing branches. We considered the possibility that the ratio could change to favor the Arg-oxidizing path with *d*₄-2OG, due to a secondary KIE that impacts the ethylene-forming path but not the Arg-oxidizing path. We carried out a steady state competition experiment with *d*₄-2OG and unlabeled 2OG and observed potentially significant discrimination between the labeled and unlabeled substrates and their partition ratios. In an independent experiment, we have looked for changes in the amplitude of the 320-nm absorption transient, which reports on the quantity of ferryl intermediate that

accumulates, upon use of either d_4 -2OG or unlabeled 2OG. Again, 2OG deuteration is associated with a potentially significant impact on ferryl accumulation.

Science priorities for the next year (2020-2021):

- Complete experiments to define stereochemistry of the ethylene-forming reaction using selectively deuterated substrate isotopologs. Write publications summarizing findings of our studies.

My major scientific area(s) of expertise is/are: Enzyme reaction mechanisms, transient enzyme kinetics, biophysics, transition-metal/dioxygen chemistry.

To take my project to the next level, my ideal collaborator would have expertise in: Synthetic organic chemistry.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years] :

1. R. A. Copeland; K. M. Davis; T. K. C. Shoda, E. J. Blaesi; A. K. Boal, C. Krebs, J. M. Bollinger, Jr. An Iron(IV)-Oxo Intermediate Initiating L-Arginine Oxidation but not Ethylene Production by the 2-Oxoglutarate-Dependent Oxygenase, Ethylene-Forming Enzyme, *J. Am. Chem. Soc.* in preparation

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Transmethylation reactions during methylotrophic methanogenesis in methanogenic Archaea

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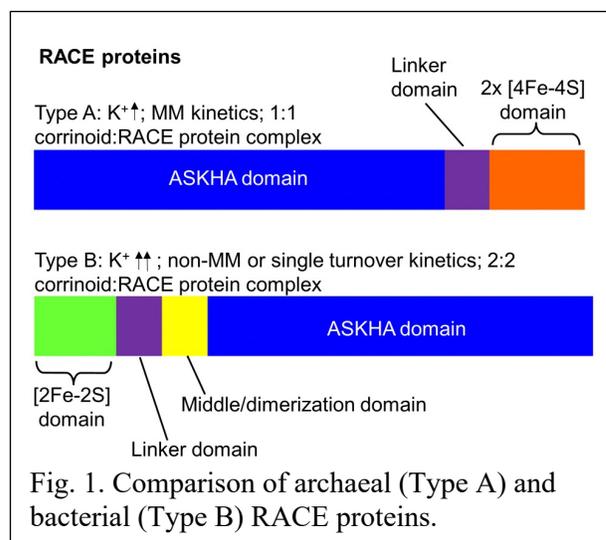
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Overall research goals:

Methanogenic archaea and anaerobically respiring bacteria employ corrinoid-dependent methyltransferase systems to methylate cellular cofactors such as CoM (methanogens) or THF (bacteria) during growth with methylated compounds. These systems are well-distributed in microbial genomes and their study continues to reveal novel insights. Each system possesses a small corrinoid binding protein that interacts with two methyltransferases. The first methyltransferase (MTI) acts to methylate the Co(I) corrinoid protein with the growth substrate generating the methyl-Co(III) state. MTII demethylates the methyl-Co(III) corrinoid protein to methylate a cellular cofactor regenerating the Co(I) state. The centrality of redox biochemistry in these methyltransferase systems is illustrated by the fact that oxidation of the Co(I)-corrinoid protein inactivates the system. The Co(II) corrinoid protein is rescued by a reductive activase. Such activases are iron-sulfur proteins (sometimes called a RACE proteins) capable of converting the energy of ATP hydrolysis into the low potential reducing power required to reduce Co(II)-corrinoid to the Co(I) state. Our long-term goals are to understand the full diversity of these systems and their biochemical mechanism(s). In this project period we have focused on one of the corrinoid reductive activases, RamA, with a view towards elucidating the mechanism by which it reduces off-cycle Co(II) corrinoid protein to the Co(I) state. Additionally, the various MTI enzymes derive from a large number of protein families and in recent years have been shown to have highly diversified active sites specific for diverse substrates. For example, the members of the TMA methyltransferase superfamily is an excellent example. Metabolism of TMA by the founding member of the family, MttB, the TMA methyltransferase, obligately depends on translation of an amber codon in the TMA methyltransferase gene as the 22nd amino acid, pyrrolysine (Pyl). With past DOE funding, we have made inroads into understanding how pyrrolysine is biosynthesized, and how it is genetically encoded. However, many TMA methyltransferase superfamily members lack pyrrolysine entirely, and have diversified for larger methylamine substrates with more functional groups. For example, glycine betaine is the substrate for TMA methyltransferase superfamily member MtgB. This has led us to comparing active sites of TMA methyltransferase family members that do or do not have pyrrolysine in an effort to understand how these distinctive active sites bind their substrates.

Significant achievements (2018-2021):

Kinetic and substrate complex characterization of RamA. RamA, the methylamine corrinoid protein activase, has a C-terminal ferredoxin domain with two [4Fe-4S] clusters from methanogenic archaea (Type A in Fig.1) and has been far less studied than the bacterial corrinoid activases (Type B, Fig. 1) bearing an N-terminal ferredoxin domain with one [2Fe-2S] cluster. In this period we have shown that the archaeal and bacterial corrinoid activases have further differences that are informative of the overall mechanism of RACE proteins. We examined RamA kinetics and the stoichiometry of the corrinoid protein:RamA complex and tested the necessity of a key residue for complex formation. Analogous to bacterial activases, K⁺ stimulates RamA up to 5-fold, whereas NH₄⁺ stimulated activity 9-fold. Potassium stimulation had been



previously been questioned for RamA by other workers due to differences in the primary structure of bacterial and methanogen activases. Unlike bacterial activases, ATP is not inhibitory allowing the first determination of apparent kinetic parameters for any corrinoid activase with a K_m value of 0.25 mM ATP. Bacterial activases interact with corrinoid proteins as dimers, but we found that single RamA monomer complexes a single corrinoid protein monomer. Previous structural studies by another group with the bacterial corrinoid activase RACo identified a key serine of RACo is complexed to the upper axial position of the corrinoid cofactor. Alanine replacement of a RamA serine residue corresponding to the position of the RACo serine led to only moderate changes in the kinetics of RamA, and no detectable changes in ability to form a complex with the Co(II)-corrinoid protein by gel permeation chromatography. In toto, these results revealed new distinctions between the two types of corrinoid activases and provide direct evidence for the proposal that corrinoid activases act as catalytic monomers, very unlike other enzymes that couple ATP hydrolysis to difficult low potential reductions.

Active site characterization of MtgB. Both MtgB and the TMA methyltransferase MttB are members of the same protein family, MtgB lacks pyrrolysine, and unlike the TMA methyltransferase MttB, does not use trimethylamine. MtgB instead uses glycine betaine and cob(I)alamin to form dimethylglycine and methyl-cob(III)alamin. How MtgB binds glycine betaine may help inform how pyrrolysine functions in MttB. Towards this end, in a preceding project period we collaborated with the Hao lab to obtain a structure of MtgB bound to glycine betaine and identified two basic and two aromatic residues in the active site interacting with glycine betaine. This year we completed triplicate kinetic analyses with respect to glycine betaine of a series of residue substitutions of the glycine betaine binding residues and the resultant variants displayed K_m and k_{cat} effects confirming residue roles in binding and/or catalysis. Comparison of the active sites of MttB (obtained in ongoing collaboration with Michael Chan) and MtgB suggests that a pyrrolysine-TMA adduct would orient a methyl group of TMA into a position close to that occupied by a glycine betaine in the MtgB active site. The similarity of methyl group placement in pyl- and non-pyl methyltransferases is further supportive of the hypothesis that a TMA-pyrrolysine adduct serves to orient the methyl group for transfer to the docked corrinoid protein. We are currently writing manuscripts describing the MtgB active site, as well as that of the TMA methyltransferase for JBC.

Science priorities for the next year (2020-2021):

- Determine the physiological electron donor to RamA.
- Determine if self-phosphorylation of RamA may occur using ATP:Pi exchange assays and radiolabeling experiments.
- Determine if binding of RamA to Co(II) changes the coordination state of Co(II) in MttC.

My major scientific area(s) of expertise is/are: Metabolism and biochemistry of anaerobic Archaea and Bacteria. Bioinformatic, proteomic, and empirical identification of novel enzyme activities, subsequent recombinant expression/isolation and characterization. C1 metabolism and biochemistry including microbial methylamine metabolism. Genetic encoding and biosynthesis of pyrrolysine. Practical genetics of methanogens..

To take my project to the next level, my ideal collaborator would have expertise in: Spectrometric methods.

Publications supported by this project [Click here to enter text.](#):

1. Huening, K.A., Jiang, R., and Krzycki, J.A. 2020. Kinetic and substrate complex characterization of RamA, a corrinoid protein reductive activase from *Methanosarcina barkeri*. FEMS Microbiology Letts. *In press.*

Understanding Selectivity in Terpene Synthases Unique Mechanisms to Generate Precursors for Biocrude and Specialty Chemicals

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Narayanan Srividya, Co-Principal Investigator

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Overall Research Goals:

Terpenoid oils and resins accumulated in plants (and sometimes microbes) are characterized by a high volumetric energy density and high degree of reduction, and are thus viable “biocrude” feedstocks for fuels in the diesel and kerosene range. Furthermore, many specialty chemicals are also based on terpenoid backbones, including polymers (e.g., rubber), solvents (e.g., limonene), and diverse small molecules (e.g., menthol). Terpenoids thus have the potential to serve as chemical feedstocks in a non-food bioeconomy based on carbon and energy capture, allocation, conversion and storage by plants, which is directly in line with the research mission of the DOE-BES program. This proposal aims to unravel the mechanistic basis for selectivity in the sophisticated enzymes, termed monoterpene synthases, that catalyze the formation of cyclic hydrocarbons as the first committed step in monoterpene biosynthesis. Such knowledge will allow us to infer the mechanistic underpinnings of how plants produce highly complex, reduced chemical scaffolds.

Significant Achievements (2017-2020):

- **Terpene synthase structure-function.** Terpene synthases convert a prenyl diphosphate of a specific chain length to the first pathway-specific (often cyclic) intermediate in the biosynthesis of a specific class of terpenoids. These enzymes are critical determinants of terpenoid chemical diversity, which is an important issue for the development of biocrude and sustainable specialty chemicals. Significant outcomes during the current funding period have been the characterization of the amino acid residues that determine enantiospecificity in monoterpene synthases. This knowledge has been used to interconvert the activities of (+)- and (-)-limonene synthases.
- **Bioenergetics of cell types with high flux toward reduced terpenoids.** We developed genome-scale mathematical models for glandular trichome and resin duct cells, and performed simulations to determine which steps are likely to be of particular relevance in terpenoid biosynthesis. In follow-up biochemical work we characterized a unique pair of Fd and FNR isoforms present in glandular trichomes. This sub-project has been completed and we are finishing up manuscripts to report on the findings.

Science Objectives for 2020-2021:

- **Elucidate the Role of Dynamic Changes in the Orientation and Conformation of Reaction Intermediates.** Molecular dynamics, combined with enhanced sampling methodologies (such as umbrella sampling and metadynamics), indicated that the α -terpinyl cation, a critical reaction intermediate, assumes preferentially a different conformation in monoterpene synthases that generate monocyclic products compared to those that produce bicyclic products. The same approaches are being used to evaluate of orientation and conformation dynamics of downstream reaction intermediates that steer the reaction to different monocyclic and bicyclic monoterpene end products. We are now conducting experiments to assess which amino acid residues are responsible for conformational preferences of the substrate and reaction intermediates that lead to these different types of end products. Our work is laying the foundation for efforts to predict (for the first time) functions of as yet uncharacterized monoterpene synthases.

- **Test Our Ability to Predict the Functions of Putative Monoterpene Synthase Genes Present in a Newly Sequenced Genome.** We recently completed a chromosome assembly of the genome of *Mentha longifolia*, a diploid mint species ancestral to both commercial spearmint and peppermint. Based on a novel computational approach that combines both sequence and structural comparisons, we have predicted the functions of all recognizable monoterpene synthases of *M. longifolia*. We are now characterizing the functions of these genes and will use the outcomes of the experiments to assess the quality of our predictions and, if necessary, make adjustments to our functional annotation model.

To take my project to the next level, my ideal collaborator would have expertise in:

- We are currently working with Simone Raugei from PNNL, which has enabled us to make progress in an area that requires unique computational expertise and resources. He continues to be our ideal collaborator.

Publications supported by this project (2017-2020):

(Note: Support by DOE-BES was acknowledged in all publications listed below)

1. Vining K.J., Pandelova I., Lange I., Parrish A.N., Lefors A., Kronmiller B., Liachko I., Kronenberg Z., Srividya N., Velasco J.A., Lange B.M. (2020) Chromosome-level *Mentha longifolia* L. genome assembly reveals gene organization underlying disease resistance and essential oil traits. *Plant J.*, submitted, August, 2020.
2. Parrish A.N., Turner G.W., Lange B.M. (2020) High contribution of canopy to oleoresin accumulation in loblolly pine trees. *bioRxiv*. doi: <https://doi.org/10.1101/2020.04.09.034264>
3. Srividya N., Lange I., Lange B.M. (2020) Determinants of enantiospecificity in limonene synthases. *Biochemistry* 59, 1661-1664. doi: <https://doi.org/10.1021/acs.biochem.0c00206>
4. Lange B.M., Srividya N. (2019) Enzymology of monoterpene functionalization in glandular trichomes. *J. Exp. Bot.* 70, 1095-1108. doi: <https://doi.org/10.1093/jxb/ery436>
5. Turner G.W., Parrish A.N., Zager J.J., Fishedick J.T., Lange B.M. (2018) Assessment of flux through oleoresin biosynthesis in epithelial cells of loblolly pine resin ducts. *J. Exp. Bot.* 70, 217-230. doi: <https://doi.org/10.1093/jxb/ery338>
6. Liu C., Srividya N., Parrish A.N., Yue W., Shan M., Wu Q., Lange B.M. (2018) Morphology of glandular trichomes of Japanese catnip (*Schizonepeta tenuifolia* Briquet) and developmental dynamics of their secretory activity. *Phytochemistry* 150, 23-30. doi: <https://doi.org/10.1016/j.phytochem.2018.02.018>
7. Johnson S.R., Lange I., Srividya N., Lange B.M. (2017) Bioenergetics of monoterpeneoid essential oil biosynthesis in non-photosynthetic glandular trichomes. *Plant Physiol.* 175, 681-695. doi: <https://doi.org/10.1104/pp.17.00551>
8. Vining K.J., Johnson S.R., Ahkami A., Lange I., Parrish A.N., Trapp S.C., Croteau R.B., Straub S.C., Pandelova I., Lange B.M. (2017) Draft genome sequence of *Mentha longifolia* and development of resources for mint cultivar improvement. *Mol. Plant* 10, 323-339. doi: <https://doi.org/10.1016/j.molp.2016.10.018>

Understanding Nitrogenase Maturation and Activity in Methanogens

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Evert C. Duin, Co-PI
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Overall research goals:

The overall goal of this project is to determine the factors that control the maturation and activity of nitrogenase in methanogens. Methanogens have a highly specialized metabolism and are the only archaea that possess nitrogenase. Methanogens lack the nitrogenase-specific Fe-S cluster assembly machinery found in bacteria. They are also not limited to cysteine as the internal source of sulfur for Fe-S clusters. Moreover, the catalytic capabilities of methanogen nitrogenases are largely unknown. Thus, methanogens serve as an untapped resource to enhance the understanding of nitrogenase biogenesis and activity to further metalloenzyme-based energy production strategies. The overarching hypothesis is that methanogens employ sulfur-specific pathways for the maturation of nitrogenases with properties distinct from bacterial nitrogenases. To test this hypothesis, we are using genetic and biochemical approaches with the model methanogen *Methanosarcina acetivorans*. *M. acetivorans* contains *nif*, *vnf*, and *anf* operons predicted to encode Mo-, V-, and Fe-nitrogenase, respectively. *M. acetivorans* lacks NifS and NifU responsible for initial Fe-S cluster biogenesis in nitrogenase within bacteria, but contains three copies of IscS and IscU, as well as two copies of SufBC, the core machinery of the ISC and SUF Fe-S cluster biogenesis systems, respectively. The specific goals are to determine the roles of IscSU and SufBC in the sulfur-specific biogenesis of Fe-S clusters in nitrogenase, to determine the catalytic properties of Mo-nitrogenase, and to understand how nitrogenase is integrated into the unique physiology of methanogens.

Significant achievements (2018-2020)

- We defined the conditions required for production of all three nitrogenases in *M. acetivorans*.
 - Only Mo-nitrogenase (Nif) is produced in cells grown in molybdate-replete medium, whereas all three nitrogenases are produced in cells grown in molybdate-deplete medium.
- We determined the effect of exogenous sulfur (cysteine or sulfide) on nitrogenase-dependent growth (diazotrophy) by *M. acetivorans*.
- We determined that IscSU2 is critical for cysteine-dependent production of Mo-nitrogenase.
- We developed a CRISPRi-dCas9 system to control gene expression in *M. acetivorans* and used the system to reveal:
 - Repression of *sufB1C1* and *sufB2C2* individually does not impact production of Mo-nitrogenase with cysteine or sulfide.
 - Repression of the *nif* operon abolishes diazotrophic growth of *M. acetivorans* in both molybdate-replete and molybdate-deplete medium.
 - Repression of *nifB* inhibits but does not abolish diazotrophic growth of *M. acetivorans*.
- We determined cysteine controls the production of a hybrid nitrogenase comprised of the Fe-nitrogenase with the M-cluster (FeMo-co) in a *M. acetivorans* strain incapable of expressing Mo-nitrogenase (**Fig. 1**).
- We developed strains and protocols to purify *M. acetivorans* Mo-nitrogenase (NifHDK):
 - Using the CRISPR-Cas9 system, we generated a *M. acetivorans* strain that produces Strep-tag NifD that allows one-step purification of active NifDK in high yield.

- We generated an *E. coli* strain capable of producing functional recombinant NifH.
- Using the acetylene reduction assay, we demonstrated that purified NifDK and recombinant NifH form active Mo-nitrogenase.
- Using CRISPR-Cas9, we have recently generated a *M. acetivorans* strain that produces Strep-tag NifH to facilitate NifH purification directly from *M. acetivorans*.

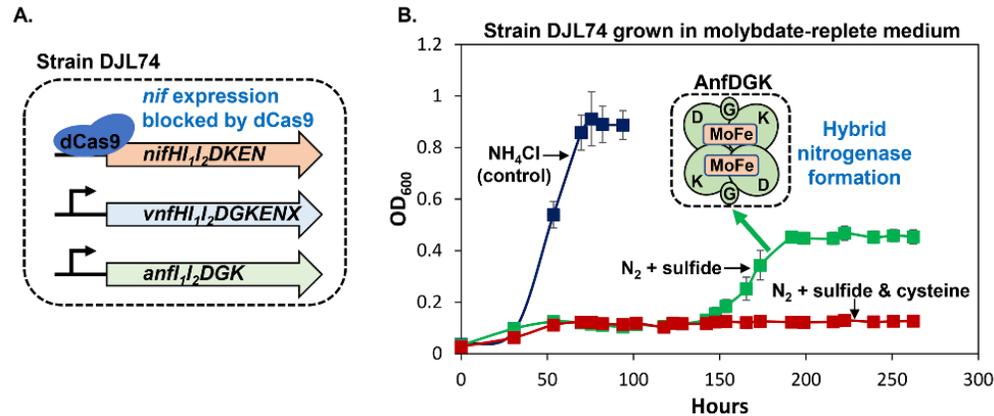


Fig. 1. dCas9-mediated repression of the *nif* operon results in production of a hybrid nitrogenase when *M. acetivorans* is grown with molybdate in the absence of cysteine. A) Diagram depicting dCas9-mediated repression of the *nif* operon in strain DJL74. B) Growth of strain DJL74 in molybdate-replete medium +/- cysteine.

Science priorities for the next year (2020-2021)

- Further define the role of IscSU and SufBC in the assembly and activity of nitrogenase.
 - Purify and characterize IscSU and SufBC complexes.
 - Generate additional IscSU and SufBC deletion or repression strains.
- Determine the biophysical and catalytic properties of *M. acetivorans* Mo-nitrogenase.
 - Scale up growth of *M. acetivorans* expression strains
 - Purify and characterize strep-NifDK and strep-NifH
 - Further develop and optimize procedures to assay nitrogenase activity
- Use CRISPR-Cas9 to strep-tag AnfD and VnfD to facilitate purification of native and/or hybrid V-nitrogenase and Fe-nitrogenase, respectively.
- Use CRISPR-dCas9 to target repression of additional nitrogenase-related genes.
- Use strep-NifH and strep-NifDK to identify redox partners to Mo-nitrogenase.
- Determine how cysteine controls production of nitrogenases in *M. acetivorans*.

My major scientific area(s) of expertise is/are: Methanogen genetics and biochemistry, Fe-S cluster proteins, redox proteins.

To take my project to the next level, my ideal collaborator would have expertise in: Nitrogenase, Bioenergetics, Structural Biology

Publications supported by this project:

A Dhamad, D.J. Lessner, "A CRISPRi-dCas9 system for archaea and its use to examine gene function during nitrogen fixation by *Methanosarcina acetivorans*." *Appl. Environ. Micro.* (2020) DOI: <https://doi.org/10.1101/2020.06.15.153874>: *accepted*.

T. M. Deere, D. Prakash, F. H. Lessner, E. C. Duin, D. J. Lessner. "*Methanosarcina acetivorans* contains a functional ISC system for iron-sulfur cluster biogenesis." *BMC Microbiology* (2020) *under review*.

Dissection of grass cell wall lignin formation and their associated dirigent proteins

Norman G. Lewis, Principal Investigator

Laurence B. Davin, John Cort Co-PI(s)

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Overall research goals:

The Objective in this proposal is aimed at resolving the biochemical basis of a long standing enigma in redox mediated plant biochemistry, and which lead to an important “electron store” in land plants, namely grass (monocot) lignins.

Objective: To establish the roles of Dir-e and monocot-specific Dir subfamilies in lignified grass cell wall formation, and their contributions to lignified cell wall mesoscale properties/lignocellulosic recalcitrance.

Significant achievements (2018 – 2020):

Biochemical activities (plant phenol C–C and C–O bond-forming processes) of dirigent proteins (DPs) known thus far give entry to diverse and complex classes of plant phenolics, these being the major contributors to lignocellulosic recalcitrance. DPs apparently began to emerge during the aquatic-to-land transition, with phylogenetic analyses revealing the presence of numerous DP subfamilies in the plant kingdom (1). The vast majority (>95%) of DPs in their large multigene sub-families, however, still await discovery of their biochemical functions. We have now solved the structures of two (phytoalexin) pterocarpan-forming proteins with dirigent-like domains, GePTS1 and PsPTS1, by X-ray crystallography to high resolution and have probed their active site biochemical mechanisms using site-directed mutagenesis of presumed key residues (2). Both PTS proteins stereospecifically convert distinct diastereomeric chiral isoflavonoid precursors to chiral phytoalexin pterocarpan, (–)- and (+)-medicarpin, respectively. NMR spectroscopy is underway to characterize the interaction of GePTS1 with substrates. The PTS1 structures have enabled comparisons with stereoselective lignan-forming DPs DRR206 (3) and AtDIR6, an aromatic diterpenoid-forming DP ortholog GhDIR4, and lignin-forming DPs in Casparian band lignification. Another pterocarpan synthase, PsPTS2, provides entry to both pterocarpan and isoflavones, with the latter perhaps undergoing subsequent metabolism to generate the opposite diastereomeric pterocarpan analogs. Each DP subfamily member with a known physiological role provides entry into diverse plant phenol natural products classes, and our experiments suggest a common biochemical mechanism in binding and stabilizing distinct plant phenol-derived mono- and bis-quinone methide intermediates during different C–C and C–O bond-forming processes. Apart from PTS, the intermediate in couplings mediated by, for example, lignan-forming DPs is a resonance-stabilized quinone methide radical whose formation *in vivo* requires an oxidase. Bottom-up proteomics (NanoPOTS) and native MS are helping unravel the architecture of a putative protein complex containing one such DP, as well as a peroxidase, and other proteins. These discoveries provide key insights into both the appearance and functional diversification of DPs, in plant phenol C–C and C–O bond formation, leading to entry into diverse plant phenol classes during land plant evolution/adaptation. The proposed biochemical mechanisms, based on our findings, provide important clues to how additional physiological roles for DPs and proteins harboring dirigent-like domains can now be rationally and systematically identified.

1. Corbin, C., Drouet, S., Markulin, L., Auguin, D., Lainé, É., Davin, L.B., Cort, J.R., Lewis, N.G., and Hano, C. (2018) *Plant Mol. Biol.* **97**: 73-101.
2. Meng, Q., Moinuddin, S.G.A., Kim, S.-J., Bedgar, D.L., Costa, M.A., Thomas, D.G., Young, R.P., Smith, C.A., Cort, J.R., Davin, L.B., and Lewis, N.G. (2020) *J. Biol. Chem.* **295**: 11584–11601.
3. Kim, K.-W., Smith, C.A., Daily, M.D., Cort, J.R., Davin, L.B., Lewis, N.G. (2015) *J. Biol. Chem.* **290**: 1308–1318.

Science priorities for the next year (2020 – 2021):

- To probe the roles of Dir-e and monocot-specific Dir subfamilies in lignified grass cell wall formation, and their contributions to lignified cell wall mesoscale properties/differing lignocellulosic recalcitrance levels. This approach employs recombinant protein expression, DP assays, and investigation of plant phenol-derived C–C and C–O bond formation, and use of CRISPR/Cas and metabolomic approaches *in vivo* as needed.

My major scientific area(s) of expertise is/are: Identifying and biochemically establishing mechanistically the biochemical mechanistic range of DP-mediated processes in land plants leading to entry into diverse plant phenol metabolic classes, these in turn being largely responsible for overall lignocellulosic recalcitrance.

To take my project to the next level, my ideal collaborator would have expertise in: Already in place.

