2018 Physical Biosciences Research Meeting

Program and Abstracts

Marriott Washingtonian Hotel
Gaithersburg, MD
October 28-31, 2018

Chemical Sciences, Geosciences, and Biosciences Division
Office of Basic Energy Sciences
Office of Science
U.S. Department of Energy
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FOREWORD...and Forward too!

This volume provides a record of the 6th biennial meeting of investigators funded by the “core” DOE-BES Physical Biosciences program, along with scientists in programmatically-relevant areas that are supported by the Office of Basic Energy Sciences (BES) through the Energy Frontier Research Center (EFRC) program. Physical Biosciences and Photosynthetic Systems are the two complimentary programs within DOE-BES that fund basic research in energy-relevant biological sciences. These two programs, along with Solar Photochemistry, comprise the Photochemistry and Biochemistry Team within the Chemical Sciences, Geosciences and Biosciences Division (CSGB) of DOE-BES.

We think we have a great meeting planned for you! As always, we'll start off with BES, Divisional and program updates from Dr. McLean (aka Gail) and me. Then it's on to our Keynote Speaker, Dr. Huilin Li from the Van Andel Research Institute, who's going to bring us all up to date on state-of-the-art cryo-EM techniques and how they can be applied to decipher some of the toughest structures Mother Nature throws at us. (Psst: He's also an excellent collaborative partner as you'll see!). We'll follow that up with a special session featuring talks by our Physical Biosciences Early Career Awardees, who are already making their mark on science! Finally, our last talk on Monday before dinner will be a special 60 minute "Perspective Talk" on the plasticity of the genetic code from Dieter Soll at Yale University. Dieter has been there from the very beginning - he postdoc'ed with H.G. Khorana - and what a perspective he offers! After dinner, it's on to the first of our two Poster Sessions - where you'll meet some of the nicest, friendliest, smartest, and most collaborative scientists anywhere!

Tuesday morning will kick off with another special 60 minute talk on bacterial microcompartments (BMCs) by Dr. Cheryl Kerfeld from the DOE-MSU-Plant Research Lab (PRL). Dr. Kerfeld will tell us how BMCs are constructed from "Lego-like" protein building blocks, and how they provide a unique capability to explore "chemistry in confined spaces". There are lots of opportunities here for both basic and applied research that are limited only by your imagination! Our last talk on Tuesday afternoon is another special 60 minute presentation titled, "How to Effectively Communicate Science – And Why It Matters". I'm delighted that Igor Houwat, the Communications Coordinator at the PRL, and Kate Bannan, who manages strategic communications for the DOE Office of Science, have agreed to give this outstanding presentation. Doing great science is the foundation for success in our world, but effectively communicating it is important as well! Question to ponder: If you publish a truly seminal paper but nobody reads and communicates it, did it really happen? As in meetings past, you can consider this our "Tools Talk". Then it's our dinner-on-your-own night, where we hope you're off somewhere exploring new ideas with new friends - and potentially new collaborators - to help take your work to the next level. Come back to the Marriott for Poster Session II though, as you won't want to miss a moment! Great science around every corner!

Of course, every day of the meeting will consist of a series of outstanding 30 minute talks from our supported PIs organized into programmatically-relevant sessions. Finally, after lunch Wednesday, we provide an open forum to provide feedback to us, ask questions, etc.

In closing this section, I want to express my appreciation to Diane Marceau at DOE-BES - now retired - for years of invaluable help in planning this meeting. Like Dr. Spock, she's been known to beam down into PI meetings so keep your eyes open! As you all know, Connie Lansdon from ORISE provides valuable assistance to all of us - especially me! Finally, can I just say I have some of the best colleagues in the world? Steve Herbert, Chris Fecko and Gail McLean for starters, but then I'd be remiss if I didn't also state that working with ALL of my BES colleagues - combined with YOU and your science - has made this job an amazing adventure in moving the frontiers of science forward...er, I mean FORWARD!

Robert J. Stack, Program Manager, Physical Biosciences, DOE-BES
Agenda
AGENDA
6th Biennial Physical Biosciences Research Meeting
Marriott Washingtonian, Gaithersburg, MD
October 28-31, 2018

Sunday, October 28, 2018

3:00 – ????  Check-in at Marriott Washingtonian, Gaithersburg, MD
3:00 – 6:00  Sunday Registration (Connie Lansdon)
6:00 – 9:00  Dinner On Your Own in Washingtonian Center, Gaithersburg, MD
8:00 – 10:00 No-Host Reception at the Marriott Washingtonian Lounge (Optional)

Monday, October 29, 2018

7:00 – 8:00 a.m. Breakfast and Ongoing Registration

Session I: Welcome, DOE News and Program Notes, and Keynote Lecture
8:00 – 8:15  Welcome, DOE-Basic Energy Sciences (BES) Updates
            Gail McLean, Team Lead, Photochemistry and Biochemistry, DOE-BES
8:15 – 9:00  Welcome, Physical Biosciences Program Notes and Updates
            Robert Stack, Program Manager, Physical Biosciences, DOE-BES
9:00 – 10:00 Invited Keynote Lecture:
            Cryo-EM reveals the evolutionary path of modern-day respiratory complexes in a heat-loving anaerobic microorganism
            Huilin Li, Van Andel Research Institute, Grand Rapids, MI
10:00 – 10:30 Break

10:30 – 11:00 Bringing Inorganic Carbon to Life: Developing Model Metalloenzymes for C1 Conversion Reactions
            Hannah Shafaat, The Ohio State University
11:00 – 11:30 Mechanistic Studies of a Primitive Homolog of Nitrogenase Involved in Coenzyme F430 Biosynthesis
            Steven Mansoorabadi, Auburn University
11:30 – 12:00 Elucidating Biological Energy Transduction from Ammonia
            Kyle Lancaster, Cornell University
12:00 – 12:30 Mechanisms of self-assembly and catalysis in protein microcompartments
            David Savage, University of California, Berkeley
12:30 – 1:30 Working Lunch
1:30 – 3:15 Free/Discussion Time (Put up all posters)
3:15 – 3:45 Coffee Break
Session III: Current Topics in Plant Cell Wall Biochemistry
3:45 – 4:30  Cell-type Specific Pectins in Plant Cell Walls: Structure, Interaction and Function
Malcolm O’Neill, Univ. of Georgia/Complex Carbohydrate Research Ctr.
Michael Crowley, National Renewable Energy Laboratory

4:30 – 5:00  Elucidating the Biochemical Mechanisms Controlling Secondary Wall Biosynthesis in Plants
Zheng-Hua Ye, University of Georgia

5:00 – 5:30  Molecular Mechanisms of Plant Cell Wall Loosening: Expansin Action
Daniel Cosgrove, Pennsylvania State University

5:30 – 6:30  A Perspective: Revealing the many faces of the genetic code
Dieter Soll, Yale University

6:30 – 7:30  Working Dinner at Marriott Washingtonian

Poster Session I
8:00 – 10:00  Poster Session, Odd Numbered Posters (No-Host)

Tuesday, October 30, 2018

Session IV: Spotlight on Carbon Dioxide Uptake
8:00 – 9:00  Natural and Engineered Organelles for the Study of Chemistry in Confinement: Carboxysomes and other Bacterial Microcompartments
Cheryl Kerfeld, DOE-MSU Plant Research Lab & Lawrence Berkeley National Lab

9:00 – 9:30  Mechanistic studies of the FdsABG formate dehydrogenase from Cupriavidus necator
Russ Hille, University of California, Riverside

9:30 – 10:00  2-Ketopropyl coenzyme M oxidoreductase / carboxylase: A unique carboxylating disulfide oxidoreductase
John Peters, Washington State University

10:00 – 10:30  Redox-regulation of electron flow in an anaerobe
Katherine Fixen, University of Minnesota
Caroline Harwood, University of Washington

10:30 – 11:00  Break

Session V: Spotlight on Hydrogenases and Methane Oxidation
11:00 – 11:30  Role of HydF in Hydrogenase Maturation
Joan Broderick, Montana State University

11:30 – 12:00  Mechanism of Proton-Coupled Electron-Transfer by [FeFe]-hydrogenase in Coupling to Dynamic Photosynthetic Energy Transduction
David Mulder, National Renewable Energy Laboratory

12:00 – 12:30  Missing links in biological methane and ammonia oxidation
Amy Rosenzweig, Northwestern University
12:30 – 1:30 Working Lunch
1:30 – 3:30 Free/Discussion Time
3:30 – 4:00 Coffee Break

Session VII: Challenging Paradigms and Pushing Fields Forward

4:00 – 4:30 Nitrogenase Reduction of N2 and CO2
   Lance Seefeldt, Utah State University
   Dennis Dean, Virginia Tech
4:30 – 5:00 Uncovering Novel Energy Paradigms in Sulfate-Reducing Bacteria and Methanogenic Archaea
   C.S. Raman, University of Maryland, Baltimore
5:00 – 5:30 Electron Transport in Methanogenesis and Reverse Methanogenesis by Methanosarcina acetivorans
   J. Gregory Ferry, Pennsylvania State University
5:30 – 6:00 How to Effectively Communicate Science – And Why It Matters
   Kate Bannan, DOE Office of Science Communications
   Igor Houwat, DOE-MSU Plant Research Lab

6:30 – 8:00 Dinner on Your Own

Poster Session II
8:00 – 10:00 Poster Session, Even Numbered Posters (No-Host)

Wednesday, October 31, 2018

7:15 – 8:00 a.m. Breakfast

Session VIII: Electron Flow at Multiple Spatial and Temporal Scales

8:00 – 8:30 Extracellular Charge Transport in Microbial Redox Chains: Linking the Living and Non-Living World
   Mohammed El-Naggar, University of Southern California
8:30 – 9:00 Flavin-Based Electron Bifurcation
   Cara E. Lubner, National Renewable Energy Laboratory
9:00 – 9:30 Regulated reductive flow through archaeal respiratory and energy production systems
   Thomas Santangelo, Colorado State University
9:30 – 10:00 Understanding redox proportioning through ferredoxins, low potential iron-sulfur proteins acting as electrical hubs to control metabolism
   George Bennett, Rice University

10:00 – 10:30 Break
**Session IX:**  *Current Topics in Plant Lipid Biochemistry. But first...*

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<td><strong>Julie Maupin-Furlow,</strong> University of Florida</td>
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<td>11:00 – 11:30</td>
<td>Elucidating the Cellular Machinery for Lipid Storage in Plants</td>
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<td><strong>Kent Chapman,</strong> University of North Texas</td>
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<td>11:30 – 12:00</td>
<td>Modification of Plant Lipids</td>
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<td><strong>John Shanklin,</strong> Brookhaven National Laboratory</td>
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<td>12:00 – 12:30</td>
<td>Intracellular Lipid Transfer in the Biosynthesis of Photosynthetic Membrane Lipids and Storage Triacylglycerol</td>
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<td><strong>Changcheng Xu,</strong> Brookhaven National Laboratory</td>
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<td>12:30 – 1:30</td>
<td>Working Lunch <em>(Reminder: Make sure all posters are down)</em></td>
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Session I
Cryo-EM reveals the evolutionary path of modern-day respiratory complexes

in a heat-loving anaerobic microorganism

Huilin Li

Structural Biology Program, Van Andel Research Institute, Grand Rapids, MI 49503
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With the recent advent of direct electron detector, cryo-EM has rapidly ascended to become the primary tool for atomic resolution structural analysis of protein complexes, particularly the membrane enzyme complexes that are recalcitrant to the crystallographic approach. In my presentation, I will first review the history, concept, and capability of cryo-EM, and then focus on an ongoing collaborative research with Dr. Michael Adams on two membrane complexes isolated from the heat-loving anaerobic *Pyrococcus furiosus*: the 14-subunit membrane-bound hydrogenase MBH and the 13-subunit membrane-bound sulfane reductase MBS.

The modern-day respiratory complex I resides in mitochondria and is the first enzyme in the pathway that converts metabolic energy into chemical energy in the form of a proton motive force by transferring electrons from cellular electron carriers to molecular oxygen. Complex I shares ancestral roots with the electrogenic H+/Na+ antiporter called Mrp (multiple resistance and pH-related antiporter), group 4 membrane bound [NiFe] hydrogenases (MBH) that couples electron transfer to H2 production and Na+ gradient generation, and the membrane-bound sulfane reductase (MBS) that couples the reduction of polysulfides derived from elemental sulfur to Na+ gradient generation. The established Na+ gradient eventually drives ATP synthesis in anaerobic microorganisms such as *P. furiosus*. Our cryo-EM structural analyses of MBH, MBS, and our homology-based structural modeling of Mrp, has put in perspective the evolutionary relationship of these complexes.

This research is a collaboration between the Li lab at Van Andel Research Institute and the Michael Adams lab at University of Georgia, Athens. Hongjun Yu in the Li Lab and Chang-Hao Wu, Gerrit Schut, and Dominik Haja in the Adams lab contributed to the research.

Li is supported by Van Andel Research Institute and National Institutes of Health. Adams is supported by a grant from the Division of Chemical Sciences, Geosciences and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy (DOE; DE-FG05-95ER20175).
Session II
Bringing Inorganic Carbon to Life:
Developing Model Metalloenzymes for C\textsubscript{1} Conversion Reactions

Hannah Shafaat, Principal Investigator
100 W. 18\textsuperscript{th} Ave., The Ohio State University, Columbus, OH 43210
Email: shafaat.1@osu.edu; Website: go.osu.edu/shafaatlab

Overall research goals:
The global carbon cycle is carefully balanced through the use of specialized enzymes in plants, algae, bacteria, and archaea. A primordial metabolic pathway for the conversion of inorganic carbon into cellular biomass uses a large, nickel-containing enzyme called carbon monoxide dehydrogenase (CODH)/acetyl coenzyme A synthase (ACS). This system fixes carbon dioxide (CO\textsubscript{2}) into carbon monoxide (CO) at the CODH site. The carbon monoxide is then used to generate acetyl coenzyme A, a biological building block, through a key carbon-carbon bond forming step at the nickel site in ACS. Despite the significance of these processes in the context of primordial life and energy conversion, the fundamental chemistry underlying these transformations has remained elusive, in part due to the complexity of the natural enzyme.

To better understand the principles governing these biological one-carbon (C\textsubscript{1}) activation reactions, the overall goals of this project are to develop functional models of CODH and ACS based on modifications to a small metalloprotein scaffold. Reactivity towards typical CODH and ACS substrates is probed using stopped-flow mixing, transient absorption and emission, and rapid-freeze-quench experiments. Comprehensive characterization of these systems using advanced spectroscopic and biophysical techniques is used to reveal key elements responsible for conferring high levels of activity to the model systems, with implications for gaining insight into the mechanisms of the natural enzymes. Moreover, the principles learned from this research can be used to guide design of robust catalysts for efficient conversion of CO\textsubscript{2} and CO into liquid fuels.

Significant achievements (2017-2018):
- We have developed semisynthetic enzyme systems based on azurin that are completely selective for CO\textsubscript{2} fixation to CO over H\textsuperscript{+} reduction to H\textsubscript{2}. This selectivity was achieved by constraining the secondary coordination sphere around the catalytic center and installing a direct intramolecular electron transfer pathway from a light-harvesting chromophore to the active site (Figure 1).
- We have used spectroelectrochemical techniques to identify that the catalytic mechanisms for CO\textsubscript{2} and H\textsuperscript{+} reduction by [Ni(cyclam)]\textsuperscript{2+} fundamentally differ, with a Ni(III) state isolated during H\textsubscript{2} evolution. This requirement may underpin the complete selectivity observed in naturally occurring CO\textsubscript{2} reducing enzymes.
- We have developed a mutant of nickel-substituted azurin (M121A NiAz) capable of accessing a Ni\textsuperscript{I} state and binding CO. Both the Ni\textsuperscript{I}Az and Ni\textsuperscript{I}-CO Az species exhibit similar electronic structure properties and binding affinities as the photolyzed A\textsubscript{red}\textsuperscript{*} and CO-bound.

![Figure 1. Cartoon structure of RuAz-[1] showing attachment sites for S78C RuAz-[1] and schematic for photoinduced CO\textsubscript{2} reduction.](image)

![Figure 2. Characterization of CO binding (\textsuperscript{12}CO in blue, \textsuperscript{13}CO in red) to M121A Ni\textsuperscript{I}Az by (A) FT-IR, (B) EPR, and (C) optical spectroscopy. (Inset, B) Zoom-in of high-field turning point clearly indicates broadening observed in the case of \textsuperscript{13}CO. (Inset, C) CO titration and binding affinity determination.](image)
states of native ACS (Figure 2).

- We have demonstrated that methyl binding to M121A NiIAz occurs via two-electron, nucleophilic attack analogous to the reactivity seen in native ACS. The resultant Ni$^{II}$-CH$_3$ species and the one-electron-reduced Ni$^{III}$-CH$_3$ species have been characterized spectroscopically using diverse techniques (Figure 3).

Science objectives for 2018-2019:

- We will determine the mechanism of light-driven CO$_2$ fixation by RuAz-[1] constructs using time-resolved, pump-probe spectroscopic techniques.

- We will develop and characterize the activity and stability of robust CO$_2$-reducing enzymes based on synthetic modifications to the nickel cyclam ligand to permit site-selective covalent attachment. These efforts are already underway in the group.

- We will install targeted secondary and tertiary sphere residues around the active site in the covalently-attached, semisynthetic CODH enzymes that can channel substrate, support transient interactions with the nickel center, and exclude solvent. Structural characterization by X-ray crystallography will facilitate second-generation protein engineering.

- We will develop reactivity of M121A NiAz towards binding both methyl and carbonyl groups as well as acyl transfer to varied nucleophiles in functional mimicry of ACS (Figure 4).

My scientific area(s) of expertise is/are: bioinorganic chemistry, nickel, protein engineering, EPR (including pulsed) spectroscopy, resonance Raman spectroscopy, electronic structure calculations.

To take my project to the next level, my ideal collaborator would have expertise in: synthetic chemistry; computational protein design software (e.g., Rosetta); paramagnetic NMR spectroscopy; X-ray techniques.

Publications supported by this project 2017-2018:


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

Steven O. Mansoorabadi, Principal Investigator
Auburn University, Department of Chemistry and Biochemistry
179 Chemistry Building, Auburn, AL 36849
Email: som@auburn.edu
Website: http://www.auburn.edu/cosam/faculty/chemistry/mansoorabadi/index.htm

Overall research goals:
This research project aims to investigate the mechanism of a primitive homolog of nitrogenase (CfbCD) that catalyzes the key step in the coenzyme F430 biosynthetic pathway. This unprecedented reaction, which converts Ni-sirohydrochlorin \( a,c \)-diamide to 15,17\(^3\)-seco-F430-17\(^3\)-acid, involves a 6-electron reduction of the isobacteriochlorin ring system, cyclization of the \( c \)-acetamide side chain to form a \( \gamma \)-lactam ring, and the formation of 7 stereocenters. Specific goals include: 1) identification of physiological electron donors to CfbCD and \textit{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 (2017-2018):
It was found that the coexpression of the coenzyme F430 biosynthesis (\textit{cfb}) genes with the \textit{sirAC} genes (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 \textit{Escherichia coli}. Sirohydrochlorin, Ni-sirohydrochlorin (the CfbA product), and Ni-sirohydrochlorin \( a,c \)-diamide (the CfbB product) could all be detected in \textit{E. coli} extracts, suggesting that CfbCD was not functioning \textit{in vivo}. CfbCD can be purified in an active form from \textit{E. coli} when it is coexpressed with the iron-sulfur cluster (\textit{isc}) biosynthetic gene cluster from \textit{Azotobacter vinelandii} and supplied with sodium dithionite as an artificial reductant \textit{in vitro}. Thus, it is likely that the \textit{cfbCD} genes must be coexpressed with the \textit{isc} operon and/or a suitable physiological reductant to be active \textit{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 15,17\(^3\)-seco-F430-17\(^3\)-acid formation \textit{in vitro}. Therefore, compatible vectors were constructed to coexpress the \textit{cfb} genes with \textit{sirAC}, spinach Fd-FNR, the \textit{isc} operon, and a nickel transporter (\textit{nixA}) from \textit{Helicobacter pylori}, which was shown to increase the \textit{in vivo} availability of Ni\(^{2+}\) for the CfbA reaction in \textit{E. coli}. 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. 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). A protocol was also developed to synthesize and purify large quantities of Ni-sirohydrochlorin \( a,c \)-diamide using an engineered strain of \textit{E. coli}. 
Science objectives for 2018-2019:

- *E. coli* cells harboring plasmids containing *sirAC, cfbABCDE, nixA*, the *isc* operon, and the genes for Fd-FNR will be tested for the heterologous production of coenzyme F430. Size-exclusion chromatographic analysis of the oligomerization state of the CfbCD complex as a function of reactant occupancy and oxidation state will be completed. EPR spectroscopy will be used to characterize the iron-sulfur cluster of CfbD and potential Ni-sirohydrochlorin *a,c*-diamide-derived radical intermediates. We will also continue our collaboration with the research groups of Dr. Cathy Drennan (MIT) and Dr. Sean Elliott (BU) on structural (X-ray crystallography and cryo-electron microscopy) and electrochemical (protein film voltammetry) studies of CfbCD, respectively.

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 (2017-2018):
Elucidating Biological Energy Transduction from Ammonia

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Overall research goals:
While most organisms metabolize carbon-based chemical fuel, a select few organisms evolved to derive sufficient biological energy from the six-electron aerobic oxidation of NH₃ to NO₂⁻. This process, referred to as nitrification, involves two remarkable steps. The first is the hydroxylation of NH₃ by the enzyme NH₃ monooxygenase (AMO) to form NH₂OH. AMO uses a Cu cofactor to activate the strong, 107 kcal/mol N–H bond of NH₃ using O₂ as the oxidant. The second step involves the oxidation of NH₂OH to NO₂⁻ by NH₂OH oxidoreductase (HAO). The oxidation of NH₂OH to NO₂⁻ by HAO is a four-electron process requiring proton management. HAO uses a heme cofactor, heme P460, which is unique in its ability to directly remove electrons from substrate bound to its Fe center.