Publications supported by this project (2016 – 2020)

- 1-11. Lewis, N.G., and Davin, L.B. (2020) *The Lignan Handbook with CD-ROM*. Taylor & Francis, Boca Raton, FL (ca. 1,400 pages). Manuscripts in press: 1) Davin, L.B., and Lewis, N.G. Redefining lignan and neolignan skeletal nomenclature with dirigent protein discovery, a historical perspective. 2) Davin, L.B., and Lewis, N.G. The occurrence and diversity of potential “lignan building blocks”. 3) Davin, L.B., and Lewis, N.G. (2020) Monomeric phenylpropanoid (C6C3) and lignan (C6C3 dimeric) natural products in plants, bacteria, fungi and animals: Structural diversity and chemotaxonomy. 4) Davin, L.B., Cort, J.R., Smith, C.A., Kim, K.W., Bedgar, D.L., and Lewis, N.G. Dirigent protein discovery and characterization in stipulating phenoxy radical coupling outcomes: 8-8' Lignan biosynthesis and its broader evolutionary ramifications. 5) Davin, L.B., and Lewis, N.G. 8-8'-Lignan biosynthesis roadmap. 6) Davin, L.B., and Lewis, N.G. 8-2'-, 8-3'- (8-5'-) and 8-O-4'-Linked lignan biosynthesis roadmap. 7) Davin, L.B., and Lewis, N.G. Allyl/propenyl phenol biosynthesis and C9/C9' deoxygenation roadmap. 8) Davin, L.B., and Lewis, N.G. Norlignan biosynthesis roadmap. 9) Davin, L.B., and Lewis, N.G. Roadmap to “mixed biochemical pathway” lignans. 10) Moinuddin, S.G.A., Davin, L.B., and Lewis, N.G. Oligomeric lignan diversity *in planta*: A biochemical roadmap for the future. 11) Davin, L.B., Ferreira, D., and Lewis, N.G. Lignan chirality roadmap: A scientific art in danger of being lost?
12. Meng, Q., Moinuddin, S.G.A., Kim, S.J., Bedgar, D.L., Costa, M.A., Thomas, D.G., Young, R.P., Smith, C.A., Cort, J.R., Davin, L.B., and Lewis, N.G. (2020) Pterocarpan synthase (PTS) structures suggest a common quinone methide-stabilizing function in dirigent proteins and proteins with dirigent-like domains. *J. Biol. Chem.* **295**: 11584-11601. <http://dx.doi.org/10.1074/jbc.RA120.012444>
13. Hwang, J.K., Moinuddin, S.G.A., Davin, L.B., and Lewis, N.G. (2020) Pinoresinol-lariciresinol reductase: Substrate versatility, enantiospecificity, and kinetic properties. *Chirality* **32**: 770-789. <http://dx.doi.org/10.1002/chir.23218>
14. Moinuddin, S.G.A., Cort, J.R., Smith, C.A., Hano, C., Davin, L.B., and Lewis, N.G. (2019) *Linum* lignan and associated biochemical pathways in human health and plant defense. In *Genetics and Genomics of Linum* (Cullis, C.A. ed.), Springer, Cham, Switzerland. pp 167-193. http://dx.doi.org/10.1007/978-3-030-23964-0_11.
15. Höhner, R., Marques, J.V., Ito, T., Amakura, Y., Budgeon, A.D., Jr., Weitz, K., Hixson, K.K., Davin, L.B., Kirchhoff, H., and Lewis, N.G. (2018) Reduced arogonate dehydratase expression: Ramifications for photosynthesis and metabolism. *Plant Physiol.* **177**: 115-131. <http://dx.doi.org/10.1104/pp.17.01766>
16. Corbin, C., Drouet, S., Markulin, L., Auguin, D., Lainé, É., Davin, L.B., Cort, J.R., Lewis, N.G., and Hano, C. (2018) A genome-wide analysis of the flax (*Linum usitatissimum* L.) dirigent protein family: from gene identification and evolution to differential regulation. *Plant Mol. Biol.* **97**: 73-101. <http://dx.doi.org/10.1007/s11103-018-0725-x>
17. Lu, D., Yuan, X., Kim, S.-J., Marques, J.V., Chakravarthy, P.P., Moinuddin, S.G.A., Luchterhand, R., Herman, B., Davin, L.B., and Lewis, N.G. (2017) Eugenol specialty chemical production in transgenic poplar (*Populus tremula* × *P. alba*) field trials. *Plant Biotechnol. J.* **15**: 970-981. <http://dx.doi.org/10.1111/pbi.12692>

Macromolecular Organization and Post-translational Regulation of Phenylpropanoid-Lignin Biosynthesis

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Overall research goals:

Phenylpropanoid metabolism represents a substantial metabolic sink for the photosynthetically fixed carbon and is a critical adaptive mechanism of land plants to cope with environmental stresses. Among diverse aromatics derived from the phenylpropanoid pathway, lignin, as one of the major cell wall structural components, accounts for about 30% photoassimilates. As an irreversible metabolic process, phenylpropanoid-lignin biosynthesis is exquisitely regulated in respect to the cellular carbon and energy status. Our research goals are to discover the biochemical and molecular mechanisms governing phenylpropanoid-lignin biosynthesis and the carbon allocation in the phenylpropanoid metabolism. Specifically, we are exploring 1) the carbon/energy signaling cascades and the related regulatory components controlling carbon or energy flux in phenylpropanoid metabolism; 2) the structural components and auxiliary proteins involved in the spatial organization and activity modulation of phenylpropanoid-monolignol biosynthesis.

Significant achievements (2018-2020):

• **Defined an electron donor protein specific for lignin biosynthesis**

In phenylpropanoid-monolignol biosynthesis, NADPH: Cytochrome P450 Reductases (CPRs) are regarded as the typical redox partners of monolignol biosynthetic P450 enzymes (C4H, C3H and F5H) to deliver electrons from reductant NADPH to the P450 catalytic center for hydroxylation of lignin monomers, thus leading to the formation of hydroxyphenyl, guaiacyl and syringyl lignin subunits. Through co-immunoprecipitation coupled with mass spectrometry analysis and the comprehensive genetic studies, we discovered that one of *Arabidopsis* cytochrome *b₅* family members, CB5D, physically associates with monolignol P450 enzymes and specifically augments F5H enzyme activity. Disruption of *CB5D* specifically depletes syringyl lignin formation, while the loss of *CPR* impairs both guaiacyl and syringyl lignin synthesis. Our study suggests that both CB5 and CPR are the indispensable electron carriers for lignin biosynthesis. While CPRs serve as the general electron donors for monolignol biosynthetic P450 enzymes, CB5D is an obligate electron shuttle protein specific for syringyl lignin formation. This study offers additional molecular tools for tailoring lignin composition and structure.

• **Revealed *Arabidopsis* Kelch domain-containing F-box proteins as the regulatory components of SnRK1-mediated carbon/energy signaling negatively regulate phenylpropanoid metabolism**

Sucrose Non-Fermenting Related Kinase 1 (SnRK1) is an evolutionarily conserved metabolic sensor that reprograms metabolism upon carbon and energy deprivation. We found that down-regulation of *Arabidopsis* SnRK1 significantly promoted the accumulation of soluble phenolics and lignin polymers and drastically increased phenylalanine ammonia lyase (PAL) cellular accumulation but did not significantly alter its transcription level, demonstrating that AtSnRK1 negatively regulates phenylpropanoid biosynthesis. Co-expression of AtSnRK1 α with PAL resulted in the severe attenuation of the latter's cellular accumulation, but protein interaction assays suggested PAL is not a direct substrate of SnRK1. Furthermore, we revealed that up- or down-regulation of AtSnRK1 positively affected the gene expression of a group of Kelch repeat domain-containing F-Box (KFB) proteins that are responsible for the ubiquitination and selective degradation of PAL. Energy starvation significantly upregulated

KFB^{PALs} expression, and the upregulation partially depends on AtSnRK1. Collectively, we conclude that *KFB^{PALs}* act as the regulatory components of the SnRK1 signaling network. They are transcriptionally regulated by SnRK1 and subsequently mediate proteasomal degradation of PAL in phenylpropanoid pathway in response to the cellular carbon/energy availability.

Science priorities for the next year (2020-2021):

- **Elucidate the *KFB^{PALs}* centered regulatory cascades in response to the cellular carbon fluctuation and light signals.** With recognition of *KFB^{PALs}* as the main players in the AtSnRK1 energy signaling network regulating phenylpropanoid metabolism, we will further explore the missing elements of the cascade, i.e. the upstream components of *KFB^{PALs}* to understand how AtSnRK1 controls the *KFB^{PALs}* gene expression in response to the cellular carbon/energy fluctuation and light signals.
- **Explore evolutionary and structural mechanisms governing the function specificity of electron donor proteins in augmenting phenylpropanoid-lignin biosynthetic P450 enzymes.** Along with the discovery of cytochrome b₅ D specifically responsible for S-lignin synthesis, we intend to explore how the electron donor cytochrome b₅ family proteins are evolved for their functional specification and what the biochemical and structural bases for distinct redox partnership of CB5D and CPR with monoglignol biosynthetic P450 enzymes.

My major scientific area(s) of expertise is/are: Biochemistry and molecular genetics of plant metabolisms, specialized in phenylpropanoid-lignin biosynthesis.

To take my project to the next level, my ideal collaborator would have expertise in: Cryo-EM based protein structure, Electrochemistry of redox proteins, Bioinformatics.

Publications supported by this project (Over The Past 3~5 Years):

1. Kim JI, Zhang X, Pascuzzi PE, Liu C-J and Chapple C “Glucosinolate and phenylpropanoid biosynthesis are linked by proteasome-dependent degradation of PAL.” *New Phytologist*, 225,154-168 (2020)
 2. Liu C-J*, Zhao Y and Zhang K “Cytokinin Transporters: Multisite Players in Cytokinin Homeostasis and Signal Distribution.” *Front. Plant Sci.*, 10, 693 (2019). doi: 10.3389/fpls.2019.00693
 3. Gou M, Yang X, Zhao Y, Ran X, Song S, Liu, C-J* “Cytochrome b5 Is an Obligate Electron Shuttle Protein for Syringyl Lignin Biosynthesis in Arabidopsis.” *Plant Cell*, 31, 1344–1366 (2019). DOI: <https://doi.org/10.1105/tpc.18.00778>
 4. Guo M, Ran X, Martin DW and Liu C-J* “The scaffold proteins of lignin biosynthetic cytochrome P450 enzymes.” *Nature Plants*, 4, 299-310 (2018). <https://doi.org/10.1038/s41477-018-0142-9>
 5. Cheng, A-X., Zhang, X., Han, X.-J., Zhang, Y.-Y., Gao, S., Liu, C.-J*, Lou, H-X.* “Identification of Chalcone Isomerase in the Basal Land Plants Reveals an Ancient Evolution of Enzymatic Cyclization Activity for Synthesis of Flavonoids.” *New Phytologist*, 217 (2), 909-924 (2018). doi: 10.1111/nph.14852
 6. Zhang, Y., Zhao L., Zhao, J., Li, Y., Wang, J., Guo, R., Gan, S., Liu, C.-J. and Zhang, K. “Arabidopsis *S5H/DMR6* Encodes a Salicylic Acid 5-Hydroxylase That Fine-Tunes Salicylic Acid Homeostasis.” *Plant Physiology*, 175, 1082-1093 (2017). DOI:10.1104/pp.17.00695
 7. Zhang, X., Abraham, C., Colquhoun, T. A., and Liu, C.-J.* “A Proteolytic Regulator Controlling Chalcone Synthase Stability and Flavonoid Biosynthesis in Arabidopsis.” *Plant Cell*, 29, 1157-1174 (2017). DOI: 10.1105/tpc.16.00855
- Gou, M., Hou, G., Yang, H., Zhang, X., Cai, Y., Kai, G., and Liu, C.-J.* “The MYB107 Transcription Factor Positively Regulates Suberin Biosynthesis.” *Plant Physiology*, 173, 1045–1058, (2017). DOI:10.1104/pp.16.01614

Mechanistic Studies of a Primitive Homolog of Nitrogenase Involved in Coenzyme F430 Biosynthesis

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Overall research goals:

Methyl-coenzyme M reductase (MCR) is the key enzyme of methanogenesis and anaerobic methane oxidation. To carry out these reactions, MCR utilizes a unique Ni-containing tetrapyrrole, coenzyme F430. This project aims to investigate the mechanism of a primitive homolog of nitrogenase (CfbCD) that catalyzes a crucial step in the coenzyme F430 biosynthetic pathway. This unprecedented reaction, which converts Ni-sirohydrochlorin *a,c*-diamide to 15,17³-seco-F430-17³-acid, involves a 6-electron reduction of the isobacteriochlorin ring system, cyclization of the *c*-acetamide side chain to form a γ -lactam ring, and the formation of 7 stereocenters. Specific goals include: 1) identification of physiological electron donors to CfbCD and *in vivo* coenzyme F430 synthesis, 2) analysis of the iron-sulfur centers, structure, and oligomerization state changes of CfbCD, and 3) characterization of transient intermediates and the intercomponent electron transfer in the CfbCD reaction.

Significant achievements (2017-2020):

It was found that coexpression of the coenzyme F430 biosynthesis (*cfb*) genes from *Methanosarcina acetivorans* with *sirAC* (which together convert the last common precursor of all tetrapyrroles, uroporphyrinogen III, to sirohydrochlorin, the substrate of CfbA) was insufficient for the production of coenzyme F430 in *Escherichia coli*. Sirohydrochlorin, Ni-sirohydrochlorin (the CfbA product), and Ni-sirohydrochlorin *a,c*-diamide (the CfbB product) could all be detected in *E. coli* extracts, suggesting that CfbCD was not functioning *in vivo*. CfbCD can be purified in an active form from *E. coli* when it is coexpressed with the iron-sulfur cluster (*isc*) biosynthetic gene cluster from *Azotobacter vinelandii* and supplied with sodium dithionite as an artificial reductant *in vitro*. Thus, it is likely that the *cfbCD* genes must be coexpressed with the *isc* operon and/or a suitable physiological reductant to be active *in vivo*. Ferredoxin (Fd) and ferredoxin-NADP⁺ reductase (FNR) from spinach together were found to reduce CfbC using NADPH as the electron donor. Furthermore, the Fd-FNR system was shown to support CfbCD-catalyzed 15,17³-seco-F430-17³-acid formation *in vitro*. However, when the *cfb* genes were coexpressed with *sirAC*, spinach Fd-FNR, and the *isc* operon, the formation of 15,17³-seco-F430-17³-acid/coenzyme F430 was still not observed. Comparative genomics was therefore utilized to identify the likely physiological electron donor to CfbCD (pyruvate:ferredoxin oxidoreductase (PFOR) from *M. acetivorans*), as well as genes that are likely involved in MCR maturation and assembly, as these may be required to bind and stabilize coenzyme F430. Compatible vectors were constructed to coexpress all of these genes together with the *cfb* pathway in *E. coli* and these are currently being tested for the heterologous production of coenzyme F430 and *holo* MCR.

A protocol was developed to synthesize and purify large quantities of Ni-sirohydrochlorin *a,c*-diamide using an engineered strain of *E. coli*, and its extinction coefficient ($\epsilon = 4.3 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at 590 nm) was determined using a combination of UV-visible spectrophotometry and inductively coupled plasma-optical emission spectroscopy (ICP-OES). Analysis of the iron and sulfide content of as-purified CfbC and CfbD was consistent with both proteins coordinating a single (partially reconstituted) [4Fe-4S] cluster at their respective homodimeric interfaces. While coexpression with the *isc* operon from *A. vinelandii* helps to improve the cluster content of CfbCD, the use of an *E. coli* cell line overexpressing the *suf* operon did not facilitate the production of *holo* CfbCD. EPR analysis of dithionite-reduced CfbC was consistent with a mixture of low- and high-spin [4Fe-4S]¹⁺ clusters. Potentiometric redox titrations of nucleotide-free CfbC gave a mid-point potential of approximately

-270 mV vs. SHE, while in the presence of ATP, the cluster potential shifts below -500 mV. Analysis of CfbC and CfbD using size-exclusion chromatography shows that both as-purified enzymes exist as mixtures of oligomerization states (dimer/monomer and dimer/tetramer, respectively). CfbCD from the thermophilic methanogen *Methanosarcina thermophila* was also prepared and found to be much more stable (i.e., did not precipitate as readily) and showed greater activity (even at room temperature) than CfbCD from the mesophilic methanogen *M. acetivorans*. Moreover, the thermophilic CfbCD purified with intact iron sulfur clusters even without coexpression with the *isc* genes. In collaboration with Dr. Cathy Drennan (Massachusetts Institute of Technology), crystallization conditions for CfbCD were identified and are being optimized for structural studies.

The observation by the Layer and Warren groups that an initial product of the CfbCD reaction is formed that slowly (and presumably non-enzymatically) converts to 15,17³-seco-F430-17³-acid was confirmed. This initial product was assumed to be Ni-hexahydrosirohydrochlorin *a,c*-diamide, which lacks the γ -lactam ring (and thus that cyclization occurs spontaneously or requires an unidentified enzyme). However, the exact structure of this species has yet to be confirmed. Another possibility is that the immediate product of the CfbCD reaction is the Ni(II)-hydride form of 15,17³-seco-F430-17³-acid, which slowly evolves hydrogen. If this is the case, in addition to facilitating cyclization, CfbCD catalyzes the addition of 8 electrons and 8 protons to Ni-sirohydrochlorin *a,c*-diamide and produces H₂ gas (analogous to the reaction catalyzed by nitrogenase) rather than the addition of 6 electrons and 7 protons (which is somewhat unusual stoichiometry). Furthermore, analysis of the stereochemistry and pseudosymmetry of the reaction product suggests that the protons could be added *anti* (one above and one below) to each of the four pyrroles within an active site containing 4-fold symmetry (e.g., at the tetrameric interface of a (CfbD)₄ core).

Science objectives for 2020-2021:

- Continue the *in vivo* studies to synthesize coenzyme F430 and *holo* MCR in *E. coli*.
- Carry out CfbCD reactions in D₂O and use NMR to determine whether deuterium/proton addition is consistent with *anti* stereochemistry. The two positions that are non-evident in the structure of 15,17³-seco-F430-17³-acid are the two (α and δ) methylene bridges, and *anti* addition predicts that the pro-*S* methylene hydrogens will be solvent derived.
- Use NMR to follow the CfbCD reaction with ¹⁵N-labeled Ni-sirohydrochlorin *a,c*-diamide to help determine if the initial product formed contains the γ -lactam ring.
- Utilize gas chromatography and/or electrochemical methods to determine whether H₂ is produced during the CfbCD reaction.
- Attempt to reduce 15,17³-seco-F430-17³-acid with sodium borohydride to generate the Ni(II)-hydride form and compare its spectral features with the immediate product of the CfbCD reaction.
- Continue our X-ray crystallographic studies to determine the high-resolution structure of the CfbCD complex.

My scientific area(s) of expertise is/are: enzymology, biophysics, functional genomics.

To take my project to the next level, my ideal collaborator would have expertise in: methanogen genetics, metabolic pathway engineering.

Publications supported by this project (2017-2020):

1. Biosynthesis of Coenzyme F430 and the Post-Translational Modification of the Active Site Region of Methyl-Coenzyme M Reductase, Mansoorabadi, S. O.; Zheng, K.; Ngo, P. D. in Metalloprotein Active Site Assembly (Johnson, M. K. and Scott, R. A., Eds.), 2017, John Wiley & Sons, Ltd, West Sussex, UK.
2. Ghebreamlak, S. M. and Mansoorabadi, S. O. (2020) Divergent Members of the Nitrogenase Superfamily: Tetrapyrrole Biosynthesis and Beyond, *ChemBioChem* 21, 1723-1728. doi:10.1002/cbic.201900782.

Redox control of ubiquitin-like modification and lysine acetylation in Archaea

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Overall research goals:

Post-translational modification (PTM) systems control metabolic flux, cell signaling, chromatin remodeling, proteostasis, redox balance and other important biological processes. Archaea and their enzymes have extremophilic properties that are desired as biocatalysts to produce renewable fuels and chemicals but are poorly characterized in terms of understanding how PTM may control redox balance, metabolic flux and carbon sequestration. The **overall aim of this project** is to determine how ubiquitin-like (Ubl) modification, proteasomes and lysine acetylation are associated with redox balance and metabolic control in archaea. The **specific goals of this project** are: 1. To establish how methionine sulfoxide reductase A (MsrA) may act as a redox 'switch' in Ubl ligation and to identify components of Ubl ligation system that appear 'missing' in the *in vitro* reconstitution assay. 2. To determine the redox-dependent interaction hubs of the Ubl proteasomal network (substrates, AAA ATPases and 20S proteasome core particles) guided by our recent finding that deletion of the 20S proteasomal $\alpha 1$ and dysregulation of the Rpt-like AAA ATPase PAN2 enhance tolerance of cells to the potent oxidant hypochlorite. 3. To determine the molecular targets of lysine acetylation that are associated with hypochlorite stress and examine how these PTMs may be coordinated with Ubl ligation to control redox balance. Our focus on lysine acetylation is based on finding that hypochlorite stress selects for lysine deacetylase *sir2* mutants and induces an increase in the abundance ratio of the lysine acetyltransferase Pat2:Pat1 homologs based on multiplex SILAC LC-MS/MS analysis.

The project relies upon biochemical, quantitative proteomic, transcriptomic and genetic approaches to examine these PTM control mechanisms in *Haloferax volcanii*, an archaeon that thrives in hypersaline conditions on glycerol (prevalent in biodiesel waste) and can be metabolically engineered. This archaeon is related to methanogens of the phylum *Euryarchaeota* and uses an unusual salt-in strategy for osmoregulation, whereby its proteins have an acidic shell. These adaptations enable biocatalytic function in concentrated salt and low water activity. These traits are beneficial to biocatalysis in organic solvent such as used to depolymerize biomass. The long-term expected outcomes of this project are to generate new knowledge related to PTMs that may be used to optimize archaea and their enzymes as biocatalysts to generate bioenergy and sequester carbon under extreme conditions.

Significant achievements (2019-2022):

- MsrA is found to stimulate Ubl ligation by an activity that is distinct from its stereospecific reduction of MSO (methionine sulfoxide).
- A new type of Moco-dependent Msr (MsrM) is discovered that reduces MSO, yet is not required for Ubl ligation. MsrM is purified to high yield and activity. The S- and R-forms of MSO are synthesized and confirmed by NMR analysis. A high throughput protocol is developed to determine the kinetic constants of Moco-dependent Msr enzymes. K_m and V_{max} values of MsrM with DMSO, MSO, MSO-R and MSO-S are now elucidated. A methionine auxotroph is found to require *msrM* for growth on MSO and specific epimers.
- The standalone rhodanese domain (RHD) protein UbaC is identified as a 'missing component' of the Ubl ligation (samylation) and sulfur mobilization pathways. UbaC is required for Ubl samp2/3ylation during oxidative stress and after proteasome inhibition. Phenotypes associated with Moco biosynthesis and 2-thiolation of wobble uridine tRNA are observed in *ΔubaC* mutants.
- Lysine acetylation and Ubl ligation have synergistic and/or antagonistic roles during redox stress in archaea. *H. volcanii* is found to have an increased abundance of Ubl modified and lysine acetylated

proteins under these conditions. A lysine deacetylase *Asir2* mutant is found hypertolerant of hypochlorite, while Ubl modification system mutants are oxidant sensitive.

- *H. volcanii* is found to form biofilms/flocs during microaerobic growth on glycerol when the lysine acetyltransferase *pat2* homolog is disrupted; the *Δpat2* mutant shifts to planktonic growth when exposed to oxidant. A Pat1 N-terminal CX₅CX₃C motif, coding sequence overlap of *usp1* and *pat2*, and two conserved lysine residues (Kx and Ky) of Usp1 on opposite faces of the 3D structural models are identified. A model for lysine acetylation and its regulation of the formation of biofilms/flocs during redox shifts is proposed. Under non-oxidizing conditions when Pat2 levels are limiting, Pat1 is thought to acetylate Usp1 at Ky, thus, signaling biofilm formation. As the level of oxidant rises, the N-terminus of Pat1 forms disulfide bonds with small thiols, itself or other protein partners. This oxidation favors the lysine acetylation of the Kx residue of Usp1 and renders cells in a planktonic state. This model will be tested by *Δpat2 Δusp1* double mutation and site directed exchange of Usp1 lysine residues to control biofilm formation in archaea.

Science priorities for the next year (2020-2021):

- Identify the molecular targets of lysine acetylation that are associated with oxidative stress and examine how these PTMs may be coordinated with Ubl ligation to control redox balance.
- Determine the redox-dependent interaction hubs of the Ubl proteasomal network (substrates, AAA ATPases and 20S proteasome core particles).

My major scientific areas of expertise are: Metabolism, stress responses, post-translational modification, biochemistry, quantitative proteomics, genetics and functional genomics of microorganisms with emphasis on archaea from hypersaline environments.

To take my project to the next level, my ideal collaborator would have expertise in: Cell imaging technologies for monitoring of protein:protein interactions and other processes related to archaeal cell biology in real-time.

Publications supported by this project (2019-present):

1. Rawat M and **JA Maupin-Furlow**. 2020. Redox and thiols in Archaea. *Antioxidants*. 9(5), 381; doi: 10.3390/antiox9050381.
2. Jiang, B, Z Adams, S Moonah, H Shi, **JA Maupin-Furlow** and J Moskovitz. 2020. The antioxidant enzyme, methionine sulfoxide reductase A (MsrA), interacts with Jab1/CSN5 and regulates its function. *Antioxidants* 9(5), 452; doi: 10.3390/antiox9050452.
3. Schulze, S, Z Adams, M Cerletti, R De Castro, S Ferreira-Cerca, C Fufezan, MI Giménez, M Hippler, Z Jevtic, R Knüppel, G Legerme, C Lenz, A Marchfelder, **J Maupin-Furlow**, RA Paggi, F Pfeiffer, A Poetsch, H Urlaub and **M Pohlschroder**. 2020. The Archaeal Proteome Project advances knowledge about archaeal cell biology through comprehensive proteomics. *Nature Comm.* 11(1):3145. doi: 10.1038/s41467-020-16784-7.
4. Montalvo-Rodríguez R and **JA Maupin-Furlow**. 2020. Insights through genetics of halophilic microorganisms and their viruses. *Genes (Basel)*. 11(4): E388. doi: 10.3390/genes11040388
5. Berglunda J, R Gjondrekaja, E Verneya, **J Maupin-Furlow** and MJ Edelmann. 2020. Modification of the host ubiquitome by bacterial enzymes. *Microbiol. Res.* 235:126429. doi: 10.1016/j.micres.2020.126429.
6. Hwang, S, NE Chavarria, RK Hackley, AK Schmid and **JA Maupin-Furlow**. 2019. Gene expression of *Haloferax volcanii* on intermediate and abundant sources of fixed nitrogen. *Int. J. Mol. Sci.* 20(19): E4784. doi: 10.3390/ijms20194784
7. Fu, X, Z Adams and **JA Maupin-Furlow**. 2019. Assays for ubiquitin-like protein ligation and proteasome function in archaea. In M. Hochstrasser (Ed). *Methods Enzymol.* 169:161-178. doi: 10.1016/bs.mie.2018.12.036.
8. Hepowitz, NL and **JA Maupin-Furlow**. 2019. Rhodanese-like domain protein UbaC and its role in ubiquitin-like protein modification and sulfur mobilization in Archaea. *J Bacteriol.* 201(15): e00254-19 doi: 10.1128/JB.00254-19. (*Journal Spotlight*).
9. Maupin-Furlow JA. 2019. Putting phage to work in deubiquitinase ligand discovery. *J. Biol. Chem.* 294(2):437-438. doi: 10.1074/jbc.H118.006803.
10. Maupin-Furlow JA. 2019. Stepping up in protein degradation. *Proc Natl Acad Sci U S A.* 116(2):350-352. doi: 10.1073/pnas.1819949116.