Mastering the fundamental chemical principles underlying these reactions will fuel the development of novel catalysts for small molecule activation and selective, proton-coupled redox transformations. Moreover, mechanistic knowledge of AMO and HAO will spur the development of improved nitrification inhibitors that would alleviate economic and ecological burdens resulting from the nitrification of nitrogenous fertilizer. However, molecular level understanding of the mechanisms involved in nitrification has remained elusive. This is partly due to the difficulty of isolating sufficient quantities of AMO and HAO from nitrifying bacteria and archaea, which are slow-growing microbes that achieve low cell densities.

The goals of this project are to establish at a molecular level of detail the reaction mechanisms underlying the complete conversion of NH₃ to NO₂⁻ by AMO and HAO. This will be achieved using stopped-flow kinetics to follow the course of reactions and rapid freeze-quench methods to trap intermediates observed during via these experiments. Resting and intermediate species will be characterized using convention spectroscopies including UV/visible absorption and electron paramagnetic resonance spectroscopy as well as using cutting-edge, synchrotron-based high-resolution X-ray spectroscopies. Studies of AMO employ a recombinant Mycobacterium smegmatis expression system based on the pMycoFos shuttle fosmid. Studies of NH₂OH oxidation employ both Nitrosomonas europaea HAO as well as recombinantly expressed N. europaea cytochrome (cyt) P460.


- We showed that our M. smegmatis expression system produces active Nitrosopumilus maritimus AMO. This AMO can be modified using site directed mutagenesis, which we have used to pinpoint residues putatively involved in metal binding and/or O₂ activation. We have shown that O₂ activation is inhibited by AMO-specific inhibitors allylthiourea and acetylene. These expression yields are low, and protein identification by SDS-PAGE has been elusive. We are moving to an alternative expression host to correct these problems. (vide infra)
- We have established key details of the mechanism for oxidation of NH₂OH by N. europaea cyt P460. We discovered that, contrary to early literature reports, cyt P460 does not enzymatically produce NO₂⁻. We have characterized by UV/vis absorption and EPR the resting Fe^{III}–OH₂ form of cyt P460 as well as intermediates in the anaerobic oxidation of NH₂OH: Fe^{III}–NH₂OH, and {Fe–NO}^⁶. We showed that {Fe–NO}^⁶ itself reacts with NH₂OH in the rate-determining step to form N₂O. These results establish the first direct link between NH₃ oxidation and environmental N₂O release.
We have established that the heme P460 cofactor is a dibasic porphyrinoid in both HAO and cyt P460. This opens the possibility of computationally-guided mechanistic study to establish relationships between heme P460 electronic structure and reactivity.

We showed that the heme-Lys cross-link in cyt P460 is essential to preventing catalyst deactivation by NO. Numerous NO-producing organisms express cyt P460 isozymes, suggesting a role for these proteins in NO tolerance.

Using insights gleaned from the study of cyt P460, we have revisited the biochemistry of HAO and disproved decades of dogma claiming that HAO enzymatically produces NO$_2^-$. This sweeping revision of bacterial nitrification establishes nitric oxide (NO) as an obligate intermediate in bacterial nitrification. Moreover, it provides an intersection between numerous nitrogen cycle metabolisms known to comprise microbial consortia.

Science objectives for 2018–2019:

- We will carry out expression trials of a new archaeal AMO construct using MethyloBricks, a recombinant expression vector for use in *Methylobacter extorquens*. These organisms have been shown to produce a high surface area internal membrane structure, making them ideally suited to express elevated quantities of AMO.

- We identified a second-sphere glutamate in cyt P460 enzymes that is absolutely required for a redox-active Fe$^{III}$–NH$_2$OH unit. We have carried out crystallographic, spectroscopic, and kinetics studies of cyt P460 variants with modifications to this second-sphere residue to establish its precise role in proton-coupled NH$_2$OH oxidation.

- We will investigate further details concerning the role of the heme-Lys cross-link in cyt P460. We showed previously that this cross-link is required for NH$_2$OH-oxidation activity, although the exact role is uncertain. We are investigating the cross-link’s influence over Fe$^{III}$ reduction potential, {FeNO}$^6$ electrophilicity, and substrate-binding properties. These studies leverage spectroelectrochemistry, kinetics, $^{57}$Fe Mössbauer spectroscopy, resonance Raman spectroscopy, and chemical titrations.

- We remain puzzled by the fact that HAO rapidly loses NO upon forming an {FeNO}$^6$, while this intermediate is robust and persistent in cyt P460. We will carry out mutagenic studies to cyt P460 to determine whether the second coordination sphere can be used to promote rapid NO loss.

My 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: Microbial ecology, particularly the use of chemostats and large-scale (> 15 L fermenters) in cell culture.

Publications supported by this project 2015–2018:


Mechanisms of self-assembly and catalysis in protein microcompartments

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Overall research goals:
The compartmentalization of biochemical reactions is a fundamental principle employed by the cell to segregate and facilitate metabolism. Understanding and engineering compartmentalization has the potential to dramatically improve the rate, yield, and toxicity of biocatalysts metabolically engineered for energy capture and conversion. Despite obvious applications, however, the predictable engineering of compartmentalized biochemical reactions within the cell remains challenging. Many bacteria use protein microcompartments to specifically encapsulate and facilitate multi-step metabolic pathways. For example, the carboxysome is a ~300 MDa capsid-like structure that compartmentalizes carbonic anhydrase and the Calvin Cycle enzyme RuBisCO in order to optimize CO$_2$ fixation. These modular, protein-based complexes could provide a unique scaffold for the development of novel biocatalysts, but, generally, their mechanism of assembly and function remains ambiguous. The goal of our work is to elucidate the molecular mechanisms which govern the self-assembly and function of the carboxysome. Ultimately, we hope to leverage this understanding in order to develop protein-based microcompartments with enhanced catalytic properties.

Significant achievements (2016-2018):
• Identified the mechanism for loading RuBisCO into alpha-carboxysomes, which involves a repetitive, intrinsically disordered protein.
• Carried out a high-throughput screen using transposon-mediated gene disruption to identify novel factors associated with alpha-carboxysome function in CO$_2$ assimilation.
• Identified and characterizes novel inorganic carbon transporters associated with CO$_2$ assimilation in the screen above.
• Characterized – including a high-resolution EM structure - the nanocompartment-forming encapsulin from $T$. maritima as novel flavin-binding protein shell that could facilitate redox chemistry of lumenal proteins.
• Identified and characterized a new nanocompartment-forming encapsulin protein involved in sulfur metabolism in cyanobacteria.

Science objectives for 2018-2019:
• Solve the high-resolution structure of carboxysomal RuBisCO with its cognate targeting element, representing a ‘trapped’ assembly intermediate.
• Finish characterizing the genes isolated from the carboxysome-focused transposon screen, with a focus on better biochemical characterization of the novel transporter.
• Finish structural analysis of the $T$. maritima encapsulin with a focus on determining the structure with the cargo enzyme ferritin bound.
• Finish biochemical characterization of the cyanobacterial encapsulin involved in sulfur metabolism.
My scientific area(s) of expertise is/are: biochemistry, microbial genetics, protein engineering.

To take my project to the next level, my ideal collaborator would have expertise in: redox chemistry and electron transfer.

Publications supported by this project 2016-2018:


Session III
Cell-type Specific Pectins in Plant Cell Walls: Structure, Interaction and Function

Malcolm A O’Neill, Principal Investigator
Michael Crowley, Principal Investigator
Maria Pena, Breeanna Urbanowicz Co-PI(s)

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Overall research goals:
Plant primary cell walls are complex macrostructures composed of cellulose, hemicellulose, and pectins together with small amounts of protein and minerals. These components interact with one another to produce an architecturally complex and dynamic biocomposite with many capabilities, including mechanical support and the determination of cell size and shape. The mechanisms that drive such interactions are poorly understood. One critical interaction involves the pectic polysaccharide rhamnogalacturonan II (RG-II), a structurally complex glycan (Fig. 1A) that exists as a borate cross-linked dimer (Fig. 1B and C) in the cell walls of all vascular plants. The RG-II dimer self-assembles through the formation of a single, site-specific borate di-ester linkage. The inability of RG-II to dimerize results in cell walls with abnormal properties and severely impacts a plant’s ability to capture, convert, and store energy. Our new data obtained using plants carrying a mutation that affects RG-II structure and enzymatically-generated RG-II structural variants provide evidence that even minor alterations to the glycosyl sequence of RG-II affects its ability to self-assemble. Our goals are to understand the unique biophysics governing RG-II self-assembly and the principles underlying the assembly of complex biocomposites. Understanding RG-II self-assembly will also provide a basis for developing polymers with unique properties based on pH- and cation-dependent tunable borate cross-links.

Significant achievements (2016-2018):
In collaboration with Harry Gilbert (Newcastle Univ, UK), we showed that the human gut bacterium Bacteroides thetaiotaomicron produces a set of enzymes that specifically hydrolyze all but one of the glycosidic bonds of RG-II (2). We used 11 recombinant Bt glycanases to selectively modify wine RG-II’s glycosyl sequence. The sidechains of the monomer are more susceptible to the hydrolases than the dimer, suggestive of extensive interactions between sidechains in the dimer. Removing L-Gal from chain A substantially reduces the rate of dimer formation \textit{in vitro}. Removal of MeFuc, Ara, and Rha from chain B increased the rate of dimer formation. Removing only Ara and Rha residues had no discernible effect, suggesting MeFuc has a role in self-assembly. Real-time $^1$H-NMR of WT dimer formation showed that the methyl-ether resonances of O-methylated sugars (MeXyl, MeGalA and MeFuc) become broader and their chemical shifts move up-field during conversion of the monomer to the dimer, implying structural changes occur in close proximity of these groups. The Ara and Rha of chain B are absent in RG-II from many plants and thus may have no role in self-assembly. Modifying chain D has a small but discernible influence on dimer formation. Together our results show that L-Gal and MeFuc have roles in controlling the rate of dimer formation. Further evidence for the importance of L-Gal was obtained in...
collaboration with Jenny Mortimer (JBEI). RNAi suppression of Arabidopsis GOLGI GDP-L-GALACTOSE TRANSPORTER 1 (GGLT1) results in dwarf plants, decreased RG-II cross-linking, and a ~50% reduction in the amounts of L-Gal present on chain A (6). The remaining L-Gal is removed by the Bt α-L-Galase confirming that gglt1 RG-II is L-Gal-deficient. Chain B of gglt1 and control plants are similar. gglt1 RG-II dimerizes more slowly than wild type. The gglt1 dimer is less stable in the presence of Ca$^{2+}$ chelators, consistent with the notion that Ca$^{2+}$ is important in RG-II cross-linking in planta (3). We hypothesize that the complexity of RG-II evolved to control the rate of dimer formation. Studies to probe dimer formation using 3-nitrophenylboronic acid (NPB) have begun as NPB forms a mono-ester with the monomer and thus inhibits dimer formation. Studies of self-assembly in vitro show that the dimer forms slowly between pH 2 and 5 in the presence of boric acid. The rate is substantially increased by adding equimolar amounts of Pb$^{2+}$. Higher [Pb$^{2+}$] inhibits dimer formation but inhibition is suppressed by adding Ca$^{2+}$, suggestive of multiple cation binding sites. The dimer is converted to the monomer at 80°C and pH 4. However, the dimer is stable at 80°C and pH 4 in the presence of added boric acid and Ca$^{2+}$.

Our studies of selected members of the Lemnoideae established that RG-II structure and cross-linking is conserved even though the structures of other pectins (5), xyloglucan and glucuronoxylan changed considerably as these plants diversified. This emphasizes that maintaining RG-II structure and cross-linking is critical for vascular plants. We have shown that the ability to form and utilize UDP-Api existed prior to RG-IIs appearance in vascular plants (1,4,7). Even though bryophytes, algae and bacteria do form UDP-Api, no Api-containing glycans have yet been identified in these organisms.

Science objectives for 2018-2021:
A collaboration between the CCRC and NREL was established to elucidate the molecular features of RG-II that are critical for borate cross-linking and to provide fundamental insight into the unique biophysics that govern the non-catalytic self-assembly of RG-II. Chemical, kinetic and spectroscopic studies of RG-II and its structural variants (CCRC) with computational modeling studies involving molecular dynamics and quantum mechanical simulations (NREL) will provide conformational models of RG-II that describe self-assembly of the borate cross-linked dimer, determine how each sidechain contributes to cross-linking, establish why specific sugars are critical for cross-linking, demonstrate how divalent cations stabilize cross-linking, and provide testable models for dimer formation in planta.

Our scientific area(s) of expertise is/are: Chemical and NMR spectroscopic studies of glycans. Production of recombinant enzymes (CCRC). Molecular dynamics and quantum mechanical simulations of glycans (NREL).

To take our project to the next level, our ideal collaborator would have expertise in: Technologies to develop chemical and chemo-enzymic synthesis of defined portions of RG-II. Identifying additional enzymes to selectively fragment RG-II backbone or release oligosaccharide sidechains. Commercial applications of glycopolymers with tunable borate cross-links. Biology of salt-tolerant plants to extend research to role of cell wall in adaptation to extreme environments.

Publications supported by this project 2016-2018:
Elucidating the Biochemical Mechanisms Controlling Secondary Wall Biosynthesis in Plants
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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 (2016-2018):
We have discovered that a rice GT61 glycosyltransferase, XYXT1 (xylan xylosyltransferase1), is a novel β-1,2-xylosyltransferase catalyzing the addition of 2-O-xylosyl side chains onto the xylan backbone (Fig. 1). When expressed in the Arabidopsis gux1/2/3 triple mutant, in which xylan was completely devoid of sugar substitutions, XYXT1 was able to add xylosyl side chains onto xylan. Glycosyl linkage analysis and comprehensive structural characterization of xylooligomers generated by xylanase digestion of xylan from transgenic Arabidopsis plants expressing XYXT1 revealed that the side chain xylosyl residues were directly attached to the xylan backbone at O-2, a substituent not present in wild-type Arabidopsis xylan. Furthermore, we showed that recombinant XYXT1 possessed an activity transferring xylosyl side chains onto xylooligomer acceptors, whereas recombinant OsXAT2, another GT61 protein, catalyzed the addition of arabinosyl side chains onto xylooligomer acceptors. Our finding uncovers a new biochemical function of members of grass-specific GT61 glycosyltransferases, which enriches our understanding of genes involved in xylan biosynthesis.
We have demonstrated that (A) nine Arabidopsis DUF231-containing proteins are xylan acetyltransferases mediating the regiospecific acetylation of xylan and that the conserved GDS and DXXH motifs are critical for their acetyltransferase activity; (B) four Arabidopsis DUF231 members and their close A. konjac homolog are mannan O-acetyltransferases catalyzing 2-O and 3-O-monoacetylation of mannosyl residues; (C) two Arabidopsis DUF231 members and four poplar close homologs are xyloglucan O-acetyltransferases catalyzing acetyl transfer onto fucosylated galactosyl (Gal) residues on xyloglucan side chains and the defucosylation of these acetylated side chains by apoplastic AXY8 fucosidase generates side chains with acetylated, non-fucosylated Gal residues; and (D) 12 xylan O-acetyltransferases from Populus trichocarpa and 14 xylan O-acetyltransferases from rice exhibit differential specificity toward the acetylation of different positions of xylosyl residues. Our findings advance our understanding of the complexity of the biochemical mechanisms controlling cell wall polysaccharide acetylation.

Science objectives for 2018-2019:
The main objective for the following year will be to investigate GT61 genes for their roles in secondary wall biosynthesis. We have identified several 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 scientific area(s) of expertise is/are: Molecular biology and biochemistry

To take my project to the next level my ideal collaborator would have expertise in: protein and carbohydrate structures

Publications supported by this project [2016-2018]:
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 unfolding 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. Our specific focus in this project is on the wall-loosening action of expansins. Expansin action underpins plant growth, yet its 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 (Cosgrove 2016). Our goal is to elucidate how expansins loosen cell walls at the molecular scale. In recent years we have learned that expansins are more diverse and their actions 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 (Cosgrove 2017). 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 (2016-2018):
- The target of β-expansin in complex plant cell walls is glucurono-arabinoxylan (GAX) (Wang et al. 2016). For this study we attached Mn^{+2} as a paramagnetic probe to ZmEXPB1 and used ssNMR to measure paramagnetic relaxation enhancement (PRE) of ^{13}C-labeled maize cell walls mixed with the ZmEXPB1-Mn probe (in collaboration with Dr. Mei Hong, MIT). By this means we discovered that ZmEXPB1 preferentially binds GAX and has negligible contact with cellulose, at least in complex walls. Moreover, EXPB1 changed GAX mobility within the cell wall in a complex manner: the rigid fraction of GAX became more rigid upon EXPB1 binding while the dynamic fraction became more mobile. Binding to cell walls and cellulose was also assessed by depletion isotherms, which demonstrated complex, electrostatic binding of EXPB1 to maize walls. These interactions differ greatly from those of bacterial expansin bound to Arabidopsis cell walls (Wang et al. PNAS 2013). Our results suggest a model for cell walls in grasses in which ZmEXPB1 weakens the noncovalent binding between two forms of GAX (high substitution, hs; and low substitution, ls).

Figure 1 (right): Conceptual scheme to account for the known CW-loosening and -binding activities of maize EXPB1. In this limited depiction of the grass CW, lsGAX binds to cellulose surfaces and hsGAX binds to lsGAX but not cellulose. EXPB1 is hypothesized to disrupt the noncovalent junctions (depicted as short black lines) between hsGAX and lsGAX. The result is solubilization of hsGAX and physical weakening of the grass CW.

- β-Expansin action alters maize wall surface texture at the nanoscale. We used atomic force microscopy (AFM) to assess 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 and in the process of detaching from microfibril surfaces. These results complement the ssNMR results (above) and further substantiate the loosening action of β-expansin.

- The molecular basis of acid growth: pH-dependent re-partitioning of expansin within the cell wall? With fluorescently-tagged probes that bind cellulose, we found that penetration into the cell wall was strongly dependent on pH of the buffer, with greater penetration at low pH, as a result of electrostatic repulsion. This effect may contribute to ‘acid growth’, that is, the increase in cell wall extensibility by expansins at low pH. Electrostatic attractions and repulsions may be a significant factor for the activity of all proteins that modify plant cell walls.

- We recognized that expansins have a structure know as a ‘Hopf link’. Topologically, the Hopf link consists of two covalent circles that are formed by disulfide bonds and that are threaded through each other. Proteins with this knotted topology are likely to be difficult to fold correctly…a possible explanation for the difficulty in expressing plant expansins in common recombinant expression systems (Many bacterial expansins lack the disulfide bonds that form the two circles in a Hopf link. This realization led to a bioinformatic assessment of expansin Hopf link structures and additional attempts to expression expansins in E. coli. These attempts did not prove successful.

Science objectives for 2018-2021:

- Test Clavibacter expansin for enzymatic activities that are suggested from the crystal structure of the protein-ligand complex.
- Assess the nano-scale actions of β-expansin on cell walls using atomic force microscopy and scanning electron microscopy coupled to nanogold probes of cell wall surfaces.
- Use computational methods to assess pH-dependent changes in α-expansin structure.
- Test the activity of a fungal expansin that appears to be intermediate in structure between bacterial expansins and plant expansins.

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;

To take my project to the next level, my ideal collaborator would have expertise in: recombinant expression of “difficult” proteins; novel methods for assessing conformation and interactions of complex polysaccharide; coarse-grain modeling of polymers; polymer biophysics;

Publications supported by this project 2016-2018:

Revealing the many faces of the genetic code

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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.


* Publications supported in part by the Department of Energy grant DE-FG02-98ER20311 are indicated by an asterisk
Engineering Selenoproteins for Enhanced Hydrogen Production

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Overall research goals:
1. Optimization of the EF-Tu-dependent system for co-translational insertion of selenocysteine at any site in a protein.
2. Engineering of hydrogenases and formate dehydrogenase with Sec-coordinated metal clusters.

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)

![Figure 1](image)

2. With the allo-tRNA\textsuperscript{UTu} methods developed above, we have restarted our collaborations on *Arabidopsis thaliana* sulfite oxidase (with Russ Hille, UC Riverside) and *E. coli* hydrogenase 1 (with Fraser Armstrong, Oxford University) aimed at replacing critical cysteine residues with selenocysteine. The plasmid-expressed enzyme (sulfite oxidase) is available in mg quantities (from a 10 L culture), while the genome-encoded enzyme (Hyd-1) yields about 75 μg (from a 10 L culture); in both cases this should allow detailed biochemical experiments. The degree of Cys to Sec conversion is almost 100% in sulfite oxidase, but is still undetermined in Hyd-1.

Science objectives for 2018-2019:
- To demonstrate that Cys to Sec conversion will work for different enzymes and proteins in order to make our approach a robust method for general selenoprotein production.

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 (2016-2018):

Poster Session I
Electron spin density distribution by $^{13}$C labeling.