**Energy conservation, electron transfer and enzymology during methane production by
Methanosarcina species**

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Overall research goals:

The long-term goal of the proposed research is to develop a comprehensive understanding of the enzymes, electron carriers and metabolic pathways that comprise the energy-conserving electron transport network of methane-producing Archaea. Using the genetically tractable *Methanosarcina acetivorans* as a model organism, we will focus on two unsolved questions that lie at the center of methanogenic metabolism. The first addresses the biosynthesis, essentiality and mechanistic function of the extensive post-translational modifications found in methyl-coenzyme M reductase (MCR), an enzyme that plays a central role in anoxic production and consumption of methane. The second examines how low potential electrons are partitioned between donors and acceptors within the cell using small iron-sulfur proteins known as ferredoxins. Our experimental approach combines genetic, biochemical and biophysical approaches to develop a holistic understanding of the processes that govern flow of electrons during CO₂ assimilation and reduction to methane, which is a central research goal of the Physical Biosciences program. The planned studies on the post-translational modification of methyl-coenzyme M reductase (MCR) will include identification of the genes required for the installation of post-translational modifications, determination of the viability of mutants that lack these post-translational modification genes, physiological characterization of all viable mutants, and structural and biochemical characterization of unmodified MCR derivatives. Our studies of the ferredoxin-dependent electron-transfer networks in *Methanosarcina* will entail examination of the viability of mutants lacking each of the thirteen *Methanosarcina* Fd-encoding genes, establishing the protein interaction network for each ferredoxin, characterizing the metal content and redox properties for each ferredoxin, and determining the structures for each ferredoxin via protein crystallography, cryoelectron microscopy or solution NMR. Our specific aims for the current funding period are: (1) biochemically characterize MCR derivatives with and without the post-translational modifications found in most methanogens; (2) identify genes required for the synthesis of remaining post-translationally modified MCR residues and characterize of mutants that lack these genes; and (3) elucidate the ferredoxin-dependent electron-transfer networks in *Methanosarcina*.

Significant achievements (2018-2020):

- We have identified the genes required for installation of thioglycine, methyl-cysteine and methyl-arginine in *M. acetivorans*. Mutants lacking these genes in all possible combinations have been constructed and phenotypically characterized.
- We have identified the gene required for installation of methyl-glutamine in other methanogens and created recombinant *M. acetivorans* strains that express this gene in the wild-type strain and in modification-minus mutants. (Note: the methyl-glutamine modification is not found in *Methanosarcina* species).
- We have determined the crystal structure of *M. acetivorans* MCR, including variants lacking thioglycine, methyl-cysteine and methyl-arginine modifications, at 1.6 angstrom resolution (*in collaboration with Dr. Satish Nair, Department of Biochemistry, University of Illinois*).

- We have constructed and characterized mutants five small ferredoxins. These include the two associated with 2-oxo-acid:ferredoxin oxidoreductase operons (MA2911, MA0033), two that are associated with geranylgeranyl reductases involved in isoprenoid biosynthesis (MA1485, MA1493) and one (MA0431) that is believed to be the major ferredoxin in the cell.
- Purified the enzyme pyruvate:ferredoxin oxidoreductase from *M. acetivorans* and determined in crystal structure at 1.9 angstroms in the presence and absence of substrates.

Science priorities for the next year (2020-2021):

- Develop a protocol for reductive activation of MCR from *M. acetivorans*
- Phenotypic characterization of *M. acetivorans* strains with methylglutamine modified MCR
- Biochemical characterization of modified MCR variants
- Establish the post-translational modification of phylogenetically diverse MCRs by mass-spectrometry
- Identify the genes required for the installation of the remaining post-translational modifications using a comparative genomic approach.
- Biochemical characterization of *M. acetivorans* five ferredoxins listed above
- Determine the structures for each ferredoxin via protein crystallography or solution NMR

My major scientific area(s) of expertise is/are: Genetic analysis of diverse microorganisms, Energy conservation in anaerobes, Natural product metabolism.

To take my project to the next level, my ideal collaborator would have expertise in: EPR spectroscopy, electrochemical characterization of enzymes.

Publications supported by this project:

1. Kulkarni, G., T.D. Mand and W.W. Metcalf. 2018. Energy Conservation via Hydrogen Cycling in the Methanogenic Archaeon *Methanosarcina barkeri*. *mBio* 9:e01256-18. PMID: 29970471.
2. Mand, T.D., G. Kulkarni and W.W. Metcalf. 2018. Genetic, biochemical, and molecular characterization of *Methanosarcina barkeri* mutants lacking three distinct classes of hydrogenase. *J. Bacteriol.* 200: e00342-18. PMID: 30012731..
3. Nayak, D.D. and W.W. Metcalf. 2018. Genetic techniques for studies of methyl-coenzyme M reductase from *Methanosarcina acetivorans* C2A. *Methods Enzymol.* 613:325-347. PMID: 30509472.
4. Mand, T.D. and W.W. Metcalf. 2019. Energy conservation and hydrogenase function in methanogenic archaea with a focus on the genus *Methanosarcina*. *Microbiol. Mol. Biol. Rev.* 83: pii: e00020-19. PMID: 31533962.
5. Nayak, D.D. and W.W. Metcalf. 2019. Methylamine-specific methyltransferase paralogs in *Methanosarcina* are functionally distinct despite frequent gene conversion. *ISME J. in press.* PMID: 31053830.
6. Zeng Z., X.L. Liu, K.R. Farley, J.H. Wei, W.W. Metcalf, R.E. Summons and P.V. Welander. 2019. GDGT cyclization proteins identify the dominant archaeal sources of tetraether lipids in the ocean. *Proc. Natl. Acad. Sci.* 116:22505-22511. PMID: 31591189.
7. Nayak, D.D., A. Liu, N. Agrawal, R. Rodriguez-Carero, S.-H. Dong, D.A. Mitchell, S.K. Nair and William W Metcalf. 2020. Functional interactions between post-translationally modified amino acids of methyl-coenzyme M reductase in *Methanosarcina acetivorans*. *PLOS Biol.* 18:e3000507. PMID: 32092071, PMC7058361.

Conformational and Chemical Dynamics for Single Proteins in Solution by Suppression of Brownian Motion

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Overall research goals:

The primary objective of this research is to study and understand the behavior of individual photosynthetic proteins involved in light capture, energy transfer, and carbon fixation, all in a solution environment without surface attachment. Single-molecule measurements have been firmly established as a powerful technique for elucidating mechanistic details of complex biological processes which are normally obscured in ensemble-averaged techniques. These measurements are most insightful when they are applied over a long enough time to watch a single protein undergo a statistically meaningful number of state transitions. Unfortunately, the immobilization of biomolecules that commonly enables this long time window is often undesirably perturbative. Unlike these tethered studies, our Anti-Brownian Electrokinetic (ABEL) trap enables the extended observation of individual proteins in free solution and is a critical tool which has led to deeper understanding as a complement to bulk studies.

Significant achievements (2017-2020):

- As an extension of our successful previous studies of single LH2 and LHCII in the ABEL trap to measure the dynamics of exciton transfer, we completed a multivariate study of one of the key pigment-protein complexes, C-phycocyanin (CPC), a three-pigment antenna protein that binds to the allophycocyanin (APC) cores to form the rods of phycobilisomes in cyanobacteria (Pub. 1). Simultaneous measurement of brightness, polarization, lifetime, and spectrum for individuals led to a deep understanding of the energy transfer pathways and states available to CPC proteins under optical irradiation.
- Going beyond indirect fluorescence reporter variables, we used the ability of the ABEL trap to measure diffusivity of single objects to quantify the oligomerization of Rubisco activase, a key enzyme in the regulation of Rubisco (collaboration with R. Wachter, Arizona State, Pub. 2). Depending upon the nucleotide available, the steady-state distribution of the proteins among various oligomeric states could be measured along with dynamics of oligomer assembly and disassembly.
- To extend the ABEL trap to additional nanoscale variables, we showed it is possible to characterize rotational diffusion of single biomolecules in solution via fluorescence lifetime anisotropy decay. This measure of rotational diffusion gives access to *volume* estimates of the trapped object as a complement to estimates of hydrodynamic radius from translational diffusion (Pub. 3, highlighted in *Nature Nanotech* **2018**, *13*, 769).
- By trapping single phycobilisomes from our collaborator, R. Blankenship, that were quenched by the Orange Carotenoid Protein (OCP) we discovered a fascinating new aspect of this unique NPQ mechanism (Pub. 4). We used the multi-variate measurement capability of the ABEL trap to identify and dissect the various quenched populations and found the presence of two distinct quenched states attributable to either one or two OCP proteins bound per phycobilisome.
- We completed the first demonstrations of an extension of the ABEL trap to grab individual objects without requiring fluorescence using interferometric scattering detection, affording a new device we term the “ISABEL” trap (Pubs. 5,6). We successfully trapped single 20 nm Au nanoparticles, semiconductor quantum dots, and fluorescent beads without fluorescence, opening up a new arena of photosynthetic assemblies to exploration.

Science priorities for the next year (2020-2021):

We will employ the ABEL and newly developed ISABEL traps to gain mechanistic insight into photosynthetic light harvesting and carbon fixation in variable environments, by observing heterogeneity from the single-molecule to membrane fragment scales. This work will provide direct measurements of the behavior of crucial photosynthetic machinery in free solution without surface attachment. Direct dissection of heterogeneous populations by combining multiparameter optical measurements and structural information from correlative cryo-electron microscopy (Pub. 7), molecule by molecule, will allow observation of the different photophysical states and correlations between parameters that allow for additional mechanistic insight. This general plan will begin by several thrusts:

- In collaboration with Doran Bennet (SMU) and Nir Keren (Hebrew Univ), when sample are available, we will begin investigation of the multifaceted role of the IsiA antenna in IsiA-PSI super complexes under iron-restricted growth conditions.
- We will build an enhanced version of the new ISABEL trap with spectrally distinct trapping and probing wavelengths, so that weak or fluctuating emissive molecules can be probed.
- Through a collaboration with David Savage (UC Berkeley), we will initiate trapping studies of individual carboxysomes in the ISABEL trap and optimize trapping conditions. Measurement of scattering cross sections will generate distributions of sizes of these critical carbon-fixing structures housing RuBisCO, in preparation for future measurements using fluorescent readouts.
- With Arthur Grossman (Stanford), we will initiate correlative fluorescent and structural studies of single thylakoid membrane patches from *Chlamydomonas*.

My major scientific area(s) of expertise is/are: physical chemistry and chemical physics of single molecules, single-molecule biophysics, photodynamics of photosynthetic protein and redox enzymes, super-resolution imaging and tracking beyond the diffraction limit in cells, correlative electron and super-resolution microscopy, and trapping of single molecules in solution.

To take my project to the next level, my ideal collaborator would have expertise in: expression and purification of photosynthetic antenna pigment-protein complexes, reaction centers, or carbon fixation assemblies, biochemistry of oligomeric protein complexes, site-specific mutation and fluorescent reporter protein labeling and validation.

Publications supported by this project 2017-2020:

1. Squires, A. H.; Moerner, W. E. "Direct Single-Molecule Measurements of Phycocyanobilin Photophysics in Monomeric C-Phycocyanin," *Proc. Natl. Acad. Sci. U. S. A.* **114**, 9779-9784 (2017). DOI: 10.1073/pnas.1705435114
2. Wang, Q.; Serban, A. J.; Wachter, R. M.; Moerner, W. E. "Single-Molecule Diffusometry Reveals the Nucleotide-Dependent Oligomerization Pathways of Nicotiana Tabacum Rubisco Activase," *J. Chem. Phys.* **148**, 123319 (2018). DOI: 10.1063/1.5005930
3. Yang, H. and Moerner, W.E. "Resolving Mixtures in Solution by Single-Molecule Rotational Diffusivity," *Nano Lett.* **18**, 5279-5287 (2018). DOI: 10.1021/acs.nanolett.8b02280
4. Squires, A. H.; Dahlberg, P. D.; Liu, H.; Magdaong, N. C. M.; Blankenship, R. E.; Moerner, W. E. "Single-molecule trapping and spectroscopy reveals photophysical heterogeneity of phycobilisomes quenched by Orange Carotenoid Protein." *Nature Commun* **10**, 1172 (2019). DOI: 10.1038/s41467-019-09084-2
5. Squires, A. H.; Lavania, A. A.; Dahlberg, P. D.; Moerner, W. E. "Interferometric Scattering Enables Fluorescence-Free Electrokinetic Trapping of Single Nanoparticles in Free Solution," *Nano Lett.* **19**, 4112-4117 (2019). DOI: 10.1021/acs.nanolett.9b01514
6. Lavania, A. A.; Squires, A. H.; Dahlberg, P. D.; Moerner, W. E. "Interferometric scattering for fluorescence-free electrokinetic trapping of single nanoparticles in free solution," *Proc. SPIE* **11246**, 112460W (2020) DOI: 10.1117/12.2546638
7. Dahlberg, P. D.; Perez, D.; Su, Z.; Chiu, W.; Moerner, W. E. "Cryogenic Correlative Single-Particle Photoluminescence Spectroscopy and Electron Tomography for Investigation of Nanomaterials," *Angew. Chemie* (in press, 2020) DOI: 10.1002/anie.202002856

Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosynthetic Reaction Centers

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Overall research goals:

The basic photosynthetic architecture consists of antenna complexes to harvest solar energy and reaction centers to convert the energy into a stable charge separated state. In oxygenic photosynthesis, the initial charge separation event occurs with near unit quantum efficiency in the photosystem II reaction center (PSII RC). PSII is the only known natural enzyme that uses solar energy to split water, making the elucidation of its design principles critical for our fundamental understanding of photosynthesis and for our ability to mimic PSII's remarkable properties. The reaction center in purple bacteria (BRC) bears many similarities to the PSII RC, but provides a spectroscopically simpler system to study. The charge separation process in the BRC is better understood, making it an appealing model system for developing new experimental and theoretical approaches. This proposal focuses on key deficits in our current understanding of the PSII RC and remaining open questions about the BRC. We propose a synergistic series of experiments on wild-type and mutant RCs and simpler dyad systems aimed at addressing the following questions:

- 1) *What is the electronic structure of the PSII RC and the BRC?*
- 2) *What are the charge separation pathways in the PSII RC?*
- 3) *Do electronic-vibrational resonances enhance energy transfer and charge separation in the PSII RC and the BRC?*

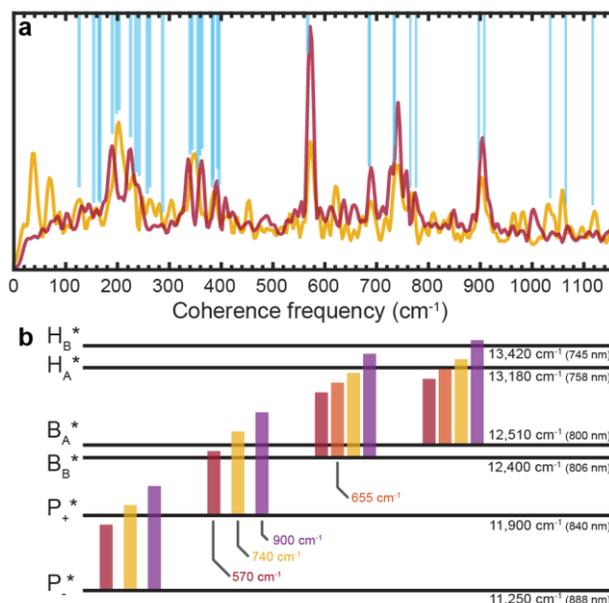
Significant achievements (2019-present):

Towards addressing question 1) we studied the electronic structure of chlorophyll a (Chl a) and bacteriochlorophyll a (Bchl a) in order to better understand vibronic states and the nature of the Q_x and Q_y states in these systems. These studies lay an important foundation for understanding the broadband 2D spectroscopy studies we have performed on the PSII RC and BRC systems. The excitonic models of the PSII RC and BRC in the Q_y region typically ignore the contributions from the vibronic progression. Bchl a and Chl a play important roles as light absorbers in photosynthetic antennae and participate in the initial charge-separation steps in photosynthetic reaction centers. Despite decades of study, questions remain about the interplay of electronic and vibrational states within the Q-band and its effect on the photoexcited dynamics. Using polarized two-dimensional electronic spectroscopic (2DES) measurements, we studied penta-coordinated Bchl a and Chl a, interpreting the results based on state-of-the-art time-dependent density functional theory calculations and vibrational mode analysis for spectral shapes¹. We found that the Q-band of Bchl a is comprised of two independent bands, that are assigned following the Gouterman model to Q_x and Q_y states with orthogonal transition dipole moments. However, we measure the angle to be 75 degrees, a finding that is confirmed by ab initio calculations. The internal conversion rate from Q_x to Q_y is found to be 11 ps. Unlike Bchl a, the Q-band of Chl a contains three distinct peaks with different polarizations. Ab initio calculations trace these features to a spectral overlap between two electronic transitions and their vibrational replicas. The smaller energy gap and vibronic state mixing yields faster internal conversion of 38–50 ps.

Towards addressing question 2) we have developed 2D electronic-vibrational (2DEV) spectroscopy and have demonstrated the method on a dye system². We have applied the method to preliminary studies of the PSII RC, using a mid-IR probe to detect charge separation. In combination with our previous data from the Q_y region, and broadband probe data across the visible region are testing excitonic models and mechanistic models of charge separation.

To address question 3) we have been focusing on the coherent dynamics of the BRC due to its better spectral separation in the Q_y region. Interestingly, we have found that the coherent dynamics of the BRC reveal previously hidden excitonic and vibronic structure. Through analysis of the coherent dynamics we can unambiguously assign the upper exciton energy of the special pair. We also observe vibronic coherence transfer processes that are frequently neglected in models of photosynthetic energy transfer. The data also reveals resonances between a number of key intramolecular pigment vibrations and electronic energy gaps in the BRC. Such resonances have been proposed to play a functional role in photosynthetic energy transfer and charge separation. We are currently working in collaboration with Tomas Mancal to model the spectroscopic signatures of coherence and their possible importance for charge separation and are currently writing up these results for publication.

Figure 1: (a) Coherence frequencies observed in 2DES studies of the M250V BRC (red) and bacteriochlorophyll a (orange). Blue lines indicate vibrational modes of BChl and Bacteriopheophytin a that lie within the 9.7 cm^{-1} resolution of the most prominent peaks from our measurement as independently identified by resonance Raman studies. It is worth noting that the BRC coherence spectrum is largely dominated by B-band contributions and that several lower signal strength modes are in good agreement with vibrational spectroscopy reported modes and exciton difference frequencies such as the $\omega_2 = 656\text{ cm}^{-1}$ mode. (b) The energy level diagram with exciton energies taken from analysis of our 2DES spectra demonstrate how prominent coherence modes bridge energetic gaps between excitonic states, revealing electronic-vibrational resonances in the BRC.



Science priorities for the next year (2020-2021):

- 2DEV experiments of the PSII RC
- Tests of excitonic models of the PSII RC and BRC using multispectral 2DES
- Multispectral 2DES measurements of BRC mutants to probe the importance of electronic-vibrational resonance on charge separation mechanism

My major scientific area(s) of expertise is/are: Multidimensional spectroscopy and nonlinear microscopy, energy transfer and charge separation processes.

To take my project to the next level, my ideal collaborator would have expertise in: Electronic structure and quantum chemical calculations, simulations of spectroscopic signals. Biochemist with the ability to make site-directed mutants.

Publications supported by this project (2019-present):¹⁻⁵

- 1 Song, Y. *et al.* Vibronic structure of photosynthetic pigments probed by polarized two-dimensional electronic spectroscopy and ab initio calculations. *Chemical Science* **10**, 8143, doi:10.1063/1.5055244 (2019).
- 2 Song, Y. *et al.* Multispectral multidimensional spectrometer spanning the ultraviolet to the mid-infrared. *Review of Scientific Instruments* **90**, doi:10.1063/1.5055244 (2019).
- 3 Wasielewski, M. R. *et al.* Exploiting chemistry and molecular systems for quantum information science. *Nature Reviews Chemistry*, doi:10.1038/s41570-020-0200-5 (2020).
- 4 Nguyen, H., Loukianov, A., Ogilvie, J. P. & Abramavicius, D. Two-dimensional Electronic Stark Spectroscopy of a Photosynthetic Dimer. *Journal of Chemical Physics* **submitted** (2020).
- 5 Cao, J. S. *et al.* Quantum biology revisited. *Science Advances* **6**, doi:10.1126/sciadv.aaz4888 (2020).

Cell-Type Specific Pectins in Plant Cell Walls: Structure, Interaction and Function

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Overall research goals:

Rhamnogalacturonan-II (RG-II) is a structurally complex pectic polysaccharide that exists as a borate ester crosslinked dimer in the primary cell walls of vascular plants. The inability to form this dimer results in abnormal cell walls, which impairs growth and substantially reduces a plant's ability to capture, convert, and store energy. We have shown that RG-II structure, pH, and divalent cations affect the rate and extent of dimer formation *in vitro* and *in planta*. We are now using experimental (chemical, enzymatic, and spectroscopic) and computational (molecular modeling and quantum mechanical calculations) methods to develop a molecular-level understanding of dimer self-assembly. We will identify critical structural features of RG-II and unravel the molecular level interactions that drive dimer self-assembly. New insight into this unique and site-specific crosslinking may facilitate the production of new, Nature-inspired, borate-diol based materials with functional properties that can be readily modulated.

Significant achievements (2018-2021):

We found that celery RG-II has the minimum naturally occurring structure required for dimer self-assembly (O'Neill et al 2020). Celery RG-II differs from many RG-IIs including wine, because its B sidechain Arap lacks the Araf-Rha- and Rha- extension at O-2 and O-3, respectively. Nevertheless, celery RG-II forms a dimer *in vitro* at a rate slightly higher than wine RG-II.

Our previous NMR studies of sidechain B generated from wine RG-II led us to propose that the 2,3-linked Arap residue interconverts between the ⁴C₁ and ¹C₄ ring pucker forms (Glushka et al 2003 Carbohydr Res 338:341). We extended these studies by comparing wine and celery RG-II dynamics using multidimensional NMR spectroscopy. Temperature-dependent conformation changes were discernible with the wine monomer but not with its celery counterpart. The 2,3-linked Arap of wine RG-II exchanges between ring conformations with ¹C₄ predominating. The Arap of celery RG-II, which lacks substituents at O-2 and O-3, adopts a ⁴C₁ conformation that is stable at different temperatures. *In silico* modeling is consistent with an increased frequency of Arap pucker-flipping at higher temperatures and when the Arap is substituted at O-2 and O-3. Moreover, the energy barrier to pucker-flipping is lower when the Arap is substituted at O-2 and O-3. Such data provides a basis for understanding the effects of ring flipping on RG-II properties and function.

The B sidechain of celery RG-II Arap lacks the Rha- and Araf-Rha- extension. Thus, it is a substrate for the *Bacteroides thetaiotaomicron* glycanases that remove the Me-Fuc and Arap residues from this sidechain (Ndeh et al 2017). Our preliminary studies indicate that removing Arap reduced the rate and extent of dimer formation. Dimer formation is further reduced by removing both Arap and MeFuc. Very little dimer was formed after removing Arap and MeFuc and the L-Gal from chain A. Thus, both the A and B sidechains may be required for normal dimer formation.

We have used *ab initio* quantum mechanical calculations and transition state theory to map the reaction energy landscape, identify barriers, and characterize reaction pathways for crosslinking two apiose (Api)

molecules with boric acid to form Api₂-borate (Bharadwaj et al *submitted*). The reaction is initiated when boric acid forms an ester linkage with the C-3 hydroxy group of Api. A second ester linkage is formed at the C-2 hydroxy group to generate Api₁-boric acid. Another ester linkage is then formed between Api₁-boric acid and a second Api. This reaction may occur at the C-2 or the C-3 hydroxy oxygen of the second Api and thus results in regio-isomers. A second chelation event culminates in the formation of Api₂-borate in which the boron is chiral. Our key insights are: (i) the first ester linkage is regiospecific for the C-3 hydroxy oxygen of Api and is rate determining; (ii) all reactions involve breaking boron-oxygen bonds and result in a condensed water molecule containing the oxygen from boric acid; and (iii) four unique, energetically similar, reaction pathways for forming the R and S stereo-isomers of Api₂-borate were identified and characterized.

Science priorities for the next year (2020-2021):

Complete experimental and computational studies of Arap ring-puckering in the RG-II monomer and dimer. Confirm that removing Arap and MeFuc reduces the rate of dimer formation *in vitro*. Generate and use apiogalacturonides to model borate ester formation and crosslinking. Complete chemical and enzymatic methods to determine sidechain spacing along backbone. Evaluate proximity-dependent labeling/TurboID (Mair et al 2019 eLife 8:e47864) to identify candidate genes for RG-II glycosyltransferases. Generate selected RG-II glycoforms and fragments using *B. thetaiotaomicron* mutants (Ndeh et al 2017). Use these glycoforms and fragments to refine RG-II structure, to probe structural features required for cross-linking, and to assess sequencing of RG-II using Fourier transform ion cyclotron resonance mass spectrometry.

Our major scientific area(s) of expertise is/are: Plant cell walls, polysaccharide structure and function, computational modeling using molecular dynamics (MD) and quantum mechanical (QM) simulations.

To take our project to the next level, our ideal collaborator would have expertise in: (i) synthetic carbohydrate chemistry to synthesize specific RG-II structures. (ii) Solid state NMR to study the walls of Rose cells grown in the presence and absence of boron.

Publications supported by this project:

Ndeh, D et al. Complex pectin metabolism by gut bacteria reveals novel catalytic functions. Nature 554, 65(2017).DOI: 10.1038/nature21725.

Shi, D-A et al. Boron-bridged RG-II and calcium are required to maintain the pectin network of the Arabidopsis seed mucilage ultrastructure. Plant Mol Biol 94, 267 (2017). DOI:10.1007/s11103-017-0606-8

Avci, U et al. Changes in the abundance of cell wall apiogalacturonan and xylogalacturonan and conservation of rhamnogalacturonan II structure during the diversification of the Lemnoideae. Planta 247, 953–971 (2018). DOI: 10.1007/s00425-017-2837-y

Sechet, J et al. Suppression of Arabidopsis GGLT1 affects growth by reducing the L-galactose content and borate cross-linking of rhamnogalacturonan-II. Plant Journal 96, 1036–1050 (2018). DOI: 10.1111/tbj.14088

Smith, JA et al. Identification of an apiosyltransferase in the plant pathogen *Xanthomonas pisi*. PLOS ONE 13: e0206187 (2018). DOI: 10.1371/journal.pone.0206187

Soto, M et al. Plant fucosyltransferases and the emerging biological importance of fucosylated plant structures. Crit Rev Plant Sci 38, 327 (2019). DOI: 10.1080/07352689.2019.1673968

O'Neill, MA et al. Locating methyl-etherified and methyl-esterified uronic acids in the plant cell wall pectic polysaccharide rhamnogalacturonan II. SLAS Technol 25, 329 (2020). DOI: 10.1177/2472630320923321

Novel microbial based enzymatic CO₂ fixation mechanisms: conformational control of enzymatic reactivity

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Overall research goals:

The broad, long term goal of the research is to provide insights into the mechanism of novel carboxylation and electron transfer reactions that use large scale conformational changes to control reactivity. These reactions generate unstable intermediates that need to be either directed down a certain reaction pathway or protected from side reactions such as decomposition reactions in aqueous solvents. In many systems, this level of control of reactivity is accomplished through large scale conformational change triggered by specific reaction events.

The project is divided into three aims to probe three different fundamental mechanisms of conformational control of reactivity. The first aim examines the mechanism of how acetone carboxylase (AC) couples ATP binding and reaction of the substrates acetone and bicarbonate with large conformational changes which protect ATP hydrolysis and the delivery of reactive intermediates to a remote metal site. This aim also investigates the identity of this metal and its involvement in catalysis. The second aim investigates how 2-ketopropyl-coenzyme M oxidoreductase/carboxylase (2-KPCC) couples the binding of substrate with conformational changes that encapsulate 2-ketopropyl coenzyme M (2-KPC), allowing the enzyme to discriminate between the substrate electrophiles CO₂ and protons from the aqueous solvent. The final aim investigates how conformational changes gate electron flow in FixABCX, an electron bifurcating member of the Etf family of enzymes.

Significant achievements (2019-2020):

The mechanism of 2-ketopropyl coenzyme M oxidoreductase carboxylase (2-KPCC)

During this grant period, we have made significant progress in investigating how the 2-KPCC structure promotes carboxylation of substrate by carrying out hydrogen-deuterium exchange (HDX) experiments and protein digest experiments in the presence and absence of various substrates and inhibitors. Through these experiments, it was determined that 2-KPCC adopts a more rigid structure upon the binding of the inhibitor bromoethanesulfonate (BES) which irreversibly binds to the active site cysteine. This more rigid form aids to exclude protons from solvent, thereby excluding the undesirable protonation reaction. To understand how 2-KPCC might exclude protons to promote carboxylation, we investigated specific differences between 2-KPCC and disulfide oxidoreductase (DSOR) enzymes. All other DSORs contain a conserved HXXXXE motif and carry out a protonation reaction while 2-KPCC contains a conserved FXXXXH (F501 and H506) motif and carboxylates its substrate. We proposed that this anion binding pocket is mechanistically important in stabilizing the developing charge incurred during acetoacetate formation and H506 appears to be a key residue in carboxylate stabilization. We solved the structures for the two 2-KPCC variants F501H and F501H_H506E and conducted biochemical investigations on these two variants to better understand the linkage between the differences in the conserved DSOR catalytic dyad residues and the unique carboxylating activity of 2-KPCC. The crystal structure of the F501H variant was determined and found to be in a different resting state than the native 2-KPCC. An additional structure of the double variant to fully mimic DSORs catalytic dyad was determined providing additional insights into the role of these key residues in carboxylating activity. These newly determined structures coupled with new biochemical data of the F501 and H506 variants, suggests that 2-KPCC functions through a different reactive intermediate than other members of the DSOR family. The deviation from the conserved catalytic dyad residues observed in all other members of the DSOR family is key in supporting coupling reductive cleavage and carboxylation.

The mechanism of ATP-Dependent acetone carboxylation by acetone carboxylase

Acetone carboxylases (ACs) are found in bacteria that can utilize isopropanol and acetone as sole carbon sources and catalyze the ATP dependent carboxylation of acetone. We have been examining acetone

carboxylases from a variety of different bacterial sources and have observed different biochemical and catalytic properties. We have recently determined the structure of an acetone carboxylase from *Xanthobacter autotrophicus* revealing the details of the Mn²⁺ containing active site (**Figure 1A**). Interestingly, previous studies indicate that the highly homologous (69% identity) ACs from *X. autotrophicus* and *Aromatoleum aromaticum* have differing metal content and catalyze acetone carboxylation with different stoichiometry with *A. aromaticum* requiring twice as many ATP equivalents for acetone carboxylation. In terms of metal content, *X. autotrophicus* consistently contains Mn²⁺ while *A. aromaticum* does not but in place contains Fe^{2+/3+} equivalents. These results were initially based on work in different labs and our first thought was that this was an artifact of expression and different preparations in different labs; however, we have reproduced the differences in our lab working with enzymes expressed, purified, and prepared for analysis in parallel. To examine the implications of this experimentally, we conducted a series of metal chelation and reconstitution experiments. For *X. autotrophicus*, we have now shown that metal chelation results in loss of activity and that only reconstitution with Mn²⁺ results in restoration of activity while reconstitution with Fe²⁺ does not restore activity and perhaps even reduces residual activity. *A. aromaticum*, on the other hand, was found to have a reduction in activity upon reconstitution with Mn²⁺ while activity was restored with Fe²⁺ (**Figure 1B**). Interestingly, only subtle structural differences are observed to explain metal variability when comparing the *X. autotrophicus* structures with *A. aromaticum* homology models. In collaboration with Simone Raugei at PNNL, we are attempting to delineate the key residues that might be involved in eliciting this unanticipated metal specificity that we can test through site specific amino acid substitution studies.

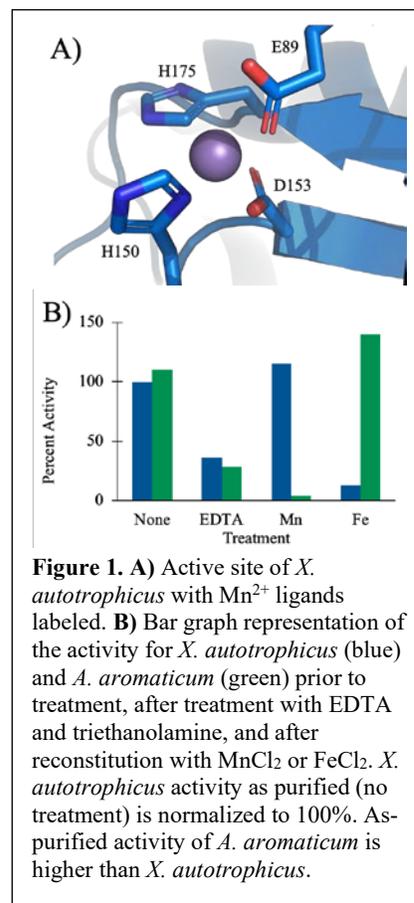


Figure 1. A) Active site of *X. autotrophicus* with Mn²⁺ ligands labeled. **B)** Bar graph representation of the activity for *X. autotrophicus* (blue) and *A. aromaticum* (green) prior to treatment, after treatment with EDTA and triethanolamine, and after reconstitution with MnCl₂ or FeCl₂. *X. autotrophicus* activity as purified (no treatment) is normalized to 100%. As-purified activity of *A. aromaticum* is higher than *X. autotrophicus*.

FixABCD conformational dynamics

The FixABCX complex is a member of the ETF family of enzymes and thus, a conformational gating of electron transfer is proposed to occur during its catalytic cycle. To define the mechanism of conformational gating in FixABCX, we have optimized the expression and purification of the FixABCX complex in *Azotobacter vinelandii*. FixABCX has successfully been expressed and we are currently in the process of purifying the complex in order to carry out structural studies. We are optimizing the detergent concentrations to solubilize the membrane protein and maintain a stable form in solution for protein purification.

Science priorities for the next year (2020-2021):

- Complete HDX experiments for 2-KPCC in the native state and with bound BES.
- Complete work demonstrating the importance of the proposed product anion binding pockets in 2-KPCC including the F501H and H506E variants.
- Determine ATP stoichiometry for *X. autotrophicus* and *A. aromaticum* AC and further investigate the metal site variability.
- Examine protein conformational changes in FixABCX using HDX mass spectroscopy.

My major scientific area(s) of expertise is/are: Protein structure function and enzyme mechanism

To take my project to the next level, my ideal collaborator would have expertise in: Simone Raugei at PNNL has been brought on board as a collaborator to help us understand AC metal dependence. Team is in place to accomplish the goals of the project.

Publications supported by this project: 2019-2020:

F. Mus, H. Wu, A.B. Alleman, K.A. Shisler, O.A. Zadvornyy, B. Bothner, J.L. Dubois, J.W. Peters, "Insights into the unique carboxylation reactions in the metabolism of propylene and acetone." *Biochem. J.*, 477, 11 (2020).

Enzymology of Methanogenesis: Mechanism Of Methyl-Coenzyme M Reductase

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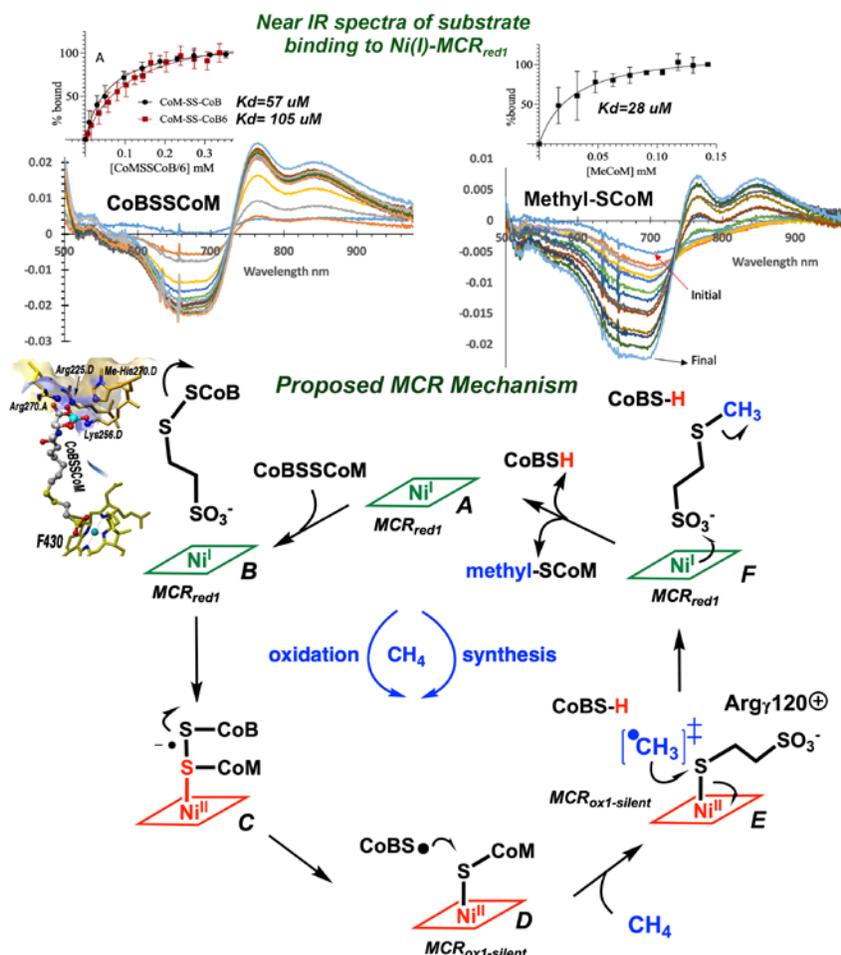
Overall research goals:

Our overall research goals are to address gaps in our understanding of the catalytic mechanism of Methyl-CoM Reductase (MCR), the key enzyme in methanogenesis and methane oxidation. We will characterize the reaction intermediates in the forward and reverse reaction by focusing on three aims:

1. Trap and characterize the proposed thiol radical intermediate, CoBS(dot), in the first step of methane synthesis by synthesizing a CoBSH analog and following the reaction by an incisive combination of spectroscopic (UV-visible, EPR, XAS, MCD), kinetic (steady-state and transient) and computational methods.
2. Trap and characterize the intermediates in reverse methanogenesis (anaerobic methane oxidation). By rapid mixing methods, we have successfully trapped what appears to be a disulfide anion radical, the final intermediate in methane synthesis. This species was trapped by reacting active Ni(I)-MCR_{red1} with a slow substrate analog (CoB₆SSCoM). We will then react that intermediate with methane in a double mixing experiment to directly observe and define the C-H activation step. That intermediate will be characterized by kinetic, spectroscopic and computational methods. In combination with Aim 1, this information will define the spectroscopic properties, rate constants and activation profiles for all intermediates in the forward and reverse reactions of MCR.
3. Characterize the mechanism and high-affinity binding site involved in methane inhibition of methanogenesis. At concentrations well below the K_m value (>10 atm) for reverse methanogenesis, we observe methane inhibition of the MCR forward reaction. Because this inhibition occurs at relatively low concentrations, MCR must contain a high-affinity binding site for methane. We are characterizing this binding site using crystallographic, spectroscopic, kinetic and computational methods.

Significant achievements (2018-2021):

We discovered that binding of CoMSSCoB, the heterodisulfide substrate for the reverse reaction, and its analog CoMSSCoB₆, a slow substrate, to the active Ni(I) state of MCR (MCR_{red1}) elicits marked changes in the near infrared (NIR) spectrum of MCR (Fig. 1, top). These are assigned to Ni d-d transitions that reflect binding of the sulfonate group of CoM to Ni(I). Similar transitions are observed for methyl-SCoM, the substrate for the forward reaction and for an analog, propyl sulfonate. K_d values were established for each of the substrates. These NIR changes take place while the enzyme remains in the Ni(I) state, retaining its 390 nm UV-visible absorption peak and Ni(I) EPR signal. The NIR studies were complemented by X-ray Absorption Spectroscopy, Quantum Mechanical Calculations and TDDFT studies to characterize the Ni ligand interaction that arises on binding. Based on these studies, we propose a model for CoBSSCoM and Methyl-SCoM binding to MCR_{red1} for the reverse and forward MCR reactions (see Figure 1, bottom).



Science priorities for the next year (2020-2021):

- Complete spectroscopic studies of the sulfonate-bound forms of Ni(I)-MCR_{red1}
- Characterize the CoBS(dot) and the methyl radical by EPR and ENDOR spectroscopy
- Determine the structure of the active Ni(I) state of MCR

My major scientific area(s) of expertise is/are: Bioinorganic chemistry, Enzyme mechanisms, anaerobic microbiology and biochemistry, Enzyme kinetics, Spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: Neutron Diffraction .

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years]:

1. Ragsdale, S. W., Raugei, S., Ginovska, B., & Wongnate, T. (2017). CHAPTER 8. Biochemistry of Methyl-Coenzyme M Reductase. In D. Zamble, M. Rowińska-Zyrek, & H. Kozłowski (Eds.), *The Biological Chemistry of Nickel* (pp. 149-169). Cambridge, UK: The Royal Society of Chemistry.
2. Wongnate, T., & Ragsdale, S. W. (2015). The reaction mechanism of methyl-coenzyme M reductase: how an enzyme enforces strict binding order. *J Biol Chem*, 290(15), 9322-9334. doi:10.1074/jbc.M115.636761
3. Wongnate, T., Sliwa, D., Ginovska, B., Smith, D., Wolf, M. W., Lehnert, N., . . . Ragsdale, S. W. (2016). The radical mechanism of biological methane synthesis by methyl-coenzyme M reductase. *Science*, 352(6288), 953-958. doi:10.1126/science.aaf0616

Redox Biochemistry of Energy Conservation in Methanogens and Their Syntrophic Partners

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Overall research goals:

The overarching objective of this proposal is to uncover and understand the extraordinary molecular mechanisms controlling cellular energy transduction. To accomplish this, we have attempted to answer the question of why strictly anaerobic methanogens within the order Methanosarcinales and their syntrophic partners, sulfate-reducing bacteria (SRB), encode multiple cytochrome oxidases, which are usually reserved for supporting life processes in aerobic organisms. A successful outcome will likely challenge existing paradigms and offer innovative solutions for seemingly intractable problems in bacterial and archaeal bioenergetics.

Significant achievements ([2018-2020]):

- We discovered and characterized an unusual membrane-integral SRB respirasome (**Fig. 1**), which spatially couples formate oxidation to O₂ reduction in a quinone-independent manner. It is comprised of a selenocysteine-tungstopterin formate dehydrogenase (Fdh2), an 11-heme cytochrome *c* (Uhc), and a previously uncharacterized *c*-heme-containing cytochrome *bd* (*cbd*) oxidase (CydAcA').
- We showed that proton motive force (PMF) generated by the Fdh2-Uhc-CydAcA' respirasome drives ATP biosynthesis in a whole-cell system. We used deletion strains of *Desulfovibrio vulgaris* Hildenborough (DvH) generated in Judy Wall's lab (University of Missouri) to validate this result.
- We found that CydAcA' has been repurposed in diverse bacterial taxa through evolutionary acquisition of novel modules for substrate oxidation and/or electron transport, as well as co-option of multiheme cytochromes *c* via gene fusion events. Consequently, the resulting respirasomes obviate the need for a freely diffusible lipophilic or water-soluble electron carrier, ensuring that direct electron transfer is persistently associated with membrane energization.
- We established that Fdh2 catalysis is not affected by O₂ (collaboration with Shelley Minter, University of Utah).
- We have solved the 2.9 Å cryo-EM structure of the Fdh2-Uhc sub-complex (collaboration with Hartmut Michel, Max Planck Institute for Biophysics, Germany).
- On the methanogen front, we revealed that *Methanimicrococcus blatticola* cytochrome *bd* oxidase (Mbl-CydAB) is highly expressed in stationary phase but not during methanogenic growth. Therefore, Mbl-CydAB does not function in O₂ detoxification.

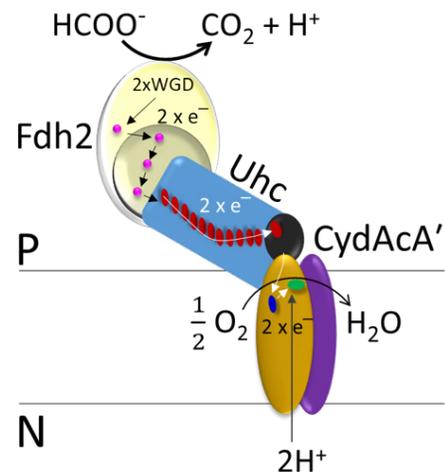


Figure 1 Model of the DvH respirasome

- We developed a novel assay and demonstrated that *M. blatticola* membranes, naturally loaded with CydAB, couple menaquinol (but not 2-hydroxyphenazine) oxidation to oxygen reduction (**Fig. 2**), thus generating a PMF.
- We identified a quinone binding site in Mbl-CydAB through chemical biology approaches. This suggests that Methanosarcinales can use quinones found in nature.

Science priorities for the next year (2020-2021):

- Determine the cryo-EM structure of Fdh2-Uhc-CydAcA' towards visualizing the complete electron transfer path from substrate oxidation to oxygen reduction.
- Investigate the diversity of electron inputs to CydAcA'.
- Assess the feasibility of functionally reconstituting Fdh2-Uhc-CydAcA' or a variant thereof in a heterologous host.
- Probe redox biochemistry of naturally occurring CydAcA' variants and CydAcA'-Uhc fusions.
- Delineate how CydAB controls function via supercomplex formation.
- Test whether CydAB is required for stationary phase exit of *M. blatticola*.

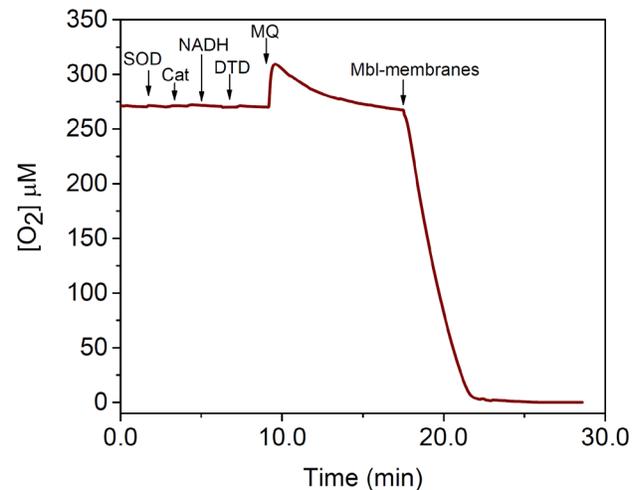


Figure 2 Aerobic respiration of *M. blatticola*. SOD, superoxide dismutase; Cat, catalase; DTD, DT-diaphorase; MQ, menaquinone.

My major scientific areas of expertise are: redox biochemistry, membrane-integral metalloprotein complexes, heme proteins, mechanistic enzymology, structural biology, bacterial respiration.

To take my project to the next level, my ideal collaborator would have expertise in: chemostat cultivation, CRISPR-Cas9 systems, whole cell electromicrobiology, single-molecule super-resolution imaging.

Publications supported by this project (2018 – present):

1. Joel E. Graham, Grant M. Zane, Jakob Meier-Credo, Qin Gui, Rayford B. Payne, Pavel Martásek, Kevin R. Sowers, Julian D. Langer, Judy W. Wall, C. S. Raman (2020) “Hardwiring respirasomes across prokaryotic lineages enabled by cytochrome oxidase neofunctionalization” In review
2. Joel E. Graham, Olivier Biner, Rayford B. Payne, Felix Elling, Kevin Becker, Jakob Meier-Credo, Jennifer Herrmann, Julian D. Langer, Pavel Martásek, Kevin R. Sowers, C. S. Raman (2020) “A Breath of Fresh Air: Energy Conservation via Quinol-Dependent Aerobic Respiration in the Methyl-Reducing Methanogen *Methanimicrococcus blatticola*.” *mBio* In submission
3. Joel E. Graham, Mengwei Yuan, Yoo Seok Lee, Grant M. Zane, Qin Gui, Judy W. Wall, Shelley D. Minter, C. S. Raman (2020) “Oxygen-insensitivity of a Se-Cys- tungstopterin formate dehydrogenase revealed by direct electrocatalysis” In preparation

Enzymatic Energy Conversion

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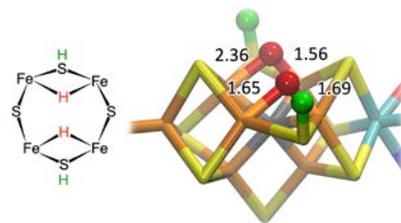
Overall research goals:

Our program aims at providing a better understanding of the core principles employed by enzymes in order to control the flow of energy and matter to achieve remarkable specificities, efficiencies, and catalytic rates. Our program integrates state-of-the-art theory and computation with experimental efforts across the DOE/BES Physical Biosciences community to fill critical gaps in knowledge about how enzymes orchestrate spatial and temporal events to direct electrons, protons, and substrates for selective conversions and allosteric regulation. These advances will contribute to the design of next-generation synthetic catalysts.

We are currently exploring a number of biological systems. The nitrogenase complex represents our prime test bed for investigating various critical biological activities (with Hoffman, Northwestern University). Along with the nitrogenase complex, the [FeFe] hydrogenase is studied to understand the inter-play between electrochemical and mechanical energy (with Peters, Washington State University). We are also investigating three other enzymes that offer a diverse set of platforms to study how nature is able to generate and selectively manipulate extremely reactive substrates (a methyl radical in methyl-coenzyme M reductase, with Ragsdale, University of Michigan; and carbocations in monoterpene synthase, with Lange, Washington State University) and selectively dehydrogenate hydrocarbon chains (desaturase, with Shanklin, Brookhaven National Laboratory).

Significant achievements (Years of Current Funding: 2018-2021):

Nature of Lowest-Energy Structure of the Nitrogenase Janus Intermediate E_4 . We carried out an extensive quantum chemical study of N_2 activation by nitrogenase. Binding and activation of N_2 takes place at the E_4 state of the catalytic cofactor, FeMo-co, which is activated by the accumulation of $4e^-/H^+$, with the concomitant reductive elimination of H_2 . Density functional theory (DFT) calculations identify a number of low-lying E_4 isomers. One of the lowest in energy, $E_4(4H)$, has two [Fe-H-Fe] bridging hydrides consistent with low-temperature ENDOR measurements by Hoffman (Northwestern University). However, limitations in both DFT and interpretations of the ENDOR data do not allow for a definitive assignment of the E_4 lowest-energy structure. To overcome these limitations, we developed an analytical point-dipole Hamiltonian model for the electron-nuclear dipolar interaction of the hydride with its 'anchoring' Fe ions, which couple ENDOR measurements to the structural DFT models. This model shows that only a structure with two bridging di-hydrides accounts for the observed presence of hydrides with rhombic anisotropic hyperfine tensors that are coaxial, but with permuted tensor components. EPR spectra indicate no other isomers below 25 K. However, calculations suggest that at ambient temperature the $E_4(4H)$ hydrides becomes fluxional, with dynamic population of multiple isomers.



DFT structure of $E_4(4H)$ state
(Selected Fe-H distances shown in Å).

Catalytic Bias in [FeFe] Hydrogenases. [FeFe]-hydrogenases all possess the same active site (H cluster). However, they display a large range of H_2 gas oxidation and proton reduction activities, with some displaying a dramatic catalytic bias, that is, the propensity of a catalyst to affect rate of acceleration in one reaction direction over the other. By combining high-resolution X-ray diffraction methods along with electrochemical and spectroscopic analyses coupled with extensive theoretical calculations that key amino acids within the H cluster environment of [FeFe]-hydrogenase I from *Clostridium pasteurianum* (CpI) adopt different conformations as a function of oxidation state. This structural flexibility makes it possible to

modulate the properties of the electronic structure of the H cluster via secondary, noncovalent interactions. We propose that the dynamic changes observed in CpI are important for imposing a neutral catalytic bias or an efficacy in catalyzing both H₂ oxidation and proton reduction at similar rates. A closer look at other Cp [FeFe]-hydrogenases (CpI and CpIII) reveals profound propensities to catalyze either H₂ oxidation or proton reduction preferentially, revealing an extreme range of catalytic biases. Through examination of the properties of these enzymes in light of our recent observations on the role of the H cluster protein environment (especially the electric field at the active site) in stabilization of oxidation states relevant to catalysis, we are able to propose a general model for catalytic bias in [FeFe]-hydrogenases invoking the relative stabilization/destabilization of key mechanistically relevant intermediate states that could presumably extend to many redox enzymes.

Science priorities for the next year (2020-2021):

Understand how dynamic confinement imposed by the enzyme scaffold controls specificity and selectivity.

My major scientific area(s) of expertise is: Computational chemistry and biophysics.

To take my project to the next level, my ideal collaborator would have expertise in: experimental characterization of enzymatic mechanisms and protein redesign. In this regard, we are already fruitfully collaborating with John Shanklin (BNL), Steven Ragsdale (U. Michigan), Michael Adams (U. Georgia), M. Lange (WSU), John Peters (WSU), Brian Hoffman (Northwestern University), and Yi Lu (U. Illinois/PNNL).

Publications supported by this project (2018-present):

1. Seefeldt, Yang, Lukoyanov, Harris, Dean, Raugei, Hoffman. Reduction of Substrates by Nitrogenases. *Chem. Rev.* 120 (2020) 5082. DOI: 10.1021/acs.chemrev.9b00556
2. Art, Zadvornyy, Mulder, Keable, Cohen, Ratzloff, Williams, Ginovska, Kumar, Song, McPhillips, Davidson, Lyubimov, Pence, Schut, Jones, Soltis, Adams, Raugei, King, Peters. Tuning catalytic bias of hydrogen gas producing hydrogenases. *J. Am. Chem. Soc.* 141 (2020) 1227. DOI: 10.1021/jacs.9b08756
3. Hoeke, Tociu, Case, Seefeldt, Raugei, Hoffman. High resolution ENDOR spectroscopy combined with quantum chemical calculations reveals the structure of the nitrogenase Janus intermediate E4(4H). *J. Am. Chem. Soc.* 141 (2019) 11984. Erratum: *J. Am. Chem. Soc.* 141 (2019) 19950. DOI: 10.1021/jacs.9b04474
4. Johnson, Ginovska, Fenton, Raugei. Chokepoints in Mechanical Coupling Associated with Allosteric Proteins: The Pyruvate Kinase Example. *Biophys. J.* 116 (2019) 1598. DOI: 10.1016/j.bpj.2019.03.026
5. Raugei, Seefeldt, Hoffman. A Critical Computational Analysis Illuminates the Reductive-Elimination Mechanism That Activates Nitrogenase for N₂ Reduction. *Proc. Nat. Acad. Sci.* 115 (2018) E10521. DOI: 10.1073/pnas.1810211115
6. Seefeldt, Hoffman, Peters, Raugei, Beratan, Antony, Dean. Energy Transduction in Nitrogenase. *Acc. Chem. Res.*, 51 (2018) 2179. DOI: 10.1021/acs.accounts.8b00112
7. Keable, Zadvornyy, Johnson, Ginovska, Rasmussen, Danyal, Eilers, Prussia, LeVan, Raugei, Seefeldt, Peters. Characterization of the P¹⁺ intermediate state of nitrogenase P-cluster. *J. Biol. Chem.*, 293 (2018) 9629. DOI: 10.1074/jbc.RA118.002435
8. Peters, Beratan, Bothner, Dyer, Harwood, Heiden, Hille, Jones, King, Lu, Lubner, Minter, Mulder, Raugei, Schut, Seefeldt, Tokmina-Lukaszewska, Zadvornyy, Zhang, Adams. A new era for electron bifurcation. *Curr. Op. Chem. Biol.* 47 (2018) 32. DOI: 10.1016/j.cbpa.2018.07.026
9. Seefeldt, Peters, Beratan, Bothner, Minter, Raugei, Hoffman. Control of electron transfer in nitrogenase. *Curr. Op. Biol. Chem.* 47 (2018) 54. DOI: 10.1016/j.cbpa.2018.08.011

Tuning Hydrocarbon Formation by Vanadium Nitrogenase via a Hybrid Approach.