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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$_H$ site of the 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 2017-2018:

**The complete spin density distribution in SQ$_A$ and SQ$_B$ by $^{13}$C labeling.** The spin density distribution of an organic or biological radical provides a unique signature of its singly occupied molecular orbital (SOMO). Determining the complete electron spin density distribution for protein-bound radicals, even with advanced pulsed EPR methods, is a formidable task. Here we report a strategy to overcome this problem combining multifrequency HYSCORE and ENDOR measurements on site-specifically $^{13}$C-labeled samples with DFT calculations. As a demonstration of this approach, pulsed EPR experiments are performed on the primary Q$_A$ and secondary Q$_B$ ubisemiquinones of the RC from Rb. sphaeroides $^{13}$C-labeled at the ring and tail positions. Despite the large number of nuclei interacting with the unpaired electron in these samples, two-dimensional X- and Q-band HYSCORE (see figure) and orientation selective Q-band ENDOR resolve and allow for a characterization of the eight expected $^{13}$C resonances from significantly different hyperfine tensors for both SQs. Six tensors with notable hyperfine anisotropy are assigned to ring carbons C$_1$ through C$_6$. The remaining $^{13}$C couplings lack a strong anisotropic component and are associated with the two carbons of the isoprenoid tail. The results of this study are then combined with previously reported $^1$H (5'-methyl, hydrogen bonds), $^{13}$C (methoxy, 5'-methyl), $^{17}$O (carbonyl), and $^{14}$N (H-bond donors) couplings to construct an extensive map of the s- and p$_\pi$-orbital spin density distribution across the quinone ring carbons, their substituents, and the hydrogen bonds for SQ$_A$ and SQ$_B$ (figure). This work introduces new techniques for mapping out the spin density distribution that are readily applicable to other systems. (Ref. 6)

**Spin density distribution around [2Fe-2S] clusters by $^{13}$C$_\beta$-cysteine labeling.** The auxotroph methodology of selective isotope labeling was extended to Fe-S clusters in metalloenzymes through the collaboration with Dr. T. Iwasaki (Nippon Medical School) for $^{13}$C labeling of Cys residues at C$_\beta$. The archaeal Rieske-type [2Fe-2S](His)$_3$(Cys)$_4$ ferredoxin from Sulfolobus solfataricus (ARF, n=2), the ISC-like [2Fe-2S](Cys)$_4$ ferredoxin from Pseudomonas putida (FdxB, n=0), and the thermophile mitoNEET [2Fe-2S](His)$_3$(Cys)$_3$ homolog from Thermus thermophiles (TthNEET, n=1) were used as representative model
proteins. We combined site-specific \(^{13}\text{C}\) labeling in three major classes of the protein-bound, reduced [2Fe-2S] (His)_\(n\)(Cys)\(_{n+1}\) \((n=0,1,2)\) clusters with HYSCORE in order to resolve all hfi of the irons with the nearby Cys\(^{13}\text{C}\_\beta\)'s. After integrating these results with hyperfine couplings for the remote N\_s of the His ligands, detailed maps of the s-spin density distribution in the immediate cluster environments relevant to biological ET are obtained (figure). We have identified a consistent trend where a significantly larger amount of unpaired spin density is transferred to the Fe\(^{2+}\) ligands than that of Fe\(^{3+}\), despite the lower \(S=2\) spin of Fe\(^{2+}\) than \(S=5/2\) of Fe\(^{3+}\). The preferred delocalization of the electron spin along the Fe\(^{2+}\) ligands correlates well with ET directionality in these proteins; redox chemistry is exclusively catalyzed at the reducible Fe\(^{2+}\) site of the cluster, suggesting this may be a mechanism for maximizing the electronic coupling with redox partners. (Refs. 6,8).

Science objectives for 2017-2018:

- \(^{13}\text{C}\) couplings provide insight into the SQ binding to the protein. We will apply a biochemical approach for selective \(^{13}\text{C}\) labeling of ring carbons in the SQ\(_H\). The quinones biochemically labeled in \(\text{bo}\(_3\)\) enzyme will also be used in studies of reaction center and \(\text{bc}\(_1\)\) complex.

- We will investigate the influence of mutations on SQ\(_S\) in RC and SQ\(_H\) in cyt \(\text{bo}\(_3\)\). We will focus on the M265I(T,S,N) mutants in RC and on two nonpolar residues Ile102 and Met78 that are part of the Q\(_H\) binding pocket in cyt \(\text{bo}\(_3\)\).

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: 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. We are open for any collaborative work requiring EPR approaches.

References to work supported by this project 2017-2018:


Expression of recombinant methyl-coenzyme M reductase in the methanogenic archaeon Methanococcus maripaludis for the examination of activation and the role of post-translational modifications

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Overall research goals:

The methyl-coenzyme M reductase (or Mcr) is a key enzyme in both the formation and anaerobic oxidation of methane. In the biosynthetic direction, it catalyzes the reduction of methyl-coenzyme M (CH₃-S-CoM) by coenzyme B (HS-CoB) to form methane and the heterodisulfide, CoM-S-S-CoB. The prosthetic group of this unique enzyme is the nickel tetrapyrrole, coenzyme F₄₃₀. When active, the metal must be in the Ni(I) oxidation state. Because the redox potential of the F₄₃₀Ni(II)/F₄₃₀Ni(I) couple is near -650 mV, the stability of the Ni(I) prosthetic group is critical for maintaining enzyme activity. The enzyme also contains unique posttranslational modifications (PTMs) near the active site that may also be important. The proposed research will address the mechanism of Mcr activation and the role of one of the PTMs. Not only will it provide basic insight in a major biogeochemical process, this research on how this enzyme functions will enable design of highly selective and efficient bio-inspired catalysts for direct activation of methane, formation of biofuels through improved biochemical pathways for methane production, and design of specific inhibitors to lower greenhouse gas production by ruminants and improve feed efficiency.

Key to advancing these core research areas is the capability to express recombinant and active Mcr. In preliminary studies, recombinant Methanothermococcus okinawensis Mcr (Mcr₉₀k) was successfully expressed in the methanogenic archaeon Methanococcus maripaludis. Although inactive, the recombinant Mcr₉₀k contained about 40% of the expected stoichiometry of coenzyme F₄₃₀ and the same 4 PTMs as present in the native enzyme. To our knowledge, this represents the first successful expression and assembly of the holoenzyme. In parallel to our studies of expression of recombinant Mcr, an in-frame deletion mutant was constructed for the gene encoding the methanogen marker 10 protein (Mmp10). Of the four PTMs found in the native Mcr from M. maripaludis, only the Me-Arg was absent. The Me-Arg PTM was restored when the mutant was complemented with the mmp10 gene on an expression plasmid. Further studies were undertaken for elucidation of the role of the Me-Arg PTM in catalysis. The Me-Arg PTM is especially interesting because it is absent in the anaerobic methanotrophs of the ANME-1 group. For this reason, it has been speculated that it plays an important role in determining the physiological direction of Mcr, either CH₄ production or oxidation.

Significant achievements (2017-2018):

1) To examine its role, MMP1554, the gene encoding Mmp10 in Methanococcus maripaludis, was deleted with a newly genetic developed tool, resulting in the specific loss of the 5-(S)-methylarginine posttranslational modification of residue 275 in the McrA subunit and a 40-60% reduction in the maximal rates of methane formation by whole cells. Methylation was restored when the gene was complemented by the wild-type gene in two separate methanococcal expression plasmids.

2) MMP0140, which encodes a putative hydrogenase maturation factor, contains an amino acid sequence very similar to the site of the Mcr posttranslational modification by Mmp10. We
hypothesized that the poor growth of the complemented strains might have resulted from mistakes in the PTM of this protein. To determine if Mmp0140 is a target of PTM, the his-tagged version of Mmp0140 was expressed in *M. maripaludis*. Upon purification and analysis of the peptides, no evidence for methylation of the specific Arg residue of Mmp0140 was found.

Science objectives for 2018-2019:
- A major objective is to purify Mmp10 and characterize the enzyme and its activity. To this end, his-tagged forms have been expressed in both *Methanococcus* and *E. coli*.

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

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

Publications supported by this project:
Expression of recombinant methyl-coenzyme M reductase in the methanogenic archaeon *Methanococcus maripaludis* for the examination of activation and the role of post-translational modifications

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**Overall research goals:**
Methyl-coenzyme M reductase (Mcr) is the key enzyme in both the biological formation of methane by methanogenic Archaea and anaerobic oxidation of methane by methanotrophic Archaea. The activity of Mcr is critically dependent on the unique nickel-containing tetrapyrrole, coenzyme F₄₃₀. There are also 5 unique post translational modifications of amino acids in close proximity of the active site channel. Recently we successfully produced recombinant MCR with low methane-forming activity. This is already a milestone, but to be able to obtain fully active recombinant MCR, we need to characterize the mechanism of protein assembly and optimize its activation. A very large protein complex is required for Mcr activation in a process that appears to involve both electron bifurcation and ATP hydrolysis. Our research is aimed at understanding the biochemical mechanism of this activation using recombinant proteins and a simplified Mcr activation assay. The long-term goals of our research are to understand the actual mechanism of methane production and the regulation of Mcr activity by the cell. A successful outcome will provide important insights into how to slow down livestock methane production and production in rice fields. Both processes contribute to climate change because methane is a potent greenhouse gas. In addition, understanding what is needed to express fully active Mcr would allow the development of designer strains of methanogenic and methanotrophic microorganisms for methane production and the conversion of natural gas to liquid fuel, respectively.

**Significant achievements (2017-2020):**
Whole cell experiments were performed to improve the activity of the recombinant Mcr using the host’s activating complex. These were successful and enzyme currently can be produced that displays 40 to 50% of the expected activity.

In parallel studies, all of the components of the activating complex have been recombinantly expressed in either *Escherichia coli* or *Methanococcus maripaludis*, depending upon cofactors expected to be required for activity. All components have now been expressed as soluble proteins, and two components appear to have very low [4Fe-4S] cluster content.

**Science objectives for 2018-2019:**
- The purification methods for the individual activating components will be improved as well as the cluster reconstitution procedures.
- One of the proteins, components A2, is very unstable. It will be coexpressed with some of the other components to determine if that increases stability.
- The recombinant proteins will be used to determine if they can activate MCR. This would allow studies of the role of each component and determine if there are components still missing.
- Since it is now possible to directly obtain active recombinant MCR, variant enzymes will be produced to test for the role of specific amino acids in the active site channel.
My scientific area(s) of expertise is/are: mechanistic enzymology, metalloenzymes, EPR/ESR.

To take my project to the next level, my ideal collaborator would have expertise in: The collaboration with Barny Whitman already brought this to the next level but the application of CRISPR might be useful.

Publications supported by this project [Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]:

   https://doi.org/10.1128/JB.00746-17

   https://doi.org/10.1101/211441
Overall research goals:
We are interested in establishing the coupling between ATP binding, hydrolysis and electron transfer in Nitrogenase and the nitrogenase-like Dark Operative Protochlorophyllide Oxidoreductase (DPOR) complex. Nitrogenase catalyzes ATP-dependent N₂ reduction and DPOR reduces protochlorophyllide to chlorophyllide, a key precursor for chlorophyll biosynthesis. Both enzymes have electron donor and electron acceptor component proteins that are arranged as two symmetrical halves. We uncovered the order of catalytic events in nitrogenase and showed that ATP is hydrolyzed after electron transfer. More interestingly, ET and ATP hydrolysis occurred first in only one half of the complex and such events in the other half were allosterically suppressed. We are currently exploring the molecular basis of this allosteric communication. We have generated half active versions of the DPOR protein where one half carries mutations in either ET or ATP hydrolysis and show that both halves need to be active to complete substrate reduction.

Significant achievements (2017-2018):
- In DPOR, we have uncovered that ATP induces a conformational compaction of the L-protein (electron donor) and specific amino acids that coordinate the sugar in ATP are essential for this structural change.
- The L-protein is a homodimer and using linked L-proteins we show that both ATP are required to enact the structural compaction.
- We have solved a crystal structure of the apo-form of the L-protein and have discovered a novel protective role for a certain region. This region caps the FeS cluster in the absence of ATP and likely serves as a regulatory switch to control DPOR activity in the cell.