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Overall research goals:

The unique ability of V-nitrogenase to catalyze the ambient reduction of CO to hydrocarbons makes it an attractive template for investigations of the chemical and mechanistic details of this unique reaction. This project aims to tune the CO-reducing activity of vanadium nitrogenase toward formation of oxygenated products and capture of mechanistically relevant, oxygenated intermediates via a novel hybrid approach. Using combined genetic and biochemical approaches, we propose to generate hybrid V-nitrogenase systems with altered proton fluxes, altered electron fluxes, and altered electron donors and perform in-depth spectroscopic and biochemical analyses of the reactions of CO-reduction by these systems. The outcome of this study will not only shed light on the reaction pathway of the enzymatic CO reduction, but also inform future designs of novel CO- and CO₂-reducing catalysts for production of oxygenate-based biofuels.

Significant achievements (2018-2020):

- Nitrogenase catalyzes the ambient reduction of N₂ and CO at its cofactor site. In the last grant period, we reported a combined biochemical and spectroscopic study of an *Azotobacter vinelandii* V-nitrogenase variant expressing a citrate-substituted cofactor (see publication #3 below). Designated VnfDGK^{Cit}, the catalytic component of this V-nitrogenase variant has an $\alpha\beta_2$ (δ) subunit composition and carries an 8Fe P*-cluster and a citrate-substituted V-cluster analogue in the $\alpha\beta$ dimer, as well as a 4Fe cluster in the "orphaned" β -subunit. Interestingly, when normalized based on the amount of cofactors, VnfDGK^{Cit} shows a shift of N₂ reduction from H₂ evolution toward NH₃ formation and an opposite shift of CO reduction from hydrocarbon formation toward H₂ evolution. These observations point to a role of the organic ligand in proton delivery during catalysis and imply the use of different reaction sites/mechanisms by nitrogenase for different substrate reductions. Moreover, the increased NH₃/H₂ ratio upon citrate substitution suggests the possibility to modify the organic ligand for improved ammonia synthesis in the future.
- The Mo- and V-nitrogenases are two homologous members of the nitrogenase family that are distinguished mainly by the presence of different heterometals (Mo or V) at their respective cofactor sites (M- or V-cluster). However, the V-nitrogenase is ~600-fold more active than its Mo counterpart in reducing CO to hydrocarbons at ambient conditions. In the last grant period, we expressed an M-cluster-containing, hybrid V-nitrogenase in *Azotobacter vinelandii* and compared it to its native, V-cluster-containing counterpart in order to assess the impact of protein scaffold and cofactor species on the differential reactivities of Mo- and V-nitrogenases toward CO (see publication #4 below). Housed in the VFe protein component of V-nitrogenase, the M-cluster displayed electron paramagnetic resonance (EPR) features similar to those of the V-cluster and demonstrated an ~100-fold increase in hydrocarbon formation activity from CO reduction, suggesting a significant impact of protein environment on the overall CO-reducing activity of nitrogenase. On the other hand, the M-cluster was still ~6-fold less active than the V-cluster in the same protein scaffold, and it retained its inability to form detectable amounts of methane from CO reduction, illustrating a fine-tuning effect of the cofactor properties on this nitrogenase-catalyzed reaction. Together, these results

provided important insights into the two major determinants for the enzymatic activity of CO reduction while establishing a useful framework for further elucidation of the essential catalytic elements for the reactivity of nitrogenase toward CO.

Science priorities for the next year (2020-2021):

- The biochemical and spectroscopic characterization of an *Azotobacter vinelandii* Mo nitrogenase variant expressing a citrate-substituted cofactor (designated NifDK^{Cit}). Preliminary data indicate that this nitrogenase hybrid is tuned towards the formation of hydrocarbons from CO, which will provide an intriguing platform for generating oxygenated products.

My major scientific area(s) of expertise is/are: Bioinorganic chemistry.

To take my project to the next level, my ideal collaborator would have expertise in: Mössbauer spectroscopy.

Publications supported by this project:

1. Hu Y, Ribbe MW. (2020) Special issue on nitrogenases and homologous systems. **ChemBiochem** 21(12):1668-1670. doi: 10.1002/cbic.202000279.
2. Jasniewski AJ, Lee CC, Ribbe MW, Hu Y. (2020) Reactivity, mechanism, and assembly of the alternative nitrogenases. **Chem Rev** 120(12):5107-5157. doi: 10.1021/acs.chemrev.9b00704.
3. Newcomb MP, Lee CC, Tanifuji K, Jasniewski AJ, Liedtke J, Ribbe MW, Hu Y. (2020) A V-nitrogenase variant containing a citrate-substituted cofactor. **ChemBiochem** 21(12):1742-1748. doi: 10.1002/cbic.201900654.
4. Rebelein JG, Lee CC, Newcomb M, Hu Y, Ribbe MW. (2018) Characterization of an M-cluster-substituted nitrogenase VFe protein. **mBio** 9(2):e00310-18. doi: 10.1128/mBio.00310-18.
5. Lee CC, Tanifuji K, Newcomb M, Liedtke J, Hu Y, Ribbe MW. (2018) A Comparative analysis of the CO-Reducing activities of MoFe proteins containing Mo- and V-Nitrogenase cofactors. **ChemBiochem** 19(7):649-653. doi: 10.1002/cbic.201800035.
6. Sickerman NS, Tanifuji K, Hu Y, Ribbe MW. (2017) Synthetic analogues of nitrogenase metallocofactors: challenges and developments. **Chemistry** 23(51):12425-12432. doi: 10.1002/chem.201702496.
7. Sickerman NS, Hu Y, Ribbe MW. (2017) Activation of CO₂ by vanadium nitrogenase. **Chem Asian J** 12(16):1985-1996. doi: 10.1002/asia.201700624.
8. Sickerman NS, Tanifuji K, Lee CC, Ohki Y, Tatsumi K, Ribbe MW, Hu Y. (2017) Reduction of C1 substrates to hydrocarbons by the homometallic precursor and synthetic mimic of the nitrogenase cofactor. **J Am Chem Soc** 139(2):603-606. doi: 10.1021/jacs.6b11633.
9. Rebelein JG, Lee CC, Hu Y, Ribbe MW. (2016) The in vivo hydrocarbon formation by vanadium nitrogenase follows a secondary metabolic pathway. **Nat Commun** 7:13641. doi: 10.1038/ncomms13641.
10. Tanifuji K, Sickerman N, Lee CC, Nagasawa T, Miyazaki K, Ohki Y, Tatsumi K, Hu Y, Ribbe MW. (2016) Structure and reactivity of an asymmetric synthetic mimic of nitrogenase cofactor. **Angew Chem Int Ed Engl** 55(50):15633-15636. doi: 10.1002/anie.201608806.
11. Hu Y, Ribbe MW. (2016) Nitrogenases—a tale of carbon atom(s). **Angew Chem Int Ed Engl** 55(29):8216-26. doi: 10.1002/anie.201600010.

Missing links in biological methane and ammonia oxidation

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Overall research goals:

This project focuses on biochemical and functional characterization of proteins encoded by the extended pMMO/AMO operons. The overarching hypothesis is that these proteins play an important role in biological methane and ammonia oxidation, and as such, represent “missing links” in our understanding of these enzymes. The operons include the three genes encoding the enzyme subunits, *pmoB* (or *amoB*), *pmoA* (or *amoA*), and *pmoC* (or *amoC*). Directly adjacent to *pmoB/amoB* is a gene denoted *pmoD/amoD* followed by three genes encoding putative copper transport proteins, *copC*, *copD*, and *pmoF*. These four genes are coregulated with the pMMO genes in a copper-dependent fashion. CopC and PmoF belong to periplasmic copper chaperone families, and CopD is a putative copper importer. PmoD does not belong to any known protein family and homologs are only found in methane- and ammonia-oxidizing bacteria. PmoD consists of an N-terminal periplasmic cupredoxin domain followed by a predicted transmembrane helix. The isolated periplasmic domain forms a homodimer bridged by an unusual Cu_A-like site. Importantly, genetic disruption experiments show that PmoD is critical for methanotroph growth during pMMO-utilizing conditions, suggesting that it is indeed a missing link. However, its function and the specific role of the observed Cu_A-like site remain unknown. The project goals are divided into two specific aims. The first aim addresses whether the Cu_A-like site forms in vivo and its relation to the growth defect observed in the $\Delta pmoD$ *Methylosinus (Ms.) trichosporium* OB3b strain. The goal of the second aim is to determine why disruption of the *pmoD* gene impairs cell growth under pMMO-utilizing conditions. Each aim incorporates both in vitro and in vivo approaches.

Significant achievements (2019-2022)

- We characterized the formation and decay of the PmoD Cu_A site using stopped-flow and electron paramagnetic resonance (EPR) spectroscopies (Fig. 1) and probed its electronic configuration and coordination via advanced paramagnetic resonance spectroscopies.
- We performed a bioinformatic analysis of PCu_AC domain-containing PmoF proteins, identified three classes in methanotrophs, PmoF1, PmoF2, and PmoF3, and showed that PmoF1 and PmoF2 bind only Cu(II), unlike typical PCu_AC domains, which bind a single Cu(I) ion (Fig. 2).
- We determined crystal structures of PmoF1 and PmoF2, revealing an N-terminal histidine brace HX₁₀H Cu(II) coordination motif (Fig. 2) resembling motifs in CopC proteins and lytic polysaccharide monooxygenases. This finding provides new insight into the biological significance of histidine brace coordination.

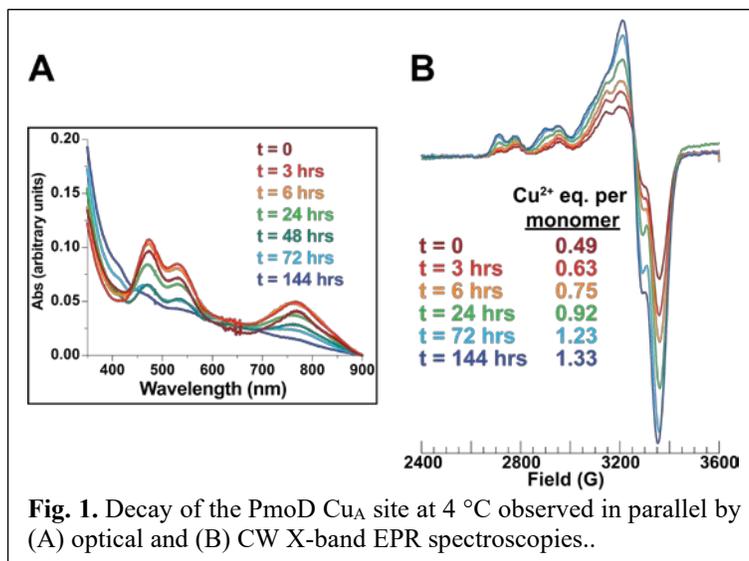
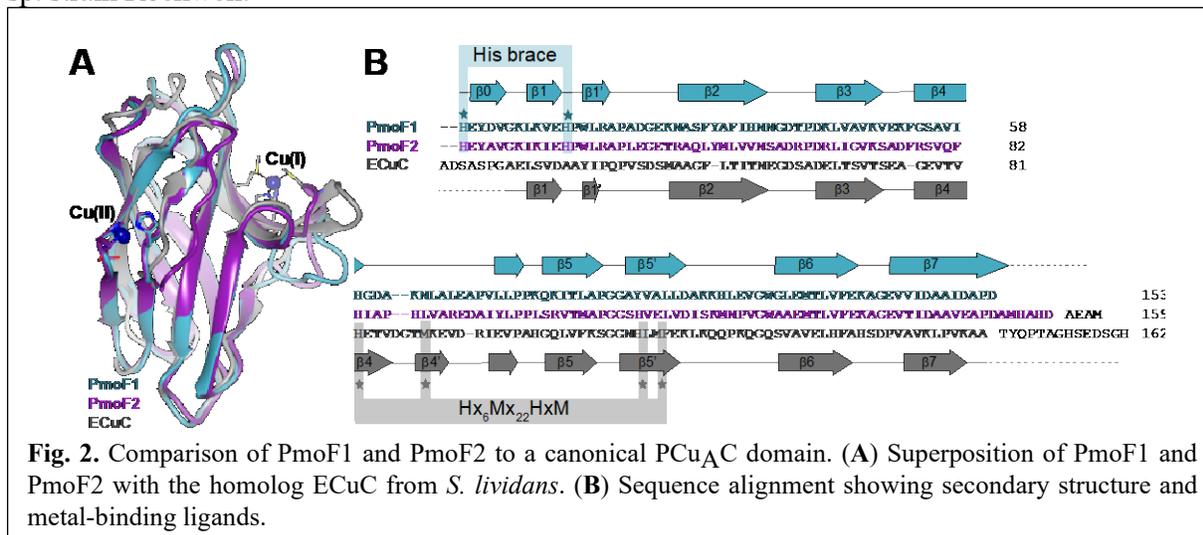


Fig. 1. Decay of the PmoD Cu_A site at 4 °C observed in parallel by (A) optical and (B) CW X-band EPR spectroscopies..

- We established a heterologous expression system for full-length PmoD, including the transmembrane domain.
- We completed the first steps toward establishing a genetic manipulation system for *Methylocystis* (*Mc.*) sp. strain Rockwell.



Science priorities for the next year (2020-2021):

- We will purify full-length PmoD, evaluate its copper binding properties, determine whether it forms the previously observed Cu_A site, and pursue crystallization and structure determination. It may be necessary to reconstitute full-length PmoD into bicelles or nanodiscs to stabilize its transmembrane helix.
- We will investigate potential interactions between full-length PmoD and pMMO as well as between PmoD and PmoF, and explore the possibilities of copper or electron transfer between PmoD and pMMO.
- We will create a $\Delta pmoD$ strain of *Mc.* sp. Rockwell and determine its phenotype via growth assays. In parallel, we will generate a Cys41Ser variant, which will disrupt Cu_A site formation, and assess its phenotype.

My major scientific area(s) of expertise is/are: bioinorganic chemistry, structural biology, copper proteins, biological methane oxidation.

To take my project to the next level, my ideal collaborator would have expertise in: microbial genetic manipulation, including CRISPR Cas9

Publications supported by this project:

1. Fisher, O. S.; Sendzik, M. R.; Ross, M. O.; Lawton, T. J.; Hoffman, B. M.; Rosenzweig, A. C. PCu_AC domains from methane-oxidizing bacteria use a histidine brace to bind copper. *J. Biol. Chem.* **2019**, *294*, 16351-16363.
2. Ross, M. O.; Fisher, O. S.; Morgada, M. N.; Krzyaniak, M. D.; Wasielewski, M. R.; Vila, A. J.; Hoffman, B. M.; Rosenzweig, A. C. Formation and electronic structure of an atypical Cu_A site. *J. Am. Chem. Soc.* **2019**, *141*, 4678-468
3. Fisher, O. S.; Kenney, G. E.; Ross, M. O.; Ro, S. Y.; Lemma, B. E.; Batelu, S.; Thomas, P. M.; Sosnowski, V. C.; DeHart, C. J.; Kelleher, N. L.; Stemmler, T. L.; Hoffman, B. M.; Rosenzweig, A. C. Characterization of a long overlooked copper protein from methane- and ammonia-oxidizing bacteria. *Nat. Commun.* **2018**, *9*, 4276.

Regulated reductive flow through archaeal respiratory and energy production systems

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Overall research goals:

The energy production strategies supporting growth of hyperthermophilic Archaea push the known limits of energy conversation mechanisms and likely mimic the energy transaction strategies present in early life on Earth. Many advances in our understanding of these systems have emerged from the genetically-tractable order *Thermococcales* wherein the roles of individual enzymes, soluble and membrane-bound respiratory complexes, regulatory factors, and competing and complementary catabolic pathways have been probed by a combination of ever-increasingly complex genetic, biochemical, and –omics approaches. **Our long-term research goal is to determine the interplay, competition and regulation of archaeal energy and redox transactions systems.**

Catabolism of marginal substrates may yield only ~one-tenth of the necessary energy ATP production. If reduction of even weakly energetic substrates can be coupled to formation of an electrochemical gradient, this gradient can be exploited for ATP production. Evolutionary competition has presumably favored mechanisms to conserve and combine multiple, relatively small ΔG gains to harness sufficient energy to drive ATP synthesis. The *Thermococcales*, a group of heterotrophic, anaerobic, hyperthermophilic marine archaea use a chemiosmotic mechanism involving an electrochemical ion gradient across the cytoplasmic membrane to drive ATP synthesis via an A_1A_0 ATP synthase. This gradient is generated via the action of a multi-subunit membrane-bound NiFe-hydrogenase (termed MBH) or a membrane-bound sulfane-reductase (MBS) which couples ferredoxin-driven reduction of protons or polysulfides (thereby generating H_2 or H_2S , respectively) to H^+ translocation across the cytoplasmic membrane. Ferredoxins (Fds) are critical to this lifestyle, given their redox potentials (E^0) are sufficiently low to not only permit reduction of protons to H_2 ($E^0 = -414$ mv), but to drive such a reaction with sufficient excess that energy is available to drive translocation of ions across the membrane.

This renewal application builds on the successes from the prior period of productive research funding that resulted in a paradigm shift regarding the roles of Fd-isoforms in directing electron flux *in vivo* through selective and distinct pathways. We aim to extend our findings to establish the rules dictating Fd-interactions, to rationally manipulate electron flux *in vivo* by altering Fd availability and interplay, and to establish the structures and redox potentials of the Fd-isoforms from *T. kodakarensis*.

Significant achievements (2016-2019; project renewed 2019-2022):

1. Sas-Chen A, Thomas JM, Matzov D, Taoka M, Nance KD, Nir R, Bryson KM, Shachar R, Liman GLS, Burkhart BW, Thalalla Gamage S, Nobe Y, Briney CA, Levy MJ, Fuchs RT, Robb GB, Hartmann J, Sharma S, Lin Q, Florens L, Washburn MP, Isobe T, Santangelo TJ, Shalev-Benami M, Meier JL, Schwartz S. 2020. Dynamic RNA acetylation revealed by cross-

- evolutionary mapping at base resolution. *Nature*. 583 (7817).
2. Gehring AM, Zatopek KM, Burkhart BW, Santangelo TJ, Gardner AF. 2020. Biochemical reconstitution and genetic characterization of the major oxidative damage base excision DNA repair pathway in *Thermococcus kodakarensis*. *DNA Repair (Amst)*. 86:102767.
 3. Turner B, Burkhart BW, Weidenbach K, Ross R, Limbach PA, Schmitz RA, de Crecy-Lagard V, Stedman KM, Santangelo TJ, Iwata-Reuyl D. 2020. Archaeosine Modification of Archaeal tRNA: Role in Structural Stabilization. *J Bacteriol*. 202(8):e00748-19.
 3. Burkhart BW, Febvre HP, Santangelo TJ. 2019. Distinct Physiological Roles of the Three Ferredoxins Encoded in the Hyperthermophilic Archaeon *Thermococcus kodakarensis*. *MBio*. 10(2): 2807-18.
 4. Liman GLS, Hulko T, Febvre HP, Brachfeld AC, Santangelo TJ. 2019. A linear pathway for mevalonate production supports growth of *Thermococcus kodakarensis*. *Extremophiles*. 23(2): 229-238.
 5. Sanders TJ, Marshall CJ, Santangelo TJ. 2019. The Role of Archaeal Chromatin in Transcription. *J Mol Biol*. 431(20):4103-4115.
 6. Speed MC, Burkhart BW, Picking JW, Santangelo TJ. 2018. An Archaeal Fluoride-Responsive Riboswitch Provides an Inducible Expression System for Hyperthermophiles. *Appl Environ Microbiol*. 84(7): 2306-17.
 7. Gehring AM, Sanders TJ, Santangelo TJ. 2017. Markerless Gene Editing in the Hyperthermophilic Archaeon *Thermococcus kodakarensis*. *Bio Protoc*. 7(22): 2604.
 8. Gehring AM, Astling DP, Matsumi R, Burkhart BW, Kelman Z, Reeve JN, Jones KL, Santangelo TJ. 2017. Genome replication in *Thermococcus kodakarensis* independent of Cdc6 and an origin of replication. *Front Microbiol*. 8:2084.
 9. Gehring AM, Walker JE, Santangelo TJ. 2016. Transcription Regulation in Archaea. *J Bacteriol*. 198(14):1906-1917.

Science priorities for the next year (2020-2021):

(1) Determine the biochemical and biophysical basis for selective electron transport through distinct Fd isoforms. Synthetic and hybrid Fds will be produced – in native and large excess – in vivo to selectively direct electron flux through desired biological routes.

(2) Describe, then alter the expression of Fd isoforms to direct electron flux through H₂-and isoprenoid-generating pathways to explore the capacities and limitations of energy production platforms for high-value bioproduct synthesis.

(3) Establish the redox potentials and structures of the three native Fd-isoforms from *T. kodakarensis*.

My major scientific area(s) of expertise is/are: Archaeal metabolisms; hyperthermophilic genetic systems; archaeal transcription and replication systems. .

To take my project to the next level, my ideal collaborator would have expertise in: X-ray crystallography of redox sensitive factors; redox potentials of ferredoxins.

Synchrotron-Based X-ray Spectroscopy Tools for Comprehensive Characterization of Metals in Biology

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Overall research goals:

Apply a combination of synchrotron-based X-ray spectroscopy techniques to study active-site electronic and structural transformations in metalloenzyme catalysis

Synchrotron-based X-ray spectroscopic methods such as hard- and tender- X-ray absorption spectroscopy (XAS), extended X-ray absorption fine structure (EXAFS), X-ray emission spectroscopy (XES) and resonant X-ray emission spectroscopy (RXES), together constitute a formidable arsenal for geometric and electronic structure determination toolset for the investigation of transition metal sites in biological systems. Over the past two decades, with support from DOE-BES, DOE-BER and NIH-NIGMS, the Structural Molecular Biology (SMB) Resource at SSRL has developed, and continues to innovate upon, methodology and instrumentation for studying sensitive biological systems with high-intensity X-rays. SSRL is currently the only laboratory providing a uniform platform for multi-energy, multi-technique applications for biological systems.

These techniques can have significant impact on the research supported by the Physical Biosciences program. Despite this, the combined biological spectroscopy toolkit at SSRL has been extensively exploited by only a handful of Physical Sciences-funded investigators and by limited (but impactful) collaborative studies by others. Another important aspect is that despite the availability of advanced X-ray spectroscopy techniques for close to two decades, their application has been largely limited to a few Fe and Mn based enzyme systems. In a recent proposal to the Physical biosciences program, one of goals is to expand standard and advanced spectroscopy for biological systems to other transition metals involved in bio-catalysis. These efforts can strongly benefit the scientific portfolio of the DOE BES Physical Bioscience program. As examples, SSRL based collaboration can enable investigation of: a) Cu- and Mo- containing CODH's and formate dehydrogenases using high-resolution XAS techniques to determine oxidation state changes and study selenocysteine variants; b) intermediates in Fe-Fe hydrogenases; c) soluble NiFe-hydrogenases; and d) methane oxidation by Cu-containing methane monooxygenases and, by extension, to others in the community interested in understanding the molecular basis for metalloenzyme transformations during catalysis.

Use a combination of X-ray spectroscopy techniques to investigate the catalytic mechanism of metalloenzymes involved in the Wood-Ljungdahl pathway for CO₂ fixation

We want to illustrate the strengths of X-ray spectroscopy with an application to the heterometallic enzyme systems involved in the Wood-Ljungdahl (WL) pathway. The WL pathway is earth's predominant anaerobic CO₂ sink and is found in strictly anaerobic bacteria, including acetogens and methanogenic archaea where it functions as the major means of energy generation and autotrophic growth. To achieve CO₂ reduction and functionalization, the WL pathway elegantly brings together a complex array of metalloenzymes, which precisely use/generate, transport and functionalize CO₂ and the otherwise toxic gas molecule, CO to acetyl Coenzyme A.

We will focus on two metalloenzyme active sites in the Wood-Ljungdahl pathway: the A-cluster in Acetyl CoA Synthase (ACS) and the C-cluster in Carbon Monoxide Dehydrogenase (CODH), in a collaborative fashion with Prof. Ragsdale and Prof. Drennan's groups, respectively. The A-cluster of ACS contains a binuclear Ni center, which is covalently linked to a [Fe₄S₄]^{2+/1+} moiety through a cysteine thiolate. During catalysis, this A-cluster forms a series of organometallic intermediates by binding CO- and methyl- and their fusion product, the acetyl- group. The C-cluster in CODH contains a Ni-Fe containing heterometallic cluster (an [Fe₄S₄Ni] cofactor comprised of a disordered [Fe₃S₄Ni] cubane attached to a ferrous component (FCII) through a cubane sulfide. The C-cluster catalyzes the first step of CO₂ reduction and involves the formation of another set of

organometallic intermediates: those that form by the interaction of CO₂ and its reduction products to the C-cluster.

At this time, the mechanism of CO₂ reduction (CODH) and CO and Me- binding and their ultimate fusion to form acetate (ACS) remains unclear. We have proposed to structurally and electronically investigate the organometallic intermediates formed by the A- and C-clusters during catalysis using a combination of Ni and Fe K-edge XAS and EXAFS and advanced spectroscopy methodologies in combination with theoretical calculations that will help us translate the experimental spectra into a molecular level understanding of the structural and electronic details of the organometallic intermediates.

Science priorities for the next year (2020-2021):

We will investigate the oxidation state and electronic structure of methylated and acetylated A-cluster in ACS by a combination of Fe and Ni based X-ray spectroscopic techniques to differentiate between two competing mechanisms and to understand how the electronic structure tunes catalysis. We will compare the local geometric structures of the resting, CO bound, methylated and acetylated A-cluster using Ni and Fe K-edge EXAFS and DFT methods and study how the nearby amino acid residues control any change in geometry upon methylation and acetylation.

My major scientific area(s) of expertise is/are:

Application and development of Synchrotron-based X-ray spectroscopy methods for biological applications.

To take my project to the next level, my ideal collaborator would have expertise in:

As a PI based at a synchrotron facility, part of my role is to bring x-ray techniques to the biochemical research community who are specifically interested in understanding the structural and electronic details of biological catalysis mediated by metal centers. My ideal collaborator would have strong expertise in biochemical characterization and would be studying mechanistic questions of poorly understood metalloenzymes, especially those that cannot be investigated by more commonly employed optical techniques for a variety of reasons.

Structure and function of carbon concentrating machinery

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Overall research goals:

Cells compartmentalize biochemical reactions as a means of improving pathway rate, yield, and toxicity. This strategy is epitomized in the bacterial microcompartment known as the carboxysome, which acts as part of a physiological strategy known as the CO₂ Concentrating Mechanism to insure fast and efficient CO₂ fixation by the enzyme Rubisco. Microcompartments are simplified, protein-based organelles. Understanding their assembly and function could provide a generalized and modular scaffold for improving the catalysis of engineered biochemical pathways. The carboxysome also plays a central role in the Calvin-Benson-Bassham Cycle - a fundamental pathway in the capture and conversion bioenergy-related molecules – and it is thought that the principles and components of the CO₂ Concentrating Mechanism could be used to improve CO₂ assimilation in many photosynthetic organisms. The overarching goal of our work is therefore to develop a mechanistic understanding of each major component of the bacterial CO₂ Concentrating Mechanism, including the carboxysome, in order to reveal how holistic function emerges from the integration of these individual components (Figure 1). We propose here to investigate three open and critical questions which have arisen through our continued work on the CO₂ Concentrating Mechanism. Firstly, we will investigate how an essential component of the carboxysome, carbonic anhydrase, is incorporated during the assembly process. Secondly, we will investigate the structure and function of the DAB, the inorganic carbon transporter which powers CCM function. Finally, we aim to reconstitute the CCM in new organisms in order to understand the evolution and improvement of CO₂ assimilation.

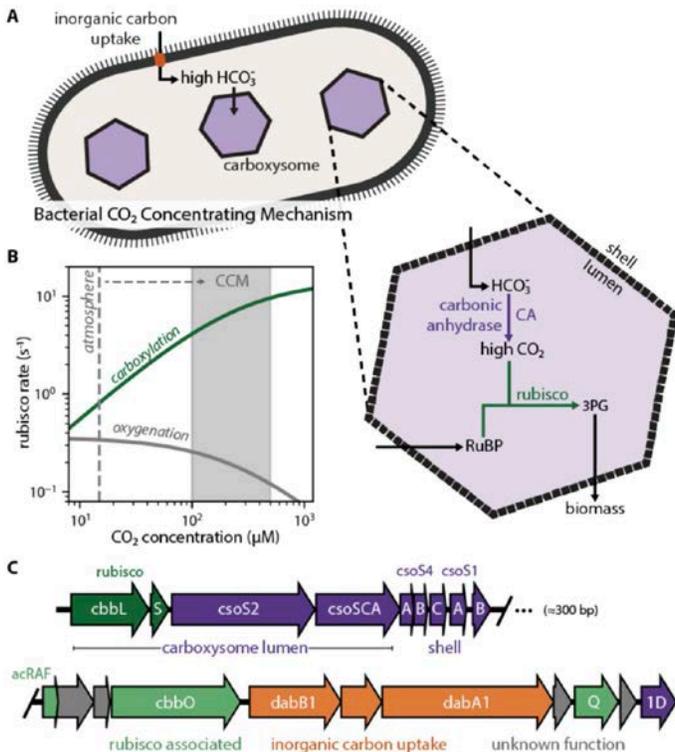


Figure 1: Twenty genes form the basis of a bacterial CCM. (A) The bacterial CCM consists of at least two essential components - energy-coupled carbon uptake and carboxysome structures that encapsulate rubisco with a carbonic anhydrase (CA) enzyme ([Mangan et al., 2016](#); [McGrath and Long, 2014](#)). Transport generates a large cytosolic HCO₃⁻ pool, which is rapidly converted to high carboxysomal CO₂ concentration by the carboxysomal CA. (B) Elevated CO₂ increases the rubisco carboxylation rate (green) and suppresses oxygenation by competitive inhibition (grey). [O₂] was set to 270 μM for rate calculations. (C) *H. neapolitanus* CCM genes are mostly contained in a 20 gene cluster ([Desmarais et al., 2019](#)) expressing rubisco and its associated chaperones (green), carboxysome structural proteins (purple), and an inorganic carbon transporter (orange).

Significant achievements (2020-2022):

- Discovered and characterized the mechanism of loading for the alpha-carboxysome carbonic anhydrase.
- Completed our initial functional reconstitution of the entire bacterial CO₂ Concentrating Mechanism in a heterologous host.

Science priorities for the next year (2020-2021):

- Structurally characterizing carbonic anhydrase loading interaction.
- Investigating the trajectory of CCM evolution using *in vivo* reconstitution.
- Initiating screening of DAB sequence-function landscape.

My major scientific area(s) of expertise is/are: biochemistry, synthetic biology, protein engineering.

To take my project to the next level, my ideal collaborator would have expertise in: plant biology, plant genetics.

Publications supported by this project: [2017-2020]

1. Flamholz, A. I., Dugan, E., Blikstad, C., Gleizer, S., Ben-Nissan, R. Amram, S., Antonovsky, N., Ravishankar, S., Noor, E., Bar-Even, A., et al. and Savage, D.F. Functional reconstitution of a bacterial CO₂ concentrating mechanism in *E. coli*. *BioRxiv* 2020 DOI: 10.1101/2020.05.27.119784. (*in revision at eLife*)
2. Oltrogge LM, Chaijarasphong T, Chen AW, Bolin ER, Marqusee S, Savage DF. 2020. Multivalent interactions between CsoS2 and Rubisco mediate α -carboxysome formation. *Nat Struct Mol Biol* 27: 281–287.
3. Lee T-H, Carpenter TS, D’haeseleer P, Savage DF, Yung MC. 2020. Encapsulin carrier proteins for enhanced expression of antimicrobial peptides. *Biotechnol Bioeng* 117: 603– 613.
4. Laughlin TG, Bayne AN, Trempe J-F, Savage DF, Davies KM. 2019. Structure of the complex I-like molecule NDH of oxygenic photosynthesis. *Nature* 566: 411–414.
5. Desmarais JJ, Flamholz AI, Blikstad C, Dugan EJ, Laughlin TG, Oltrogge LM, Chen AW, Wetmore K, Diamond S, Wang JY, et al. 2019. DABs are inorganic carbon pumps found throughout prokaryotic phyla. *Nat Microbiol*. 4: 2204-2215.
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7. Welkie DG, Rubin BE, Diamond S, Hood RD, Savage DF, Golden SS. 2019. A Hard Day's Night: Cyanobacteria in Diel Cycles. *Trends in Microbiology* 27: 231–242.
8. Chaijarasphong T, Savage DF. 2018. Sequestered: Design and Construction of Synthetic Organelles. In *Synthetic Biology*, pp. 279 - 306, Wiley-Blackwell.
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Quantitative Analysis of Central Metabolism in Seed Storage Synthesis

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Overall research goals:

Plant biomass is of increasing importance as renewable resource for the production of fuels and for chemical feedstocks that replace petroleum-based materials. The goal of this project is to increase the basic understanding of the functioning of storage metabolism in plants as a basis for rational engineering of seeds and other plant storage organs. To do this, different genotypes and transgenics of oilseed rape (*Brassica napus*) and related oilseed crop plants (*Thlaspi arvense*) will be created and studied. Seeds/embryos in the stage of seed development and storage synthesis are biochemically characterized by quantitation of enzyme activities, metabolites and transcripts. Stable isotope tracers are used to quantitatively analyze pathway usage (metabolic flux). Computational methods like flux balance analysis and enzyme kinetic models are used to analyze and interpret the measured data resulting in a dynamic view of the metabolic process. The plasticity of the central metabolism network is revealed and new hypotheses on regulation and function of central metabolism can be derived and tested by additional genetic and physiological perturbations. Taken together, this approach will increase understanding of the biochemical processes involved in partitioning carbon and nitrogen into seed storage compounds. It is aimed at integrated understanding of regulatory processes as well as to test the effect of transgenic alterations with regards to carbon partitioning and storage synthesis.

Significant achievements (2018-2020):

- The architecture of the central carbon metabolism network in plants is complex with parallel cytosolic and plastidic glycolytic pathways. The relative contribution of different enzyme isoforms in central carbon metabolism to oil synthesis are still far from being completely understood.
- WRINKLED1(WRI1) is known as a transcriptional master regulator that orchestrates the conversion of sucrose into triacylglycerols during seed development by upregulation of specific enzyme isoforms.
- We hypothesized that: 1) Many of the direct targets of WRI1 in central carbon metabolism are still unknown. 2) This challenge can be addressed by identifying cis-regulatory elements that bind WRI1. 3) Since oil synthesis pathways and the amino acid sequences of the DNA binding domains of WRI1 are highly conserved, such WRI1 binding sites tend to be conserved across species.
- To that end we developed a Phylogenetic Footprinting bioinformatics approach to identify cis-regulatory elements that are conserved in orthologous upstream regions. This identified conserved putative conserved WRI1 binding sites in gene promoters across 12 Brassicaceae species. Such conserved sites are over-represented in the glycolysis and fatty acid biosynthesis pathways.
- For about 150 such identified putative WRI1 DNA binding sequences in *Arabidopsis thaliana*, binding to recombinantly expressed WRI1 was confirmed by *in-vitro* by Microscale Thermophoresis.
- Mapping of putative direct WRI1 gene targets onto central carbon metabolism revealed a genetic blueprint of how the conversion of sucrose to triacylglycerols is controlled by WRI1. Several gene targets were revealed that had formerly not been implicated to be relevant in oil synthesis.

Science priorities for the next year (2020-2021):

- Further validation of 10 direct WRINKLED1 targets by Chromatin immunoprecipitation assays.
- Genetic knock out of two gene orthologs in *Thlaspi arvense*.
- The KO lines will be characterized by metabolic flux analysis, metabolomics and other biochemical assays of cultured developing embryos.

My major scientific area(s) of expertise is/are: Plant central metabolism; Metabolic Flux Analysis.

To take my project to the next level, my ideal collaborator would have expertise in: targeted proteomics for quantification of proteins/enzymes in plant tissues.

Publications supported by this project:

1. J. Schwender, I. Hebbelmann, N. Heinzl, T. Hildebrandt, A. Rogers, D. Naik, M. Klapperstuck, H.P. Braun, F. Schreiber, P. Denolf, L. Borisjuk, H. Rolletschek “Quantitative Multilevel Analysis of Central Metabolism in Developing Oilseeds of *Brassica napus* during in vitro culture.” *Plant Physiol.* 168: 828-848 (2015). <https://doi.org/10.1104/pp.15.00385>
2. H. Shi, J. Schwender, "Mathematical models of plant metabolism." *Curr. Opin. Biotechnol.* 37:143-152 (2016). <http://dx.doi.org/10.1016/j.copbio.2015.10.008>
3. X.H. Yu, R.E. Cahoon, P.J. Horn, H. Shi, R.R. Prakash, Y. Cai, M. Hearney, K.D. Chapman, E.B. Cahoon, J. Schwender, J. Shanklin, “Identification of bottlenecks in the accumulation of cyclic fatty acids in camelina seed oil.” *Plant Biotechnol J* 16, 926-938 (2018). <http://dx.doi.org/10.1111/pbi.12839>
4. X.H. Yu, Y. Cai, J. Chai, J. Schwender, J. Shanklin, “Expression of a Lychee PHOSPHATIDYLCHOLINE:DIACYLGLYCEROL CHOLINEPHOSPHOTRANSFERASE with an *Escherichia coli* CYCLOPROPANE SYNTHASE Enhances Cyclopropane Fatty Acid Accumulation in Camelina Seeds.” *Plant Physiol* 180, 1351-1361 (2019). <http://dx.doi.org/10.1104/pp.19.00396>
5. H. Liu, Z. Zhai, K. Kuczynski, J. Keereetaweep, J. Schwender, J. Shanklin, “WRINKLED1 Regulates BIOTIN ATTACHMENT DOMAIN-CONTAINING Proteins that Inhibit Fatty Acid Synthesis.” *Plant Physiol* 181, 55-62 (2019). <http://dx.doi.org/10.1104/pp.19.00587>
6. U. Schluter, A. Brautigam, J.M. Droz, J. Schwender, A.P.M. Weber, “The role of alanine and aspartate aminotransferases in C4 photosynthesis.” *Plant Biol (Stuttg)* 21 Suppl 1, 64-76 (2019). <http://dx.doi.org/10.1111/plb.12904>
7. K. Kuczynski, S. McCorkle, J. Keereetaweep, J. Shanklin, J. Schwender, “An Expanded Role for WRINKLED1 Metabolic Control Based on Combined Phylogenetic and Biochemical Analyses.” *bioRxiv*: 2020.2001.2028.923292 (2020). <https://doi.org/10.1101/2020.01.28.923292>
8. H. Rolletschek, J. Schwender, C. König, K.D. Chapman, T. Romsdahl, C. Lorenz, H.P. Braun, P. Denolf, K.V. Audenhove, E. Munz, N. Heinzl, S. Ortleb, T. Rütten, S. McCorkle, T. Borysyuk, A. Gundel, H. Shi, M. Vander Auwermeulen, S. Bourrot, L. Borisjuk, “Cellular Plasticity in Response to Suppression of Storage Proteins in the *Brassica napus* Embryo.” *Plant Cell*, accepted manuscript (2020). <https://doi.org/10.1105/tpc.19.00879>
9. T.J. Clark, L. Guo, J. Morgan, J. Schwender, “Modeling Plant Metabolism: From Network Reconstruction to Mechanistic Models.” *Annu Rev Plant Biol* 71, 303-326 (2020). <http://dx.doi.org/10.1146/annurev-arplant-050718-100221>

Nitrogenase Reduction of N₂ and CO₂

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Overall research goals:

The research objectives of our joint project are to reveal molecular level insights into the mechanism for how the enzyme nitrogenase catalyzes the reduction of N₂ to NH₃ and the reduction of CO₂ and CO to formate and hydrocarbons. Further, we seek to gain insights into the complex array of proteins and steps involved in the installation of the active site metal clusters of nitrogenase. The outcomes of these studies are expected to provide foundational information about the assembly and reactivity of the complex metal clusters of nitrogenase, giving guidance to the design of next generation N₂, CO₂, and CO reduction catalysts.

Significant achievements (2017-2020):

Progress during this three-year funding cycle (2017-2020) has resulted in 20 peer reviewed publications (a few of these publications are listed below). A main thrust for our studies was to gain an understanding of the mechanism of N₂ binding and H₂ release at the E₄ state of the Mo-nitrogenase active site FeMo-cofactor. By utilizing an array of approaches, including amino acid substitutions, advanced paramagnetic spectroscopies, and theory, we have been able to detail events at this critical step in the nitrogenase mechanism. In short, we now know that accumulation of four electrons and 4 protons on FeMo-co is achieved by the formation of two Fe-hydrides and two protons bound to sulfides. The reductive elimination of these two hydrides as H₂ is mechanistically coupled and required to achieve N₂ binding and reduction. Our mechanistic insights suggest that the exergonic release of H₂ is coupled to the endergonic binding of N₂, creating an overall favorable reaction for this extremely difficult step in N₂ reduction.

During this funding period, our team developed a strep-tag purification system for all three nitrogenase isozymes (Mo-, V-, and Fe-), allowing for the first time a comprehensive comparison of the substrate reactivity for these isozymes. Earlier work suggested that the three isozymes might follow different N₂ reduction mechanisms. We showed that all three isozymes, in fact, follow the same reductive elimination mechanism for N₂ activation, with variations in the rate constants between H₂ release and N₂ binding accounting for the observed differences among the enzymes. These findings, made possible by the development of a robust kinetic model, thus reveals a universal mechanism for N₂ reduction used by all known biological catalysts.

Finally, we have made significant progress in understanding key steps in the maturation and insertion of the active site metal clusters in Mo-nitrogenase. We are now in a position to gain insights into fundamental steps in the activation pathway and the roles of key protein partners.

Science priorities for the next year (2020-2021):

- Develop kinetic models that describe the reactivity of all three nitrogenase isozymes for CO and CO₂ reduction to hydrocarbon products.
- Utilize QM/MM calculations to predict the reactivity of the three nitrogenase isozymes toward CO and CO₂, and to test these predictions by a combination of amino acid substitutions and product profile analysis.

- Expand the existing reaction pathway for N₂ conversion to NH₃ by characterizing the three nitrogenase isozymes with a combination of spectroscopic and substrate-product kinetic studies.
- Exploit the unique EPR characteristics of the V- and Fe-nitrogenase isozymes to gain insights into all of the E reaction mechanism intermediates.
- Employ electrochemical methods developed during the current funding cycle to advance our mechanistic understanding for substrate reduction.
- Explore the protein factors involved in the delivery of Fe, Mo, S and homocitrate during the maturation of the MoFe protein.

Our major scientific area(s) of expertise is/are: Mechanistic enzymology, metalloenzyme mechanism, electron transfer, FeS clusters, kinetics, electrochemistry, spectroscopy, microbial genetics.

To take this project to the next level, ideal collaborators would have expertise in: Application of robust QM/MM calculations on the metal cofactors and protein surroundings in nitrogenase to guide new studies to advance our mechanistic understanding and expertise in X-ray crystallography and scanning EM .

Publications supported by this project (2017-2020) (Selected of 20 total)

1. Seefeldt, L. C., Yang, Z.-Y., Lukoyanov, D. A., Harris, D. F., Dean, D. R., Raugei, S., and Hoffman, B. M. (2020) Reduction of substrates by nitrogenases. *Chem. Rev.* *120*, 5082–5106. 10.1021/acs.chemrev.9b00556
2. Van Stappen, C., Davydov, R., Yang, Z.-Y., Fan, R., Guo, Y., Bill, E., Seefeldt, L. C., Hoffman, B. M., and DeBeer, S. (2019) Spectroscopic description of the E1 state of Mo nitrogenase based on Mo and Fe X-ray absorption and Mössbauer studies. *Inorg. Chem.* *58*, 12365–12376. 10.1021/acs.inorgchem.9b01951
3. Lukoyanov, D., Krzyaniak, M. D., Dean, D. R., Wasielewski, M. R., Seefeldt, L. C., and Hoffman, B. M. (2019) Time-resolved EPR study of H₂ reductive elimination from the photoexcited nitrogenase Janus E4(4H) intermediate. *J. Phys. Chem. B* *123*, 8823–8828. 10.1021/acs.jpccb.9b07776
4. Jimenez-Vicente, E., Yang, Z.-Y., Campo, J. S. M. del, Cash, V. L., Seefeldt, L. C., and Dean, D. R. (2019) The NifZ accessory protein has an equivalent function in maturation of both nitrogenase MoFe protein P-clusters. *J. Biol. Chem.* *294*, 6204–6213. 10.1074/jbc.RA119.007905
5. Hoeke, V., Tociu, L., Case, D. A., Seefeldt, L. C., Raugei, S., and Hoffman, B. M. (2019) High-resolution ENDOR spectroscopy combined with quantum chemical calculations reveals the structure of nitrogenase Janus intermediate E4(4H). *J. Am. Chem. Soc.* *141*, 11984–11996. 10.1021/jacs.9b04474
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7. Raugei, S., Seefeldt, L. C., and Hoffman, B. M. (2018) Critical computational analysis illuminates the reductive-elimination mechanism that activates nitrogenase for N₂ reduction. *Proc. Natl. Acad. Sci. U. S. A.* *115*, E10521–E10530. 10.1073/pnas.1810211115
8. Lukoyanov, D. A., Khadka, N., Yang, Z.-Y., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2018) Hydride conformers of the nitrogenase FeMo-cofactor two-electron reduced state E2(2H), assigned using cryogenic intra electron paramagnetic resonance cavity photolysis. *Inorg. Chem.* *57*, 6847–6852. 10.1021/acs.inorgchem.8b00271
9. Harris, D., Yang, Z., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2018) Kinetic understanding of N₂ reduction versus H₂ evolution at the E4(4H) Janus state in the three nitrogenases. *Biochemistry* *57*, 5706–5714. 10.1021/acs.biochem.8b00784
10. Harris, D. F., Lukoyanov, D. A., Shaw, S., Compton, P., Tokmina-Lukaszewska, M., Bothner, B., Kelleher, N., Dean, D. R., Hoffman, B. M., and Seefeldt, L. C. (2018) Mechanism of N₂ reduction catalyzed by Fe-nitrogenase involves reductive elimination of H₂. *Biochemistry* *57*, 701–710. 10.1021/acs.biochem.7b01142

Bringing Inorganic Carbon to Life: Developing Model Metalloenzymes for C₁ Conversion

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Overall research goals:

The carbon monoxide dehydrogenase (CODH)/ acetyl coenzyme-A synthase (ACS) enzyme is a critical component of the Wood-Ljungdahl metabolic pathway, catalyzing the reduction of CO₂ to CO and a subsequent coupling reaction between CO and a methyl group to form the energy-rich cofactor acetyl-CoA. The Wood-Ljungdahl pathway is thought to be the oldest mechanism for fixing CO₂ into cellular biomass and remains a dominant metabolic process in acetogens, methanogens, and other classes of anaerobic organisms; thus, the CODH/ACS enzyme is implicated in the chemoautotrophic theory of the origins of life. Moreover, the reaction catalyzed by CODH/ACS generates a new carbon-carbon bond under net redox-neutral conditions, with relevance to production of liquid fuels from simple precursors. Because of the potential applications of CODH/ACS-type chemistry, understanding the native enzyme is an important objective; however, much remains unknown about the mechanism by which CO₂ is selectively reduced or acetyl-CoA is synthesized from its C₁ constituents.

The active site of ACS contains a [4Fe-4S] cluster bound to a binuclear nickel center. Despite this complexity, only one of the nickel ions is thought to be involved in catalysis, and the pertinent oxidation states of the catalytically relevant nickel center remain debated. To better characterize the landscape of accessible chemical reactivity at a biological nickel center, we are developing a functional model of ACS within the active site of the azurin protein scaffold. Building from preliminary results indicating nickel-substituted azurin (NiAz) can access a Ni^I state that is suggested to closely resemble the geometric and electronic structure of ACS, we are installing reactivity towards substrate binding and conversion. Using an array of spectroscopic, analytical, and computational tools, we will probe effects on reduction potential, active-site geometry, and spin state as the primary and secondary coordination spheres are varied. Reactivity of the site towards natural and engineered ACS substrates will be explored, and the scope of carbon-carbon bond forming reactions will be investigated and optimized using rational protein design. We are also developing structural and functional models of CODH using Az and other protein scaffolds, based on a semisynthetic approach, to understand the role of the secondary and tertiary sphere on catalytic activity and selectivity. Comparison between the protein-based models and the native enzyme will address long-debated questions about the mechanisms of CODH and ACS and shed insight into critical elements for effective CO₂ reduction and C-C bond coupling reactions.

Significant achievements (2017 - 2020):

- We have characterized the binding of both ACS substrates, CO and CH₃⁺, to a nickel-substituted azurin mutant, M121A Ni^IAz, showing physiologically relevant affinities and redox transformations.
- We have demonstrated, for the first time, the capacity of a biological nickel center to support a two-electron addition of a cationic methyl group to the Ni^I center, generating a formal Ni^{III}-CH₃ species.

- We have interrogated the bioorganometallic Ni^I-CO and Ni^{III}-CH₃ species using advanced pulsed EPR techniques to resolve the geometric and electronic structures of these states. In conjunction with quantum chemical calculations and MO analysis, the formal Ni^{III}-CH₃ species is suggested to utilize an inverted ligand field, retaining a ⁺CH₃ ligand bound to Ni^I. This inverted ligand field is suggested to be a mechanism by which to control further reactivity and is proposed to be operative in native ACS, allowing stabilization of the otherwise unstable Ni-CH₃ species until the appropriate conditions are achieved.
- We have identified novel reactivity performed by M121A Ni^IAz when both CO and ⁺CH₃ are added in the presence of excess reductant. The products formed mirror those observed in native ACS under analogous conditions. A distinct product distribution is observed depending on the initial state, Ni^I-CO or Ni^{II}-CH₃ Az.
- We have expressed, purified, and reconstituted a structural model for the CODH active site within a model protein scaffold and demonstrated the reversible accumulation of 3 reducing equivalents while remaining inert towards H⁺ reduction. Preliminary data indicate the interaction of this model system with CODH substrates.

Science priorities for the next year (2020-2021):

- Quantify products formed by M121A Ni^IAz and characterize spectroscopic signatures of protein-based intermediate species produced during the reaction.
- Develop an azurin-based model ACS that can be regenerated to catalytically produce acetic acid and/or thioesters.
- Develop catabolic reactions with thioesters to establish biochemical precedence for organometallic reversibility.
- Establish reactivity of structural CODH model with CO and CO₂ as a function of applied electrochemical and solution-phase potential and characterize intermediates formed using spectroscopic techniques.

My major scientific area(s) of expertise is/are: Bioinorganic chemistry, pulsed EPR, resonance Raman spectroscopy, inorganic electronic structure, protein electrochemistry, protein engineering.

To take my project to the next level, my ideal collaborator would have expertise in: Enzymology assays (e.g., radiolabels, LC-MS/MS), paramagnetic NMR, anaerobic X-ray crystallography.

Publications supported by this project 2017 - 2020 :

1. Schneider, C.R.; Lewis, L.C.; Shafaat, H.S.* (2019) The good, the neutral, and the positive: buffer identity impacts CO₂ reduction activity by nickel(II) cyclam. *Dalton Trans.*, 48, 15810-15821. DOI: 10.1039/C9DT03114F. ***Selected as Dalton Transactions HOT Article.**
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4. Schneider, C.R.; Manesis, A.C.; Stevenson, M.J.; Shafaat, H.S.* (2018) A Photoactive Semisynthetic Metalloenzyme Exhibits Complete Selectivity for CO₂ Reduction in Water. *Chem. Commun.* 54, 4681 - 4684. DOI: 10.1039/C8CC01297K.
5. Manesis, A. C.; O'Connor, M. J.‡; Schneider, C. R.; Shafaat, H. S.* (2017) Multielectron Chemistry within a Model Nickel Metalloprotein: Mechanistic Implications for Acetyl-CoA Synthase. *J. Am. Chem. Soc.* 139 (30), 10328 - 10338. DOI: 10.1021/jacs.7b03892.

Modification and Regulation of Plant Lipids

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Y. Cai, Postdoctoral Research Associate, Z. Zhai and J. Keereetaweep Assistant Biochemists
Biology Dept. Brookhaven National Lab

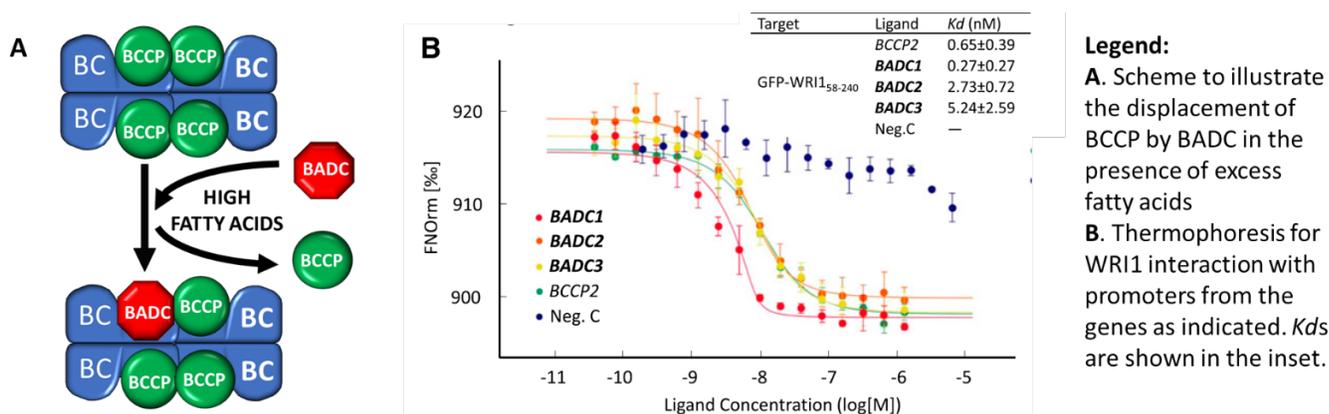
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Overall research goals:

To understand the mechanism(s) of regulation of lipid homeostasis in plants, specifically how cells reprogram metabolism when cellular conditions are appropriate for making carbon- and reductant-demanding fatty acids and triacylglycerols. Another main thrust is to understand mechanisms underlying the functional diversity of diiron enzymes.