Science objectives for 2018-2019:
- Single molecule studies of Nitrogenase and DPOR. We are working towards introducing non-canonical amino acids in Azotobacter vinelandii towards generating fluorescently labeled nitrogenase for single molecule studies. tRNA and its cognate tRNA synthetase for 4-azodophenylalanine have been engineered into Av and we are currently establishing procedures for expressing and isolating the 4AZP carrying Fe-protein.
- Using TIRF microscopy, we will be visualizing assembly and disassembly of the DPOR and nitrogenase protein complexes, substrate binding and product release.

My scientific area(s) of expertise is/are: Transient-state kinetics; non-canonical amino acids; single molecule fluorescence.

Publications supported by this project 2017-2018:
Macromolecular Organization and Post-translational Regulation of Phenylpropanoid-Lignin Biosynthesis

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Overall research goals:

Phenylpropanoid biosynthesis leads to a myriad of aromatic products, including cell wall structural polymer lignin. Lignin represents a substantial metabolic sink of photosynthetically reduced carbon, accounting for up to 30% photoassimilates. Since the formation of lignin (i.e., lignification) is an irreversible process, the carbon allocation into cell wall lignin is tightly controlled during plant growth and development and in response to the environmental stresses. Our long-term goals are to discover the biochemical and molecular mechanisms governing carbon flux and electron shuttling to the phenylpropanoid metabolism. Specifically, our researches are to 1) explore structural components and the related spatial organization mechanism governing monolignol biosynthesis and affecting carbon precursors and electrons specifically channeled into the biosynthetic process at the molecular and subcellular levels; 2) define the energy sensors and regulators as well as the related regulatory networks controlling carbon allocation into the phenylpropanoid metabolism.

Significant achievements (2016-2018):

• A molecular machine was identified that organizes monolignol biosynthetic reactions and dominates the channeling of reduced-carbon precursors to lignin
  Many vital processes in eukaryotes are optimized by spatial organization of reactions into distinct compartments. Although monolignol biosynthesis is commonly thought to take place in cytoplasm, its three cytochrome P450 enzymes (C4H, C3'H and F5H) reside on the endoplasmic reticulum membranes, which are postulated to serve as the anchor points of a metabolon for monolignol biosynthesis. Adopting a set of biochemical approaches, we discovered that three monolignol synthetic P450 enzymes localize near one another on the ER membrane but they don’t act as what was previously reported to interact directly; instead, all three P450 enzymes commonly interact with two ER-resident proteins, MSBP1 and MSBP2. Moreover, MSBPs themselves can form homomers or heteromers, suggesting that they may serve as scaffold for organizing P450-catalzed reactions. Suppressing the genes for MSBPs in plant substantially reduces lignin biosynthesis but does not impair the formation of flavonoids, another closely related class of phenolics whose synthesis requires one of the three P450 enzymes, suggesting MSBP-mediated enzyme organization specifically facilitates lignin formation.

• Demonstrated that cytochrome b5, in addition to cytochrome P450 reductase, is an obligate electron shuttle intermediate for lignin biosynthesis
  In phenylpropanoid-monolignol biosynthesis, NADPH: Cytochrome P450 Reductases (CPRs) typically serve as the redox partner of P450 enzymes to deliver electrons from reductants to the P450 catalytic center for hydroxylation of phenolic substrates. Our biochemical and genetic study discovered that cytochrome b5 (CB5) physically associates with monolignol P450 enzymes and specifically augments F5H enzyme activity thus controlling syringyl lignin subunit formation in Arabidopsis. The data suggest that both CB5 and CPR are the indispensable electron carriers for lignin biosynthesis.

• Elucidated that F-box protein as negative regulator controls flavonoids/anthocyanin biosynthesis in response to light and sugar signals
  Following our previous characterization of four F-box proteins, the AtKFB\textsuperscript{PALS}, we further identified an additional F-box protein, AtKFB\textsuperscript{CHS}, that interacts with chalcone synthase (CHS), a committed enzyme diverting metabolic flux from general phenylpropanoid pathway to a variety of flavonoid metabolites, and
mediates CHS’ ubiquitination and degradation. AtKFB\textsuperscript{CHS} is differentially responsive to the different quality of lights and to carbon supply, further demonstrating that the identified F-box proteins, via directly targeting on the phenylpropanoid enzymes, may function as the key regulators in response to light and sugar signals to control carbon allocation into phenylpropanoid metabolism.

Science objectives for 2018-2020:

- We suspect that the MSBP-mediated P450 enzyme complex might be involved in the spatial organization of monolignol biosynthesis in a subdomain of endomembrane. Our Co-immunoprecipitation-mass spectrometry analysis reveals a set of proteins potentially associated with monolignol P450 enzymes, including the proteins involved in forming microdomain of membrane. We will further elucidate and confirm the potential subdomain organization of monolignol biosynthesis.

- With the discovery of CB5 as an electron shuttle protein specific for S-lignin synthesis in planta, we will further explore the molecular basis for its specificity in augmenting F5H but not C4H and C3’H-catalyzed reactions. We will also define and differentiate biological functions of different CB5 and CPR family members in monolignol biosynthetic redox reactions.

- Since the identified KFB\textsuperscript{PALs} and KFB\textsuperscript{CHS} are transcriptionally responsive to the sugar and light signals, we will further discover the molecular players and the potential regulatory network/cascade controlling the KFB genes expression and phenylpropanoid-lignin synthesis in sugar and light signaling.

My 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(s) would have expertise in: Single molecular imaging, Cryo-EM based protein structure, Electrochemistry of redox proteins.

Publications supported by this project 2017-2018:

**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. Figure 2 indicates that 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 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 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).

**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).

**Figure 2**. rgs1 mutants have impaired photosynthesis efficiency in variable light environment. Top. Light environment showing on/off as for a typical growth chamber, “natural” mimicking natural daylight by incremental increase to decrease, “natural w/ fluctuations” mimicking natural plus fluctuations to mimic a day with shadows, flowed by on/off again. The heat map shows the difference between the rgs1 mutant and wildtype. Blue is lower, black is same, and red is greater than wildtype. Calculated parameters are Photosynthetic efficiency (PS II), nonphotochemical quenching (NPQ), reversible quenching (qE), photobleaching (qI). Modified from Liao et al 2017

**Significant Achievements 2017-2018:** We made the most remarkable discovery toward this Aim. Plants lacking the AtRGS1 protein were monitored for photosynthesis efficiency under different light conditions and compared to wildtype plants. When grown in a chamber where the lights come on at the beginning of the day then off at the end, the photosynthesis efficiency of the rgs1 mutants behaved like wildtype (**Fig. 2, on/off**). However, when the light increased...
slowly to noon then decreased to mimic a natural day (“natural”), the rgs1 mutant was not able to optimize photosynthesis. When the “natural lighting” was altered with fluctuations to mimic shadows and clouds, the photosynthetic efficiency of the rgs1 mutant became even worse compared to wild type plants. Specifically, the reversible component (qE) of nonphotochemical quenching (NPQ) was increased over time. rgs1 mutants do not photobleach more easily than wildtype. Therefore, we mathematically modeled the data and discovered one particular form of AtRGS1 changed at the light→dark and dark →light transitions in a way that was consistent with this form being a change detector. The mathematical model revealed that the AtRGS1/G complex enables the plant to detect shadows, filter noise such as flickering light, and impart memory such as the timing of the last shadow to pass over the leaf. These properties are crucial for plant fitness. Because neighboring plants are competing with each other for photons, maximizing energy capture provides a competitive advantage. On the other hand, under bright light, plants must be able to dissipate energy to avoid photobleaching and death. Dissipating energy involves, in part, decreasing photosynthesis efficiency. Thus, rapidly adjusting photosynthesis efficiency in a dynamic light environment is paramount for success. What we have learned from our investigations is that plants can distinguish a shadow from the end of day in order to make a decision to keep efficiency low (in the case of a transient shadow) or to maximize efficiency (in the case of a trend in decrease in solar irradiation-dusk).

Science Objectives for 2018-2019:

Aim 1: AtRGS1 in energy perception: Glucose binding and mapping. How does glucose initiate G activation? Building on our work from the last cycle, we will produce biochemical amounts of AtRGS1 in liposomes and in nanodiscs for glucose-binding experiments. We anticipate that the binding affinity to be low thus limiting our approaches to nonequilibrium methods. We will photolabel AtRGS1 and map the location with saturation-difference NMR.

Aim 2: Glucose-induced AtRGS1 conformation changes. Two approaches will be taken simultaneously. Two-dimensional electron microscopy (Cryo EM) will be attempted using AtRGS1 in nanodiscs prepared at UNC. Solution and solid-state NMR with liposome/nanotubes and nanodiscs, respectively in collaboration with DOE contractor Alex Smirnoff (NCSU) and Patrick Reardon (OSU).

Aim 3: Glucose-induced AtRGS1-AtGPA1 interaction. Is AtGPA1 in dynamic equilibrium between bound states with its competing partners: the G βγ dimer (AGB1/AGG1) and AtRGS1 and does glucose affects this equilibrium? We will reconstitute these molecules in vitro and test association partners, rate constants, and affinities at a range of glucose. We will determine the structural requisites for association and the glucose effect. We will use 19F-NMR to detect long-range constraints. We will quantitate the effect of glucose on intermolecular interactions within the complex.

2017-2018 Publications acknowledging BES support:


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

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Overall research goals:
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. We will use computational modeling, genetic analysis, and X-ray structural determination to determine the structural and energetic features of the Asc1 from *S. aciditrophicus* that allow it to function in the ATP-forming direction. We know very little about the enzymes used to reoxidize NADH and reduce electron transfer flavoprotein generated during syntrophic metabolism. We hypothesize that *S. aciditrophicus* uses NADH-linked hydrogenases and formate dehydrogenases to reoxidize NADH and a membrane-bound NADH:ferredoxin oxidoreductase to make reduced ferredoxin from NADH. The objectives of this project are to determine whether: (1) the reoxidation of NADH occurs by NADH-linked hydrogenases and formate dehydrogenases, (2) the production of reduced ferredoxin occurs by an ion-pumping membrane-bound NADH:ferredoxin oxidoreductase, and (3) the Acs1 of *S. aciditrophicus* has an altered active site compared to known Acs enzymes that allows the Acs1 of *S. aciditrophicus* to function in the ATP-forming direction.

**Figure 1.** Bioenergetic model for pyrophosphate cycling and ATP synthesis by Acs1 in *S. aciditrophicus*. Acs1 uses pyrophosphate made during substrate activation or by membrane-bound pyrophosphatases. The chemiosmotic energy needed for pyrophosphate synthesis is generated by ATP hydrolysis by ATP synthase.

**Significant achievements (2016–2018):**
- We discovered a new mechanism for substrate-level phosphorylation in *S. aciditrophicus*. This specialized bacterium uses pyrophosphate, an important prebiotic energy source, and an acetyl-CoA synthetase (Acs1) to produce ATP (Fig. 1). Pyrophosphate formed during substrate activation and by a membrane-bound pyrophosphatase is used by Acs1 to make ATP. ATP hydrolysis by a sodium-dependent ATP synthase creates the chemiosmotic energy needed for pyrophosphate formation. We have shown that a sodium-dependent ATP synthase and a sodium-dependent pyrophosphatase are active in *S. aciditrophicus* membranes.
- Proteomic analysis of syntrophic butyrate metabolism by *Syntrophomonas wolfei* identified a protein conduit of electron flow from butyryl-CoA dehydrogenase to a membrane-bound hydrogenase involving an electron transfer flavoprotein and a membrane-bound iron-sulfur oxidoreductase, postulated to interact with the menaquinone. We recently showed that *S. aciditrophicus* has a similar protein conduit of electron flow but uses a membrane-bound, formate dehydrogenase rather than a hydrogenase.
- Stable isotope analyses showed the incorporation of $^{13}$C from $^{1-13}$C-labeled acetate into crotonate, benzoate, and cyclohexane-1-carboxylate during the degradation of these substrates. Thus, syntrophic metabolism involves reversible enzyme systems that operate close to thermodynamic equilibrium, but conserve energy in a highly efficient manner.
• The enzyme systems involved in NADH reoxidation generated during syntrophic metabolism are not well understood. We characterized the [FeFe] hydrogenases (Hyd) from *S. wolfei* and *S. aciditrophicus* and showed that both function as NADH-dependent hydrogenases. These enzymes produce hydrogen from NADH without the need of reduced ferredoxin. The *S. wolfei* Hyd1ABC forms a trimeric complex (~120 kDa) while the *S. aciditrophicus* HydAB is a dimer of an αβ heterodimer (~ 280 kDa). The *S. aciditrophicus* β subunit appears to be a fusion of the smaller gamma subunit to the N-terminal end of the β subunit. The production of hydrogen from NADH without ferredoxin involvement avoids the energetically costly reaction to produce reduced ferredoxin. However, continual NADH-dependent hydrogen production depends on the presence of a hydrogen-consuming organism to maintain a low hydrogen partial pressure, thus explaining, in part, the obligate requirement for a hydrogen-consuming microorganism during syntrophy.

• The genome of *Methanospirillum hungatei* strain JF1 is the first completely sequenced genome of the family *Methanospirillaceae*. The large genome of *M. hungatei* JF1 (3.5 MB) suggests the presence of unrecognized biochemical/physiological properties that likely extend to the other *Methanospirillaceae* and include the ability to form the unusual sheath-like structure and to successfully interact with syntrophic bacteria. The core machinery of *M. hungatei* to produce methane from hydrogen and carbon dioxide and/or formate is typical of other hydrogenotrophic methanogens except that *M. hungatei* has genes for three H⁺ or Na⁺-translocating A0A1-type ATP synthases.

**Science objectives for 2018-2019:**

- We will use homology modeling/docking and X-ray crystallography to determine the position of amino acid side chains in the *Sa*Acs1 active site and to identify the conformation of *Sa*Acs1 in the presence and absence of ATP and/or acetate. Furthermore, amino acid substitutions will be utilized to determine the role of amino acids important for interactions with ATP in the active site of *Sa*Acs1.
- 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 2016-2018:**


Photosynthetic Energy Capture, Conversion and Storage: From Fundamental Mechanisms to Modular Engineering

Christoph Benning, Principal Investigator
Federica Brandizzi, Danny D. Ducat, Sheng-Yang He, Gregg A. Howe, Jianping Hu, Cheryl A. Kerfeld, David M. Kramer, Beronda L. Montgomery, Thomas D. Sharkey., Co-PI(s)
Michigan State University-DOE Plant Research Laboratory, East Lansing, Michigan 48824
Email: benning@msu.edu; Website: https://prl.natsci.msu.edu/

Overall research goals:
The conversion of sunlight into chemical energy by photosynthesis is the basic biological process driving life on earth. Photosynthesis sustainably provides food, feed and energy rich molecules, and has led to the formation of fossil fuels over geological times. Collectively, we are “Exploring the biological solar panel” at scales ranging from photosynthetic complexes and bacterial microcompartments, the thylakoid membrane and its biophysical properties, the interaction of chloroplasts with other organelles, to the overall integration of photosynthesis in cells and leaves. Importantly, we strive to gain a comprehensive understanding of “real life photosynthesis”, i.e. its limitation and regulation under dynamic conditions in the natural environment. The long-term goal is to explore basic mechanisms of oxygenic photosynthesis, carbon fixation and partitioning in cyanobacteria and plants from nano- to mesoscales. Gaining a multiscale mechanistic photosynthetic knowledge will allow us to improve photosynthetic efficiency and, therefore, plant productivity, and it will enable us to develop photosynthetic modules that can be recombined in novel ways to expand the production of photosynthesis-based bioproducts.

Beyond the proven reductionist approach, this project embraces a non-invasive approach towards the analysis of photosynthetic performance in close to natural environments. Moreover, following principles of synthetic biology, after initially gaining a fundamental mechanistic understanding of the structure and function of photosynthetic modules and their communication, recombining these modules in novel ways will allow us to discover new principles to improve the capture and conversion of light energy.

The project is conceptually divided into three focus areas: A. Robust photosynthesis in dynamic environments, B. Construction and operation of the biological solar panel, and C. Characterization and engineering of subcellular and cellular modules for photosynthetic productivity.

Our Expertise
To achieve these goals, a multidisciplinary team covers expertise ranging from cell biology, biophysics, biochemistry, synthetic biology and bioengineering to advanced genomics and protein structural biology in plant and microorganisms.

Significant achievements (Highlight slides provided to DOE BES or in preparation* 2017-2018):
1. Chloroplast lipid breakdown products help plants protect against complex stresses
2. How algae’s photosynthetic solar panels change size in response to environmental changes
3. Detecting how light regulates the number and size of carbon-fixation structures in F. diplosiphon
4. Additional families of Orange Carotenoid Protein in the photoprotective system of cyanobacteria.
6. The ATP synthase as the safety brake of photosynthesis
7. A new tracking method for plant lipids on the move
8. *PGI regulation, G6P stromal levels and redox regulation of G6PDH
9. *Engineering scaffolds based on BMCs
10. *New in vitro method for assembling BMC architectures
11. *Carboxysomal localization of RubisCO activase-like homologs in multiple types of cyanobacteria
12. *Discovery of a rhomboid protease affecting thylakoid lipid biosynthesis
13. Discovery that JAZ proteins promote carbon partitioning to growth and reproduction by preventing catastrophic effects of an unrestrained defense response

14. A new approach to produce and select hybrid algal strains with increased photosynthetic productivity under multiple conditions.

PRL Publications directly supported by this grant of 50 total mentioning DOE BES support 9/17 – 9/18


Overall research goals:
The long-term research plans of the Walker lab at the PRL are to reveal how photosynthetic fluxes interact with plant metabolism and the environment to drive energy capture, carbon fixation and growth by coupling computational models with advanced *in vivo* and biochemical approaches. Specifically, the Walker lab investigates the flux of carbon throughout central metabolism generally and related processes, like the mechanisms governing carbon transport from the leaf intercellular airspace into the chloroplast (mesophyll conductance). Our research places special emphasis on the under-appreciated role of photorespiration in central carbon and energy metabolism. Our end goal is to integrate current knowledge and original discovery into higher-order system-scale models of carbon fixation that can be used to better model and optimize plant productivity to dynamic environmental conditions.

Since starting at the DOE-Michigan State University Plant Research Laboratory almost three months ago, the Walker lab has been setting up the advanced infrastructure needed to accomplish our science objectives. We have begun construction of a next-generation membrane inlet mass spec system capable of resolving gross fluxes of oxygen and carbon dioxide under a greater variety of controlled steady-state conditions than previously investigated. These gross fluxes of carbon and oxygen can then be used to better constrain flux models of photosynthesis built from the labeling kinetics of central metabolism. Additionally, we have begun integration of advanced laser trace gas monitors with leaf measurement systems to determine more independent $^{13}$CO$_2$-based estimates of mesophyll conductance.

Science objectives for 2018-2019:
- Revealing non-dogmatic carbon fluxes during photorespiration
- Elucidating mechanisms governing mesophyll conductance
- Building a platform for measurements of carbon flux through central metabolism

My scientific area(s) of expertise is/are: Biochemical flux models of carbon fixation from the cellular-to-canopy scale and measurements of carbon dioxide and oxygen exchange.

To take my project to the next level, my ideal collaborator would have expertise in: Mass-Action metabolic modeling and metabolic flux measurements.
Cellulose and the control of growth anisotropy

Tobias I. Baskin, Principal Investigator
Eri Kamon, Postdoctoral Research Associate

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Overall research goals:
For land plants, the cell wall is a high-capacity sink for fixed carbon. In the economy of the plant, the cell wall can be considered as equivalent to a battery. Yet when the cell wall is first synthesized, the cell is growing, increasing the volume of cell and cell wall even thousands of times. This project seeks to better understand the relationship between cell wall synthesis and growth. At the heart of this relationship are cellulose microfibrils, long crystalline polymers of glucose, around which the rest of the cell wall is organized. 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 will investigate cellulose and the control of growth anisotropy at several levels. On a biochemical level, the project will study the catalytic enzymes (CESAs) responsible for cellulose synthesis. The CESAs from one species (Arabidopsis thaliana) will be replaced with those of another, somewhat distantly related species (the grass, Brachypodium distachyon) and the consequences will be assayed for growth anisotropy and cellulose synthesis. On a cellular level, the project will test a hypothesis that growth anisotropy is controlled by the organization of microfibrils across the organ. This will be done altering growth anisotropy of maize roots (by defined inhibitors, cool temperature, and dry conditions) and characterizing growth and microfibril organization across the growth zone. As part of this effort, the PI will collaborate with scientists at Brookhaven and Oak Ridge National Labs to develop methods where cellulose orientation can be quantified in intact, hydrated roots by using scattering from X-rays and neutrons. Finally, to simplify both biological and physical issues, the project will develop a system where cellulose synthesis and growth anisotropy can be studied in single cells. For this, polarized light microscopy will be developed to quantify cellulose orientation in an intact cell through novel instrumentation based on light-field imaging. 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 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 and neutron scattering, providing key 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 (2017-2018):
Developed successful protocol for quantifying the amount of cellulose in cell walls from arabidopsis roots and from tobacco BY-2 cells. While protocols of this kind are standard in the literature, they are new for my lab. Because of the relatively small amount of mass in our starting samples we found that considerable optimization was needed to get reproducible data.

Attempted to resolve cellulose microfibril orientation in living, hydrated roots with X-ray scattering by using the LiX beamline of NSLS-II at Brookhaven National Labs. Although some promising scattering patterns were recovered, they were not reproducible in repeated sessions. In general, this approach struggles against the background from water being too high and the amount of overall cellulose organization being too low.

Confirmed that several arabidopsis lines, mutant for a given CESA and expressing that CESA’s putative ortholog from *B. distachyon*, have not only a wild-type morphology but also wild-type levels of cellulose. This implies that the diverged genes are in fact orthologous.

In collaboration with Rudolf Oldenbourg at Marine Biological Laboratory, took steps toward development of three-dimensional polarized-light microscopy. This involved generating BY-2 cells that can be induced to form tracheary elements through conditional expression of the master transcription factor VND7 (imaged at right). Tracheary elements were stained and polarized fluorescence was imaged through a light field camera and alternatively with a dual light-sheet system (“diSPIM”). These images are being used by computational scientists to develop and validate the required algorithms.