Significant achievements:

During the current funding cycle, we investigated the role biotin attachment domain-containing proteins (BADCs) on lipid homeostasis. BADCs are inactive homologs of biotin carboxy carrier protein that lack a biotin prosthetic group and are therefore inhibitory to acetyl-CoA carboxylase (ACCase), the rate limiting enzyme in fatty acid synthesis (see panel A below). Our group previously showed that excess fatty acids reversibly inhibit ACCase in the short term by binding the terminal product of the ACP track of fatty acid biosynthesis, oleoyl-ACP. Prolonged exposure to excess fatty acids resulted in irreversible inhibition of ACCase that is mediated by the incorporation of BADCs into ACCase (Keereetaweep et. al., 2018). While BADCs are responsible for irreversible inhibition of ACCase, a knockout of BADC1 and BADC3, showed that under normal growth conditions, BADC1 and BADC3 also inhibit ACCase, because *badc1, badc3* double mutant seeds accumulate approximately 33% more TAG. In subsequent work we determined that WRI1, the transcriptional activator of approximately 50 genes involved in fatty acid synthesis also activates inhibitors of the same process by binding to AW sites upstream of the transcriptional initiation site (Liu et. Al 2019). To verify their transcriptional control microscale thermophoresis was used to evaluate *K_d*s of WRI1 binding to the promoters of BADC1 2 and 3 which showed high affinity dissociation constants equivalent to that of BCCP2, a strongly WRI1-induced gene (see panel B. below). Consistent with this observation, both expression of *BADC1*, *BADC2*, and *BADC3* genes and BADC1 protein levels were reduced in the *wri1-1* mutant relative to the wild type and elevated upon *WRI1* overexpression. That WRI1 positively regulates genes encoding both FA synthesis and BADC proteins (i.e. conditional inhibitors of FA synthesis), represents a coordinated mechanism to achieve lipid homeostasis in which plants couple the transcription of their FA synthetic capacity with their capacity to biochemically downregulate it.



Science objectives for 2020-2021:

- Use biophysical techniques to determine the mechanism of replacement of BCCP with BADC
- Test whether BADC inhibition is responsible for ACCase inhibition upon the expression of unusual fatty acids such as ricinoleic acid.
- Use computational theory and molecular dynamics simulations to understand desaturase regioselectivity and chemoselectivity.
- Model modes of binding of acyl chains to various ACPs and see how this affects desaturation and thioesterase substrate specificity.

My scientific area(s) of expertise is/are: enzymology of desaturases, mechanisms of metabolic regulation.

To take my project to the next level, my ideal collaborator would: Expression of proteins in animal cells.

Publications supported by this project 2018-2020:

- Keereetaweeep J, Liu H, Zhai Z, Shanklin J. 2018. Biotin Attachment Domain-Containing Proteins Irreversibly Inhibit Acetyl CoA Carboxylase. *Plant Physiol.* 177(1):208-215. <https://doi.org/10.1104/pp.18.00216>
- Zhai Z, Keereetaweeep J, Liu H, Feil, R., Lunn, J.E., Shanklin J. 2018. Trehalose 6-phosphate positively regulates fatty acid synthesis by stabilizing WRINKLED1. *The Plant Cell* 30: 2616–2627. <https://doi.org/10.1105/tpc.18.00521>
- Cai Y., Yu, X-H., Liu, C.J., and Shanklin, J. 2018. Two clusters of residues contribute to the activity and substrate specificity of a bifunctional oleate- linoleate and linolenate- desaturase Fm1. *J. Biol. Chem.* 293(51) 19844 –19853. <https://doi.org/10.1074/jbc.RA118.005972>
- Yu, X-H., Cai, Y., Chai, J., Schwender, J., and Shanklin, J. 2019. Expression of a Lychee PDCT with *E. coli* CPS Enhances Cyclopropane Fatty Acid in Camelina Seeds. *Plant Physiol.* 180, pp. 1351–1361. <https://doi.org/10.1104/pp.19.00396>
- Liu, H., Zhai, Z., Kuczynski, C., Keereetaweeep, J., Schwender, J., and Shanklin, J. 2019. BIOTIN ATTACHMENT DOMAIN-CONTAINING proteins, inhibitors of ACCase, are regulated by WRINKLED1. *Plant Physiol.* 177, pp. 208–215. <https://doi.org/10.1104/pp.19.00587>
- Fan, J., Zhou, C., Yu, L., Li, P., Shanklin, J., and Xu, C. 2019. Diversion of Carbon Flux from Sugars to Lipids Improves the Growth of an Arabidopsis Starchless Mutant. *Plants* 2019, 8(7), 229. <https://doi.org/10.3390/plants8070229>
- Whittle, E.J., Cai, Y., Keereetaweeep, J., Chai, J., Buist, P.H., Shanklin, J. 2020. Castor Stearoyl-ACP Desaturase Can Synthesize a Vicinal Diol by Dioxygenase Chemistry. *Plant Physiol.* 182, pp. 730–738. <https://doi.org/10.1104/pp.19.01111>
- Cai Y., Yu, X-H., Chai, J., and Shanklin, J. 2020. A conserved evolutionary mechanism permits Δ^9 desaturation of very long chain fatty acids. *J. Biol. Chem.*, In Press. doi: 10.1074/jbc.RA120.014258.
- Yu, X-H., and Shanklin, J. 2020. Solving a furan fatty acid biosynthesis puzzle. *J. Biol. Chem.* (2020) 295(29) 9802–9803. <https://www.jbc.org/content/295/29/9802>

Understanding redox proportioning through ferredoxins, low potential Fe-S proteins acting as electrical hubs to control metabolism

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Overall research goals:

Iron-sulfur (Fe-S) cluster containing ferredoxins (Fd) function as electron carriers in biochemical pathways important for energy transduction, with roles ranging from hydrogen and alcohol production to carbon and nitrogen fixation. These low potential (high energy) proteins behave as central energy-conserving redox hubs, serving as conduits between diverse redox donors and acceptors. While it is clear that Fds are abundant across the tree of life, with individual microbes frequently using multiple Fd paralogs to control electron flow, we do not yet fully understand what controls the proportion of electrons relayed by individual Fds among the diverse oxidoreductases found within cells. Our goal is to elucidate the physicochemical parameters that underlie Fd control over electron flow sufficiently so that we can use Fd sequence and structure to anticipate the proportion of electrons that colocalized Fds deliver to their various natural redox partners. We posit that studies that characterize large numbers of natural and non-natural protein-protein interactions involving structurally-diverse Fds will be critical to establishing the rules that underlie sequence-structure-ET relationships in the Fd family.

Significant achievements (2019-2020):

Prochlorococcus phage Fd: structural characterization and interactions with cyanobacterial oxidoreductases. Bioinformatic analysis revealed that phage Fds display the highest similarity with cyanobacterial Fds that transfer electrons from photosystems to oxidoreductases involved in nutrient assimilation. Structural analysis of myovirus P-SSM2 Fd, which infects *Prochlorococcus marinus*, yielded a crystal structure of this protein, which displays high similarity to cyanobacterial Fds. Electrochemical analysis of P-SSM2 Fd also revealed a low midpoint reduction potential (-336 mV vs. SHE), which is similar to Fds that couple with photosystems. However, this phage Fd presented unusually low thermostability ($T_M = 29^\circ\text{C}$). When expressed in an *Escherichia coli* strain with a sulfite assimilation defect, P-SSM2 Fd complemented growth when coexpressed with a *P. marinus* SIR, revealing that P-SSM2 Fd can transfer electrons to host SIRs. The high structural similarity with cyanobacterial Fds and cross reactivity with sulfite reductase suggests that phage Fds evolved to transfer electrons between both phage- and cyanobacterial-encoded oxidoreductases.

Recombination of Fds reveals differences in the inheritance of thermostability and midpoint potential. To investigate how recombination affects the properties of an oxidoreductase that transfers electrons in cells, we created Fd chimeras by recombining distantly-related cyanobacterial and cyanomyophage Fds that present similar midpoint potentials but distinct thermostabilities. Fd chimeras having a wide range of amino acid substitutions retained the ability to coordinate an iron-sulfur cluster, although their thermostabilities varied with the fraction of residues inherited from each parent. The midpoint potentials of chimeric Fds also differed. However, all of the synthetic Fds exhibited midpoint potentials outside of the parental protein range. Each of the chimeric Fds could also support electron transfer between Fd-NADP reductase and sulfite reductase in *Escherichia coli*, although the chimeric Fds required distinct expression levels to support similar levels of cellular electron transfer. These results show how recombination can be used to rapidly diversify the properties of ferredoxins and reveal differences in the inheritance of thermostability and electrochemical properties.

Flavodoxin electron carriers: bioinformatic analysis and electron transfer to assimilatory sulfite reductases. To investigate if flavodoxins (Flds) can support ET to Fd-dependent SIRs, we examined their ability to support the growth of an *E. coli* auxotroph that requires ET from corn FNR to a SIR to synthesize an essential metabolite, sulfide. Like corn Fd, *Synechocystis sp.*, *Acaryochloris marina*, and *Nostoc sp* Fld complemented growth. To assess the relative ET efficiencies of Fds and Flds in this strain, we created fusions of these proteins with RFP and have begun quantifying the relative expression of these proteins under different inducer conditions where the selection was performed. FNR and SIR are constitutively expressed while Fd-RFP (and Fld-RFP) expression is induced by anhydrotetracycline. When we calculated the fluorescence/OD ratio across a range of aTc levels, which is proportional to the relative ET flux/Fd under conditions where ET is rate limiting for growth, we observe a consistent ratio of growth to expression for individual proteins. These results provide the first evidence that Flds support ET to SIR and show how Fld-RFP fusions can be used with selections to quantify the relative efficiencies of cellular ET through a defined linear pathway.

Science priorities for the next year (2020-2021):

- Use cellular assays to analyze how Fds from diverse organisms vary in their electron cycling between pairs of donor/acceptor proteins that require ET across distinct midpoint potential ranges. One assay will evaluate Fd ET from a strong reductant, pyruvate Fd reductase (PFOR) to Fd NAD(P) reductase (FNR), while the other will evaluate ET from a weaker reductant, FNR to sulfite reductase (SIR). Determine how ET variation in the assay depends upon Fd midpoint potential.

My major scientific area(s) of expertise is/are: metabolism, microbial genetics, protein electron carriers, protein design, redox cofactors, and synthetic biology.

To take my project to the next level, my ideal collaborator would have expertise in: (i) computational docking of electron transfer proteins, (ii) high-throughput biophysical analysis, such as analysis of midpoint potentials, and (iii) quantitative MS for determining intracellular protein concentrations.

Publications supported by this project [2018-2020] :

1. Campbell, I.J., Kahanda, D., Atkinson, J.T., Sparks, N.O., Kim, J., Tseng, C.P., Verduzco, R., Bennett, G.N., and Silberg, J.J., Recombination of 2Fe-2S ferredoxins reveals differences in the inheritance of thermostability and midpoint potential, *ACS Synth. Biol.*, *in revision* (2020) [BioRxiv DOI: 10.1101/2020.05.29.122317]
2. Campbell, I.J., Olmos, J., Xu, W., Kahanda, D., Atkinson, J.T., Sparks, N., Phillips, G.N., Bennett, G.N., and Silberg, J.J., Prochlorococcus phage ferredoxin: structural characterization and interactions with cyanobacterial oxidoreductases, *J. Biol. Chem.*, *in press* (2020). [DOI: 10.1074/jbc.RA120.013501]
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5. Mutter, A.C., Tyryshkin, A.M., Campbell, I.J., Poudel, S., Bennett, G.N., Silberg, J.J., Nanda, V., and Falkowski, P.G., De novo design of symmetric ferredoxins that shuttle electrons in vivo, *PNAS*, 116(29): 14557-14562 (2019). [DOI: 10.1073/pnas.1905643116]
6. Atkinson, J.T., Campbell, I.J., Thomas, E.E., Bonitatibus, S.C., Elliott, S.J., Bennett, G.N., and Silberg, J.J., Metalloprotein switches that display chemical-dependent electron transfer in cells, *Nat. Chem. Biol.*, 15(2), 189-195 (2019). [DOI: 10.1038/s41589-018-0192-3]
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Nanotube-Confined Lipid Bilayers and Redox-Active Enzymes

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Overall research goals:

The overarching goal of this project is to attain fundamental understanding of local structure and electron spin density distribution in reaction centers/catalytic sites of redox-active enzymes associated with cellular membranes. We will employ advanced EPR methods and nanotechnology to (1) further improve resolution of EPR methods, (2) derive orientations of magnetic tensors with respect to membrane, and (3) investigate effects of lipid matrix, nanoscale confinement, as well as manmade bio-nano interfaces on electron spin density distribution. For the current funding period our specific goals will be to demonstrate the utility and novel capabilities of the lipid nanotube array and advanced pulsed EPR technologies developed in the preceding phase of this DOE project to provide unique insights into local structure and electron spin density distribution in Photosynthetic Reaction Centers.

Significant achievements (2020-2021):

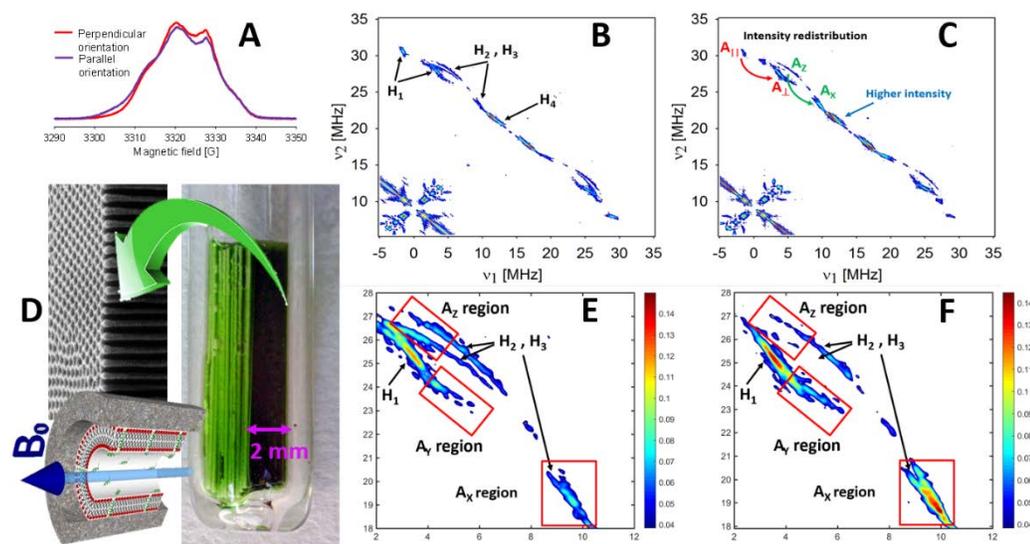


Figure 1. Macroscopic alignment of 750 kDa PSII reconstituted into DOPC lipids after deposition into ordered nanoporous substrates with ca. 400 nm pore diameter (D) and subsequent echo-detected (A) and HYSCORE X-band experiments at two sample orientations (B, C, E, F).

Here we provide the *initial demonstration of new capabilities* of the lipid nanotube array technology developed in the preceding phases of this DOE project to study redox active enzymes on example of Photosystem II (PSII). After lipid reconstitution and deposition into 400 nm pores (Fig.1D) the sample was illuminated for 10 min and shock frozen to generate and trap the Y_D radical, which we used to test deposition and orientation of PSII inside the AAO nanopores. Echo detected field sweep EPR spectra of Y_D^\bullet exhibit well resolved differences induced by orientation selection (small peak at ~ 3328 G is from quartz E' centers). The low field shoulder is well pronounced for the parallel orientation but strongly suppressed for perpendicular one. The low field region corresponds to B_0 along the largest g - and hyperfine tensor components. Those orientations are absent if the bilayer director is perpendicular to B_0 .

AAO-aligned samples allowed us to obtain data on the orientation of the hyperfine tensors with respect to bilayer normal for lipid embedded PSII *for the first time* by measuring 4-pulse HYSCORE X-band spectra at two AAO orientations. All experimental parameters were kept identical and only the orientation was varied from parallel (B) to perpendicular (C). While the overall patterns are similar, (i) there is a clear intensity redistribution for the strongly coupled β -methylene proton (H_1 in the Figure) from the cross-peaks corresponding to larger A_{\perp} to smaller A_{\parallel} , (ii) cross-peaks corresponding to A_z , the largest hyperfine component of either 3- or 5-ring (H_2 and H_3) protons (the differences between them

remain spectrally not assigned up to this date) disappear nearly completely for perpendicular orientation while are well pronounced for the parallel one; (iii) in contrast, cross-peaks corresponding to the smallest A_X and A_Y components are much more intense for perpendicular vs. parallel orientation; (iv) intensity of the cross-peaks corresponding to the H-bonded proton of His189 (predominantly from A_{\parallel} orientation) is significantly higher for the perpendicular orientation. See Fig. 1E-F for zoom in of the ring ^1H features.

Well resolved orientation effects on the echo detected field sweep EPR spectra provide direct evidence for the efficient *PSII* deposition inside the AAO nanopores. The observed changes of HYSORE spectra are well in line with the field sweep spectra, both indicative of orientation effects caused by decreased contributions of large hyperfine couplings in perpendicular vs. parallel orientations. The most pronounced differences observed in HYSORE spectra for either the 3- or 5-ring proton can only be explained by orientation of the z-axis of the hyperfine tensor being approximately along the lipid membrane normal. Similarly, axes corresponding to A_{\parallel} of the β -methylene and A_{\perp} of the H-bonded protons must be directed at small angles with respect to the membrane normal.

Science priorities for the next year (2020-2021):

- Analyze HYSORE spectra by simulations to deduce orientation of hyperfine tensors vs. membrane.
- Optimize lipid reconstitution and deposition procedures to increase EPR signal.
- Apply AAO method to elucidate electronic structure of the primary electron donor $\text{P}_{700}^{+\bullet}$ in *PSI*.
- Resolve protonation patterns of S_3 and S_0 intermediate states of tetra-nuclear Mn cluster of *PSII*.

My major scientific area(s) of expertise is/are: electron paramagnetic resonance, double resonance and pulsed methods such as DEER and HYSORE, solid state NMR, biophysics of lipid bilayers and membrane proteins, nanoporous substrates, hybrid lipid-inorganic nanostructures.

To take my project to the next level, my ideal collaborator would have expertise in: biochemistry of redox-active enzymes and electrochemistry of such enzyme systems.

Publications supported by this project (2019-2020):

1. L. Cha, S. Milikisiyants, M. Davidson, S. Xue, T. Smirnova, A. I. Smirnov, Y. Guo, and W.-C.n Chang, Alternative Reactivity of Leucine 5-Hydroxylase Using an Olefin-2 Containing Substrate to Construct a Substituted Piperidine Ring, *Biochemistry*, 59, 1961-1965 (2020); DOI: 10.1021/acs.biochem.0c00289
2. E. G. Kovaleva, L. S. Molochnikov, D. P. Tambasova, A. Marek, M. Chestnut, V. A. Osipova, D. O. Antonov, I. A. Kirilyuk, A. I. Smirnov, Electrostatic Properties of Inner Nanopore Surfaces of Anodic Aluminum Oxide Membranes upon High Temperature Annealing are Revealed by pH Spin Probes and Labels, *Journal of Membrane Science*, 604 (2020) 118084; doi: 10.1016/j.memsci.2020.118084
3. V. Perelygin, M. A. Voinov, A. Marek, E. Ou, J. Krim, D. Brenner, T. I. Smirnova, and Alex I. Smirnov, Dielectric and Electrostatic Properties of Silica Nanoparticle - Water Interface by EPR of pH-Sensitive Spin Probes, *J. Phys. Chem. C* 2019, 123, 49, 29972-29985 (2019); DOI: 10.1021/acs.jpcc.9b08007.
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5. J. Ma, Y. Lin, Y.-W. Kim, Y. Ko, J. Kim, K. H. Oh, J.-Y. Sun, C. B. Gorman, M. A. Voinov, Alex I. Smirnov, J. Genzer, M. D. Dickey, Liquid Metal Nanoparticles as Initiators for Radical Polymerization of Vinyl Monomers, *ACS Macro Lett.* 8, 1522-1527 (2019); DOI: 10.1021/acsmacrolett.9b00783
6. T. I. Smirnova and A. I. Smirnov, Chapter 7 - EPR Studies of Bionanomaterials, in *Experimental Methods in the Physical Sciences*, v. 50, "Electron Magnetic Resonance - Applications in Physical Sciences and Biology" edited by A. K. Shukla, Academic Press, Elsevier, Cambridge, MA, pp. 129-159 (2019), DOI: 10.1016/B978-0-12-814024-6.00007-8
7. D. B. Good, M. A. Voinov, D. Bolton, M. E. Ward, I. V. Sergeev, M. Caporini, P. Scheffer, A. Lo, M. Rosay, A. Marek, L. S. Brown, Alex I. Smirnov, V. Ladizhansky, A Biradical-tagged Phospholipid as a Polarizing Agent for Solid-state MAS Dynamic Nuclear Polarization NMR of Membrane Proteins, *Solid State Nuclear Magnetic Resonance*, 100: 92-101 (2019); DOI: 10.1016/j.ssnmr.2019.04.003

Engineering Selenoproteins for Enhanced Hydrogen Production

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Overall research goals:

1. Engineering of hydrogenases and formate dehydrogenase with Sec-coordinated metal clusters.
2. Method for making designer selenoproteins in mammalian cells.

Significant achievements (2016-2019):

1. Development of a very efficient, designed tRNA that allows site-specific insertion of up to five selenocysteine residues into proteins (e.g., *E. coli* formate dehydrogenase H)

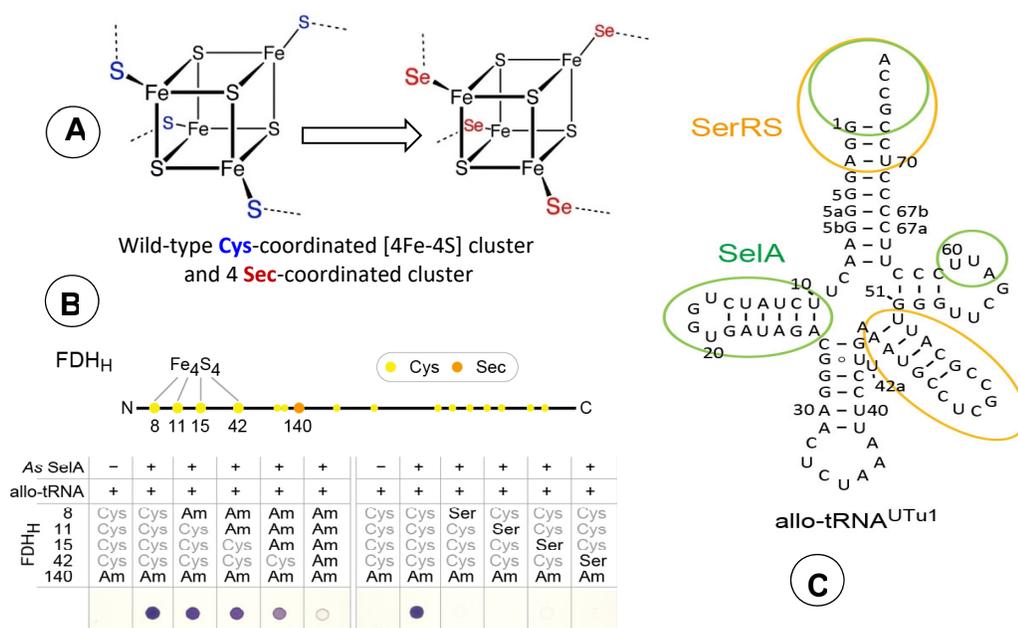


Fig 1. A novel tRNA for improved selenoprotein synthesis. **A)** Replacement of the Cys-coordinated 4Fe-4S cluster in *E. coli* formate dehydrogenase H (FDH_H). **B)** *E. coli* FDH_H encoded by *fdhF* with a catalytic Sec residue (140) and four Cys residues accommodating the iron sulfur cluster. FDH_H expression in *E. coli* *ΔselABC ΔfdhF* cells with the allo-tRNA^{UTu}, *Aeromonas salmonicida* (As) SclA, and an *fdhF* gene variant carrying mutations at codon positions 8, 11, 15, 42, and 140. “Am” indicates the UAG codon. FDH_H activity is monitored by formation of a purple dye (at the bottom of the panel) in response to benzyl viologen reduction. **C)** The very efficient new allo-tRNA^{UTu}. Regions important for SclA or SerRS identity are indicated.

2. Collaborative studies of Cys to Sec conversions in hydrogenases or dehydrogenases are continuing with (1) Russ Hille (UC Riverside), (2) Fraser Armstrong (Oxford University), and (3) David Mulder (NREL).

(1) We have produced an active-site Sec variant of the *Cupriavidus necator* NAD⁺-dependent formate dehydrogenase. This should provide the missing link for understanding the hydride transfer mechanism. (2) We have generated a Sec variant of *E. coli* hydrogenase-1 which shows extraordinary oxygen tolerance over that of its corresponding Cys enzyme. (3) We plan to insert Sec into the FeS cluster of a model Fe-Fe hydrogenase. These variants will be the driving force for understanding the mechanistic model of H₂-catalysis.

Science objectives for 2020-2021:

- To demonstrate that Cys to Sec conversion will work for different enzymes and proteins in order to make our approach a robust method for selenoprotein production in *E. coli*, yeast, and mammalian cells.

My scientific areas of expertise are: Translation and the Genetic Code.

To take my project to the next level, my ideal collaborator would have expertise in leading-edge bioinformatics.

Publications supported fully or in part by this project (2016-2019):

1. T. Mukai, M. Englert, H.J. Tripp, C. Miller, N.N. Ivanova, E.M. Rubin, N.C. Kyrpides, D. Söll (2016) Facile recoding of selenocysteine in nature. *Angew. Chem. Int. Ed. Engl.* **55**, 5337-5341.
2. A. Crnković, T. Suzuki, D. Söll, N.M. Reynolds (2016) Pyrrolysyl-tRNA synthetase, an aminoacyl-tRNA synthetase for genetic code expansion. *Croat. Chem. Acta* **89**, 163-174. doi:10.5562/cca2825.
3. T. Mukai, M. Englert, H.J. Tripp, C. Miller, N.N. Ivanova, E.M. Rubin, N.C. Kyrpides, D. Söll (2016) Leichte natürliche Neukodierung von Selenocystein. *Angew. Chem.* **128**, 5423-5427.
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9. T. Suzuki, C. Miller, L-T. Guo, J.M.L. Ho, D.I. Bryson, Y-S, Wang, D.R. Liu, D. Söll (2017) Crystal structures reveal an elusive functional domain of pyrrolysyl-tRNA synthetase. *Nat. Chem. Biol.* **13**, 1261-1266.
10. A. Crnković, O. Vargas-Rodriguez, A. Merkuryev, D. Söll (2018) Effects of heterologous tRNA modifications on the production of proteins containing noncanonical amino acids. *Bioengineering*, **5**, 11.
11. T. Mukai, A. Sevostyanova, T. Suzuki, X. Fu, D. Söll (2018) A facile method for producing selenocysteine-containing proteins. *Angew. Chem. Int. Ed. Engl.* **57**, 7215-7219.
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18. X. Fu, D. Söll, A. Sevostyanova (2018) Challenges of site-specific selenocysteine incorporation into proteins by Escherichia coli. *RNA Biol.* **15**, 461-470.
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Regulation of cell wall assembly: Myosin and exocyst involvement in cellulose synthase delivery to the plasma membrane

DOE-BES Award No. DE-FG02-04ER15526

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Overall research goals:

Cellulose is the most abundant biopolymer on the planet and is produced in the primary and secondary cell wall of terrestrial plants by a plasma membrane (PM)-localized, multimeric protein complex. The catalytic enzyme, or cellulose synthase, belongs to a multigene family known as CESA. It is generally accepted that the behavior and trajectories of cellulose synthase complexes are oriented by the position of cortical microtubules, but how exactly these complexes are delivered and recycled from the PM remains poorly understood. Limited evidence suggests that microtubules determine the site for delivery of new complexes; however, abolishing microtubules with the inhibitor oryzalin has absolutely no effect on rates of delivery. Thus, there is a pressing need to explore the contribution of another component of the cortical cytoskeleton, actin filaments and the associated motor protein myosin, to the delivery and dynamics of CESA at the PM.

In the current proposal, we will test the *central hypothesis* that myosin XI and the exocyst complex cooperate to tether CESA-containing secretory vesicles at the PM and facilitate vesicle fusion. The *specific aims* include:

- 1) testing whether actin filament organization and individual myosin XI isoforms are necessary for CESA vesicle tethering and fusion, using a powerful combination of genetics and quantitative live-cell imaging; and
- 2) determining whether myosin–exocyst interactions are necessary for vesicle tethering and fusion during the delivery of CESA complex to the PM.

In general, the Staiger laboratory aims to understand dynamic control of the cortical actin cytoskeleton and how it is used to deliver materials to and from the PM and cell wall. Although it is commonly accepted that actin filaments serve as tracks for exocytosis, there is little direct evidence that secreted cargo traffics along single filaments or bundles. Moreover, which dynamic properties of the cortical cytoskeleton regulate vesicle trafficking remain poorly understood. We recently demonstrated that class XI myosins from *Arabidopsis* participate in cellulose deposition during primary cell wall formation. Capitalizing on the identification of a new small molecule inhibitor, pentabromopseudilin or PBP, we implicated myosin in trafficking of CSCs and CESA dynamics. We will pursue further analysis of myosin activity and its role in cell wall assembly by combining high spatiotemporal live-cell imaging with molecular genetic analysis of class XI myosins.

Significant achievements (2019-2020):

- Through chemical and genetic inhibition of plant myosin XI, demonstrated a role for this molecular motor in cellulose deposition via the delivery of cellulose synthase complex (CESA) to the plasma membrane.

- Demonstrated a role for myosins XI in tethering and/or fusion of CESA-containing vesicles at the plasma membrane, for the first time implicating actomyosin in the late stages of secretory vesicle trafficking in plants.
- Identified Myosin XI isoform as the major isoform involved in CSC vesicle trafficking.
- Provided *in vitro* and *in vivo* evidence for myosin XI–exocyst complex interactions, specifically between the globular tail domain (GTD) of myosin XI and the SEC5b subunit of exocyst.
- Using chemical and genetic inhibition, demonstrated the dependence of exocyst dynamics and lifetime on myosin activity.
- Demonstrated that myosin XI and exocyst subunits transiently colocalize with CESA at the PM during vesicle tethering and that myosin activity is necessary to stabilize exocyst complex at tethering sites.
- Reported that the plant phytohormone auxin signals to actin cytoskeleton remodeling in root epidermal cells via the auxin transporter AUX1.
- In collaboration with Chunhua Zhang (Purdue) identified a new chemical inhibitor of cellulose synthase, endosidin 20 or ES20, that inhibits CESA catalytic activity and perturbs vesicle trafficking.

Science priorities for 2020-2021:

- Complete studies of exocyst–myosin interactions and publish paper.
- Investigate the role of cortical actin in secretory vesicle tethering by chemical and genetic inhibition of the ARP2/3 complex.

My major scientific area(s) of expertise is/are: quantitative cell biology, imaging, cytoskeleton.

To take my project to the next level, my ideal collaborator would have expertise in: chemical genetics

Publications supported by this project (2019-2020):

Zhang^P, W., C. Cai, and C.J. Staiger. 2019. Myosins XI are involved in exocytosis of cellulose synthase complexes. *Plant Physiology* 179: 1537-1555 doi:10.1104/pp.19.00018

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Huang, L., X. Li, W. Zhang^P, N. Ung, N. Liu, X. Yin, Y. Li, R.E. McEwan, B. Dilkes, M. Dai, G.R. Hicks, N.V. Raikhel, C.J. Staiger, and C. Zhang. 2020. Endosidin20 targets the cellulose synthase catalytic domain to inhibit cellulose biosynthesis. *Plant Cell* 32: 2141-2157 doi.org/10.1105/tpc.20.00202

Zhang, W., L. Huang, C. Zhang, and C.J. Staiger. Arabidopsis myosin XI interacts with the exocyst complex to facilitate vesicle tethering during exocytosis. *BioRxiv* <https://doi.org/10.1101/2020.08.18.255984>

Molecular Mechanism of Energy Transduction By Plant Membrane Proteins

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Overall research goals:

The plasma membrane proton pump is comprised of a 100,000 Dalton protein in the group of energy transducing ATP fueled enzymes known as P-type ATPases. In plants and fungi, it is an abundant protein that creates the proton-motive force that powers the transport of practically all solutes into and out of the cells. In this manner, it performs the same function in plants and fungi that the sodium pump (Na^+ , K^+ -ATPase) performs in animal cells. In higher plants especially, this protein not only plays an essential role in mediating the influx and efflux of solutes but also, by regulating the pH of the cell wall, is hypothesized to play a fundamental role in regulating the rate of expansion of cells. Because they are sessile, this control of cell expansion critically controls the overall growth of the plant. For example, to evade pathogens or predators the plant shoot can only grow faster or slower or bend and all of this is controlled via pump mediated cell expansion. Because of this, this protein is at the nexus of diverse signaling pathways by which hormones and environmental effectors such as light and pathogens regulate plant growth. In rapidly dividing yeast and transport specialized cells like root hairs, guard cells and phloem companion cells, this enzyme is the single major ATP consumer.

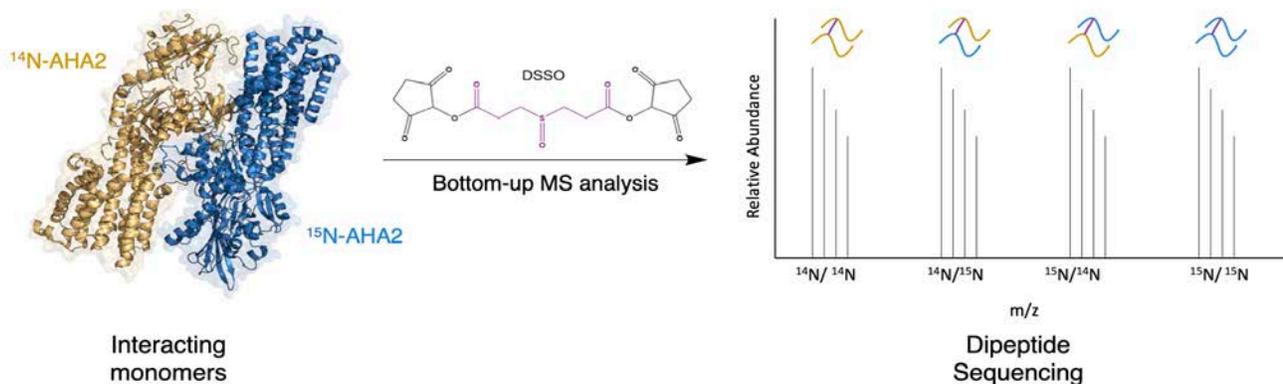
The plant and fungal proton pump creates the largest membrane potential observed in nature (exceeds 250 mV) because of its steep reversal potential of ca. minus 450 mV (inside negative). The protein is highly hydrophobic, containing ten transmembrane domains, some of which are involved in the process by which a proton is ejected out of the cell. Very little of the protein resides on the outside of the plasma membrane. Several large well studied soluble domains on the inside surface of the plasma membrane are involved in the hydrolysis of ATP. Importantly, the mechanism by which these two processes are coupled, i.e., ATP hydrolysis and proton efflux, is unknown. A crystal structure for the protein lacking the 100 amino acid water soluble regulatory C-terminal domain has been obtained, and provides important information on specific amino acids involved in ATP hydrolysis and possibly, proton movement. However since the X ray structural data is incomplete and cryo-EM has not yet been successful, definitive data on how these two processes occur and especially, how they are coupled to perform energy transduction, is missing. The C-terminal domains is a major focus of this project since it appears to be disordered in vitro, and its phosphorylation and de-phosphorylation at several serine and threonine residues in vivo are key aspects of growth regulating signaling pathways both in yeast and higher plants.

There are two main goals of this project. The first is to decipher the structure and function of the regulatory C-terminal 100 amino acids and in particular, how it interacts with the rest of the protein. The second is to understand how ATP hydrolysis is coupled to proton efflux. These goals are pursued mainly using mass spectrometric based technologies such as crosslinking, covalent labeling (e.g., hydroxyl radical footprinting) and deuterium-hydrogen exchange, as well as site directed mutagenesis via plant enzyme heterologously expressed and purified from yeast.

Significant achievements (2018-2021):

Our major achievement recently was the discovery that the protein is being regulated, at least in part, by the water soluble C-terminal 100 amino acids via an inter-molecular, rather than intra-molecular, mechanism. In other words, contrary to prior expectations, we have found that the C-terminus of one pump polypeptide is interacting with N-terminal residues in a second identical polypeptide, in a head-to-tail formation of dimers and higher order polymers. This discovery was made

possible by utilizing heavy isotope (^{15}N) labeling of one cell, and mixing the detergent solubilized protein from that cell, with identical protein derived from a second cell produced with only the ambient, light isotope (^{14}N) lacking the extra neutron. We observed that over a few hours, the pump exchanged polypeptides and with crosslinking reagents, we were able to identify specific amino acids



present at the peptide surfaces of the two polypeptides. We observed both dimers and trimers and from this, we created a 'head-to-tail' model for how the proteins were positioned, with potentially important ramifications for how the C-terminal domain may be regulating ATP hydrolysis and/or proton efflux (see Figure below).

Science priorities for the next year (2020-2021):

- Determining how the precise sites of interaction of the two monomers present in a homodimer or homotrimer, are altered during the various catalytic states of the enzyme.
- Determining whether the C terminal domain regulates the coupling stoichiometry (i.e., number of protons ejected per ATP hydrolyzed) via covalent trapping and quantification of the phosphorylated aspartyl residue in mutant enzymes with altered proton efflux capabilities.
- It has been suggested that lipids as well as water soluble domains in the N-terminal cytoplasmic surface of the protein are involved in regulating the enzyme and we will be initiating experiments to evaluate their potential roles.

My major scientific areas of expertise are: mass spectrometry, protein chemistry, genomic technology development.

To take my project to the next level, my ideal collaborator would have expertise in: cryo-EM and/or X Ray crystallography based structural analysis of hydrophobic transmembrane proteins.

Publications funded by DOE:

1. Nguyen TT, Blackburn MR, Sussman MR. Intermolecular and intramolecular interactions of the *Arabidopsis* plasma membrane proton pump revealed using a mass spectrometry cleavable cross-linker. *Biochemistry*. 2020 Jun 23;59(24):2210-2225.
2. Phillips GN Jr., Sussman MR. Plant hydraulics and agricultural genomics. *Science*. 2019 Oct 25;366(6464):416-417.
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Intracellular Lipid Transfer in the Biosynthesis of Photosynthetic Membrane Lipids and Storage Triacylglycerol

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Overall research goals: Our long-term research goal is to understand the molecular mechanism underlying intracellular lipid transfer essential for photosynthetic membrane biogenesis and to dissect the factors and principles that regulate lipid metabolism and fatty acid flux between membrane lipids and storage triacylglycerol. The specific objectives are to: (1) understand whether and how starch and lipid metabolic pathways interact in plants; 2) characterize Arabidopsis mutants defective in lipid trafficking between the endoplasmic reticulum and the plastid and functionally analyze the encoded proteins; (3) understand the factors that regulate the flux of fatty acids between membrane lipids and storage triacylglycerols. The results from this study will provide new mechanistic insights into lipid metabolism, homeostasis and trafficking in plants and create the knowledge base to enhance our ability to manipulate the processes that regulate photosynthetic membrane biogenesis, carbon partitioning between lipids and carbohydrates and lipid storage in plants.

Significant achievements (2018-2020):

We carried out a detailed biochemical, genetic and cell biological analysis of the role of autophagy, a major metabolic pathway whereby cellular materials are delivered to the vacuole for degradation and recycling, in plant lipid metabolism. Taking advantage of Arabidopsis mutants defective in lipid trafficking, we demonstrate that autophagy contributes to triacylglycerol synthesis and lipid droplet accumulation under normal growth conditions, but to the degradation of lipid droplets under carbon starvation. We show that lipophagy, the autophagy-mediated degradation of lipid droplets, is induced under starvation. Unlike lipid droplets, chloroplasts are degraded by processes largely independent of autophagy under carbon starvation conditions. We additionally show that lipophagy occurs in a process morphologically resembling microlipophagy and requires the core components of the macroautophagic machinery.

Additional work focused on analyzing the interplay between starch and lipid metabolic pathways in plant growth, development and biomass production. Our results demonstrate that lipids can partially compensate for the lack of function of transient starch during normal growth and development in Arabidopsis. Disruption of starch synthesis resulted in a significant increase in fatty acid synthesis via posttranslational regulation of the plastidic acetyl-coenzyme A carboxylase and a concurrent increase in the synthesis and turnover of membrane lipids and triacylglycerol. We also found that the combined disruption of starch synthesis and fatty acid turnover resulted in increased accumulation of membrane lipids, triacylglycerol, and soluble sugars and altered fatty acid flux between the two lipid biosynthetic pathways compartmentalized in either the chloroplast or the endoplasmic reticulum. Collectively, our findings provide insight into the role of fatty acid β -oxidation and the regulatory network controlling fatty acid synthesis, and reveal the mechanistic basis by which starch and lipid metabolic pathways interact and undergo cross talk to modulate carbon allocation, energy homeostasis and plant growth.

Science priorities for the next year (2020-2021):

- Characterization of the *rlt3* (*reduced lipid transport3*) lipid trafficking mutant isolated from a forward genetic screen. The *rlt3* displays an increase in the rate of fatty acid synthesis, changes in membrane lipid fatty acid composition indicative of a decrease in lipid transport from the endoplasmic reticulum to the plastid. Detailed genetic and biochemical experiments will be performed to identify the gene, characterize the mutant and establish the function of the protein.
- Analysis of *trigalactosyldiacylglycerol1* (*tgdl1*) lipid trafficking mutants with respect to the role of lipid metabolism and trafficking in thylakoid membrane remodeling during light acclimation. Our recent results indicate that *tgdl1* mutants are sensitive to high light stress. Further genetic, biochemical and cell biological studies will be carried out to examine how defects in lipid transfer between the endoplasmic reticulum and the plastid impacts plant adaptation to changes in light conditions.
- Analysis of the cross talk between sterol and glycerolipid metabolism. We recently found that disruption of sterol synthesis in *tgdl1* and other genetic backgrounds caused drastic decreases in triacylglycerol and lipid droplet accumulation in leaves. Since both sterol and fatty acid synthesis shares the same precursor acetyl-CoA, additional experiments will be performed to test the effect of sterol deficiency on fatty acid synthesis and membrane lipid metabolism in Arabidopsis. Sterols are key regulators of physical properties of biological membranes and recent studies have suggested a critical role of the bilayer lipid composition and surface tension in lipid droplet formation. Therefore, further experiments will be carried out to determine whether and how sterol deficiency affects lipid droplet biogenesis.

My primary expertise is in: molecular genetics, cell biology and biochemistry of plant lipids

To take my project to the next level, my ideal collaborator would have expertise in: cryo-electron microscopy and membrane protein crystallography

Publications supported by this project 2016-2018:

1. J. Fan, L. Yu and C. Xu, "Dual role for autophagy in lipid metabolism in Arabidopsis." *Plant Cell* 31: 1598-1613 (2019).
2. J. Fan, Z Chao, L. Yu, P. Li, J. Shanklin and C. Xu, "Diversion of carbon flux from sugars to lipids improves the growth of an Arabidopsis starchless mutants." *Plants* 2019, 8, 229.
3. L. Yu , J. Fan, C. Xu, "Peroxisomal fatty acid β -oxidation negatively impacts plant survival under salt stress." *Plant Signal Behav.* 14:1561121 (2019).
4. L. Yu , J. Fan, C. Xu, "Starch deficiency enhances lipid synthesis and turnover in leaves." *Plant Physiol.* 178: 118-129 (2018).
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Elucidating the Biochemical Mechanisms Controlling Secondary Wall Biosynthesis in Plants

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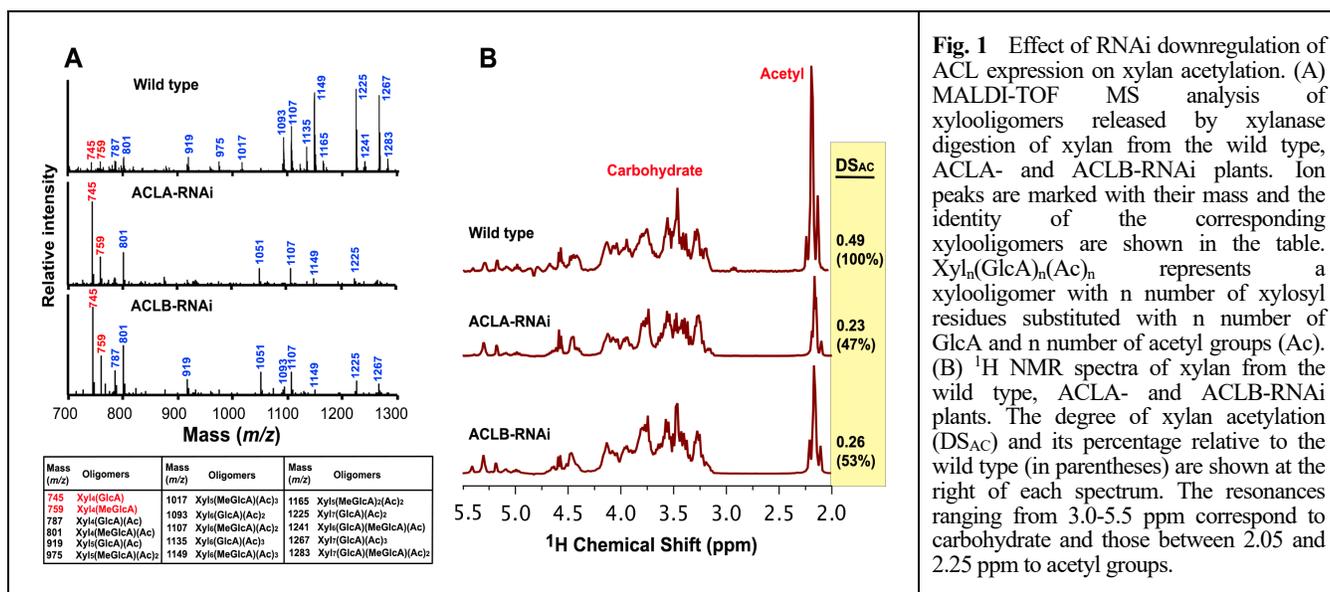
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Overall research goals:

The major goal of this DOE-funded project is to carry out biochemical characterization of enzymes involved in the biosynthesis of xylan, the second most abundant polysaccharide in secondary cell walls. Secondary walls in the form of wood and fibers are the most abundant stored energy in plant biomass. Understanding how secondary walls are synthesized will provide fundamental insight into how plants convert the fixed carbon through photosynthesis into a long-term stored energy. Xylan is composed of a linear backbone of β -1,4-linked xylosyl (Xyl) residues substituted with various side chains and often acetylated at *O*-2 or *O*-3. In grass xylans, the side chains include 2-*O*- and/or 3-*O*-linked arabinofuranose (Ara), 3-*O*-linked Ara substituted at *O*-2 with another Ara [Ara-(1->2)-Ara] or Xyl [Xyl-(1->2)-Ara], and 2-*O*-linked glucuronic acid (GlcA)/4-*O*-methylglucuronic acid (MeGlcA) residues. The biochemical mechanisms controlling the substitution of xylan with these various side chains and acetyl groups remain to be investigated. The specific aims of this proposed research are to carry out a comprehensive biochemical characterization of glycosyltransferases catalyzing the substitutions of xylan and acetyltransferases responsible for wall polymer acetylation, the results of which will shed light on the biochemical mechanisms controlling secondary wall biosynthesis.

Significant achievements (2019-2022):

We have investigated roles of ATP-citrate lyase (ACL) that generates cytosolic acetyl-CoA in cell wall polysaccharide acetylation and effects of simultaneous mutations of four Reduced Wall Acetylation (RWA) genes on acetyl-CoA transport into the Golgi in Arabidopsis. We analyzed the expression of genes involved in the generation of acetyl-CoA in different subcellular compartments and found that the expression of several ACL genes responsible for cytosolic acetyl-CoA synthesis was elevated in interfascicular fiber cells and induced by secondary wall-associated transcriptional activators. We demonstrated that simultaneous downregulation of the expression of ACL genes resulted in a substantial decrease in the degree of xylan acetylation and a severe alteration in secondary wall structure in xylem vessels. In addition, we revealed that the degree of acetylation of other cell wall polysaccharides, including glucomannan, xyloglucan and pectin, was also reduced. Moreover, we found that Golgi-enriched membrane vesicles isolated from the *rwa1/2/3/4* quadruple mutant exhibited a drastic reduction in acetyl-CoA transport activity compared with the wild type. These findings indicate that cytosolic acetyl-CoA generated by ACL is essential for cell wall polysaccharide acetylation and RWAs are required for its transport from the cytosol into the Golgi, which provides the first line of evidence regarding the source of acetyl donors for cell wall polysaccharide acetylation.



We have found that cell walls of moss and *Selaginella* contain acetylated glucomannans and that recombinant proteins of a group of DUF231 members from moss, *Selaginella*, pine, spruce, poplar and rice possess *O*-acetyltransferase activities catalyzing 2-*O*- and 3-*O*-acetylation of mannan. Our findings suggest that the recruitment of DUF231 members as mannan *O*-acetyltransferases occurred as early as in bryophytes and their biochemical functions are conserved throughout different taxa of land plants, which enriches our understanding of evolutionary origins of acetyltransferases involved in mannan acetylation.

We have demonstrated that recombinant proteins of a group of DUF231 members from rice and tomato are xyloglucan backbone 6-*O*-acetyltransferases (XyBATs). We further showed that XyBAT-acetylated celohexaose oligomers could be readily xylosylated by AtXXT1 (Arabidopsis xyloglucan xylosyltransferase1) to generate acetylated, xylosylated cello-oligomers. Heterologous expression of a rice XyBAT in Arabidopsis led to a severe reduction in cell expansion and plant growth and a drastic alteration in xyloglucan xylosylation pattern with the formation of acetylated XXGG-type units, including XGG, XGGG, XXGG, XXGGG, XXGGG (G denotes acetylated Glc). Our findings provide new insights into the biochemical mechanism underlying xyloglucan backbone acetylation and indicate the importance of maintaining the regular xyloglucan xylosylation pattern in cell wall function.

Science objectives for 2020-2021:

The main objective for the following year will be to investigate the evolutionary origins of xylan acetyltransferases and GT61 genes for their roles in secondary wall biosynthesis. We have identified 17 and 16 DUF231 encoded proteins from moss and *Selaginella* and will examine if they possess xylan acetyltransferase activities. We have found several novel GT61 genes that are expressed during secondary wall biosynthesis and we propose that they are involved in xylan biosynthesis. We will employ both gain-of-function and biochemical analyses to dissect their roles in xylan biosynthesis. The study of xylan biosynthesis will provide knowledge foundation for genetic modification of biomass with altered wall composition.

My major scientific area(s) of expertise is/are: Biochemistry and molecular genetics

To take my project to the next level, my ideal collaborator would have expertise in: protein and carbohydrate structures; carbohydrate analysis expertise from Dr. P. Azadi and cell wall biophysics expertise from Dr. D. Cosgrove

Publications supported by this project [2018-2020]:

1. Zhong, R., Cui, D., Phillips, D.R., Richardson, E.A. and Ye, Z.-H. (2020) A group of *O*-acetyltransferases catalyze xyloglucan backbone acetylation and can alter xyloglucan xylosylation pattern and plant growth when expressed in Arabidopsis. *Plant Cell Physiol.* 61, 1064-1079.
2. Zhong, R., Cui, D., Richardson, E.A., Phillips, D.R., Azadi, P., Lu, G. and Ye, Z.-H. (2020) Cytosolic Acetyl-CoA generated by ATP-citrate lyase is essential for acetylation of cell wall polysaccharides. *Plant Cell Physiol.* 61, 64-75.
3. Zhong, R. and Ye, Z.-H. (2020) XND1 regulates secondary wall deposition in xylem vessels through inhibition of VND functions. *Plant Cell Physiol.* (accepted for publication)
4. Zhong, R., Cui, D. and Ye, Z.-H. (2019) Evolutionary origin of *O*-acetyltransferases responsible for glucomannan acetylation in land plants. *New Phytol.* 224, 466-479.
5. Zhong, R. and Ye, Z.-H. (2019). Tansley review: Secondary cell wall biosynthesis. *New Phytol.* 221, 1703-1723.
6. Zhong, R., Cui, D. and Ye, Z.-H. (2018) Members of the DUF231 family are *O*-acetyltransferases catalyzing 2-*O*- and 3-*O*-acetylation of mannan. *Plant Cell Physiol.* 59, 2339-2349.
7. Zhong, R., Cui, D. and Ye, Z.-H. (2018) Xyloglucan *O*-acetyltransferases from *Arabidopsis thaliana* and *Populus trichocarpa* catalyze acetylation of fucosylated galactose residues on xyloglucan side chains. *Planta* 248, 1159-1171.
8. Zhong, R., Cui, D., Dasher R.L. and Ye, Z.-H. (2018) Biochemical characterization of rice xylan *O*-acetyltransferases. *Planta* 247, 1489-1498.
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10. Zhong, R., Cui, D., Phillips, D.R. and Ye, Z.-H. (2018). A novel rice xylosyltransferase catalyzes the addition of 2-*O*-xylosyl side chains onto the xylan backbone. *Plant Cell Physiol.* 59, 554-565.

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