Science objectives for 2014-2015:
- Complete the characterization of arabidopsis CESA mutants transformed with the putatively orthologous gene from *Brachypodium distachyon*.
- Construct at tobacco BY-2 cell line that expresses a tagged CESA protein.
- Image CESA motility at high resolution in BY-2 cells.
- Use polarized fluorescence microscopy to test our hypothesis on the relation between cellulose organization and growth anisotropy.

My scientific area(s) of expertise are: Cellulose synthesis; polarized light microscopy; scanning electron microscopy, plant genetics; microtubules.

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

Publications supported by this project 2017 – 2018:


[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]
Overall Research Goals: The Complex Carbohydrate Research Center (CCRC) of the University of Georgia is a national resource for the study of complex carbohydrates. The goal of the DOE Center is to enable CCRC to provide collaborations, services and training to academic, government, and industrial researchers who study the complex carbohydrates of plants and microbes by providing support of experts who operate, maintain, and assist in interpreting the data obtained from a variety of scientific instrumentation. The expanding need for expertise in studies of the structure/function of complex carbohydrates is rapidly growing as the importance of carbohydrate research in areas such as biomass conversion to biofuels, bioproducts biomedical glycobiology and vaccine development is being recognized.

Significant Achievements: The DOE Center has enabled several types of collaboration and services to be offered to researchers. Scientists who request analytical services (see below) receive a written report containing a description of (i) the analytical procedures used, (ii) publishable quality results (data) of the analyses of their samples, and (iii) an authoritative interpretation of the results. CCRC personnel also provide collaborative service by becoming involved in “in depth” scientific research projects with individuals from other laboratories. One hallmark of the collaboration and services offered is the continued addition of new technologies originally developed in the CCRC research laboratories. During Sept 2016-Sept 2018, the DOE Center Grant at CCRC has processed over 291 projects from 178 outside investigators in analytical or collaborative services. The service and collaborative activities of the Center has resulted in over 100 peer-reviewed publications where the DOE Center Grant has been cited either through co-authorship with the DOE Center scientists or the work that has been carried out by the DOE Center. The CCRC’s website (www.ccrc.uga.edu) provides freely accessible, internet-searchable databases in aspects of carbohydrate science, a scheduler for shared use of instrumentation, and descriptions of the CCRC’s various research projects.

Training students and scientists in various fields of carbohydrate science is a very important part of the CCRC’s mission. Training occurs when undergraduate students, graduate students, postdoctoral fellows, and visiting scientists undertake research projects with or take formal courses from CCRC faculty and staff. In addition, several annual one-week training courses and one- and/or two-day specialized courses are offered for individuals from academic institutions, government laboratories, and private industry. In the last two years 127 scientists have taken part in our hands-on training courses in carbohydrate analysis from 49 different national and international institutions.

Science Objectives:

CCRC will continue to provide "high demand" collaboration and analytical services to the scientific community as a result of the funding by the DOE Center. The major areas of collaboration and analytical services will include:
1. Purification and analysis of plant and microbial polysaccharides
2. Purification and characterization of plant and microbial glycoproteins
3. Molecular weight determination by SEC, MALDI-MS, or ESI-MS
4. Glycosyl composition analysis: GC-MS and HPAEC
5. Glycosyl linkage analysis
6. Determination of absolute configuration
7. Structural characterization by mass spectrometry
8. 1-D and 2-D NMR spectroscopy
9. Lignin analysis using Py-MBMS and Py-GC-MS
10. CarboSource Services: production of rare nucleotide-sugars, and monoclonal antibodies reactive against plant cell wall epitopes
11. Glycome profile analysis

We will continue to offer hands-on training courses in the upcoming year:

Course 1. Techniques for Characterization of Carbohydrate Structure of Polysaccharides
Course 2. Separation and Characterization of Glycoprotein and Glycolipid Oligosaccharides
Course 3. Analytical Techniques for Structural Analysis of Glycosaminoglycans
Course 4. Mass Spectrometry of Glycoproteins
Course 5. Tools for Data Interpretation in Glycomics and Glycoproteomics

My scientific areas of expertise are: Isolation, purification and structural characterization of plant and microbial polysaccharides, glycoproteins and glycolipids using NMR and MS.

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

Selected list of publications supported by this project (2016-2018):


Unraveling the regulation of terpenoid oil and resin biosynthesis for the development of biocrude feedstocks

Bernd Markus Lange, Principal Investigator
Narayanan Srividya, Senior Research Associate; Joshua Polito, Graduate Student
Institute of Biological Chemistry & M.J. Murdock Metabolomics Laboratory, Washington State University; Email: lange-m@wsu.edu; Website: https://public.wsu.edu/~lange-m/
Simone Raugei, Collaborator, Pacific Northwest National Laboratory

Overall Research Goals:
Terpenoid oils and resins from plants are characterized by a high volumetric energy density and high degree of reduction, and are thus viable “biocrude” feedstocks. Biocrude has potential applications as a source of commodity monoterpenes and fuels (e.g., pinene and limonene), and as precursors for higher value fragrances and plastics. This proposal aims to provide the scientific basis for improving terpenoid feedstocks by (i) exploring structure-function relationships of monoterpene synthases, which are responsible for terpene structural diversity and (ii) investigating a unique pair of ferredoxin/ferredoxin NADP⁺ reductase isoforms linked to reductive reactions in plant cell types with exceptionally high flux through terpenoid biosynthesis.

Significant Achievements (2017-2018):

- **Monoterpene 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 sustainable specialty chemicals. We are taking a two-pronged approach to improving our understanding of the catalytic specificity of monoterpene synthases: (1) site-directed mutagenesis of active site residues to adjust catalytic outcomes (recent achievement: reversing stereochemistry by converting a (-)-limonene synthase into a (+)-limonene synthase) and (2) advanced ab initio molecular dynamics to begin to understand mechanistic details of monoterpene synthase catalysis with unprecedented accuracy.

- **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. Follow-up biochemical work has established the presence of a unique pair of Fd and FNR isoforms, the biochemical properties of which we are now characterizing.

Science Objectives for 2018-2019:

- **Terpene synthase structure-function.** Generate mutations that results in the conversion of a monoterpene synthase that produces a monocyclic product (limonene) into an enzyme that generates (1) a different monocyclic product (γ-terpinene), (2) an acyclic monoterpene (myrcene), or a bicyclic monoterpene (sabinene). Develop a hierarchical set of tools from modern computational biophysics, ranging from flexible docking methodologies to force-field-based (“classical”) molecular dynamics (MD) simulations and hybrid ab initio/molecular mechanics (QM/MM) approaches (augmented with statistical mechanics for free energy calculations) to characterize the role of active site residues in determining function and product specificity.

- **Evaluating ferredoxin (Fd) and ferredoxin-NADP⁺ reductase (FNR) to facilitate reductive metabolism in non-green cell types.** Terpenoid oils and resins are often accumulated in cells of anatomical structures such as glandular trichomes or resin ducts. We recently identified a pair of Fd and FNR isoforms that occurs uniquely in these specialized cell types, which we hypothesize enables
particularly high flux through reductive reactions involved in terpenoid biosynthesis. We are currently characterizing the midpoint potentials of these Fd and FNR isoforms and the impacts of the formation of ternary complexes with enzymes of terpenoid biosynthesis.

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

- Terpene synthase structure-function – quantum mechanical modeling of catalysis involving carbocationic intermediates. We are now working with Simone Raugei from PNNL, which has enabled us to make progress in an area that required unique computational expertise and resources.

Publications supported by this project (since submission of the most recent DOE proposal in 12/2016):

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


2. 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. (This work involved the characterization of a (+)-limonene synthase gene, which then served as a sequence template for our efforts to convert a (-)-limonene synthase into a (+)-limonene synthase)


Overall research goals:
Pentaheme cytochrome c nitrite reductase (NrfA) catalyzes the remarkable six-electron reduction of \( \text{NO}_2^- \) to \( \text{NH}_4^+ \). The long term goals of this project are to ascertain the enzymatic mechanism of NrfA, elucidate the strategy for the storage and flow of electrons within the protein scaffold, and determine how the flow of electrons is regulated. The specific objective for this two-year grant is to establish the intellectual framework for mechanistic studies of NrfA. To accomplish this objective, we have established cloning, expression, and purification protocols for NrfA from \textit{Geobacter lovleyi}, a DNRA bacterium recently identified for its environmental relevance, in both native and heterologous hosts. We are employing a synergistic combination of kinetic, spectroscopic, and electrochemical methods to interrogate reaction intermediates, and coupled with computational experiments, these \textit{in vitro} studies will provide insights into transition state structures. To aid interpretation of the \textit{in vitro} model, we are also employing \textit{in vivo} studies in the native bacterium, \textit{G. lovleyi}. Successful completion of this project will enable us to elucidate how electron storage and flow can be controlled in a multiheme protein circuit, and will provide detailed mechanistic knowledge of how this unique pentaheme enzyme orchestrates the challenging multi-electron and multi-proton reduction of \( \text{NO}_2^- \) to \( \text{NH}_4^+ \).

Significant achievements (2017-2018):
\textit{Geobacter lovleyi} NrfA was successfully cloned and overexpressed in \textit{Shewanella oneidensis} with a yield of 7-9 mg purified protein per liter of culture. The purified enzyme contains a full complement of 5 hemes and has a \( k_{\text{cat}} \sim 970 \text{ NO}_2^-/\text{sec} \). We demonstrated that NrfA can be reduced quantitatively by titanium(III) citrate and europium(II) diethylenetriaminepentaacetate (DTPA), and we analyzed by UV-visible and electron paramagnetic resonance (EPR) spectroscopies a redox titration of NrfA (Figure 1). Using a series of mutations, we further demonstrated that we are able to perturb the NfrA monomer-dimer equilibrium.

We have also developed microbiological and genetic tools to (i) overexpress NrfA in the native model representative \textit{G. lovleyi} and (ii) gain insights into the metabolic constraints that regulate the flow of electrons to NrfA \textit{in vivo}. Our studies revealed that low nitrate, rather than a high carbon-to-nitrogen ratio, induces the DNRA pathway. Using genome-wide transcriptomics, we are elucidating the regulatory network that allows the cells to synthesize and mature all of the enzymes of the pathways and use NrfA as a sink of electrons to gain energy for growth under conditions of nitrate limitation.

![Figure 1. Redox titrations of NrfA. UV-visible spectra showing a redox titration of NrfA with Ti(III) citrate (left panel) and Eu(II) DTPA (center panel). Right panel: EPR spectra of the NrfA redox titration with Ti(III) citrate.](image-url)
Science objectives for 2018-2019:

- The process by which the active site heme is incorporated into *G. lovleyi* NrfA is not known. In the next year we plan to identify the enzyme responsible for insertion of this unique heme and further elucidate NrfA maturation.

- We will fully characterize purified *G. lovleyi* NrfA both biochemically and spectroscopically. Towards this end, we will optimize the stoichiometric chemical reduction of *G. lovleyi* NrfA to help characterize various redox states of the enzyme and determine the midpoint potentials of each heme.

- To better understand key protein-protein the interactions at the NrfA dimer interface, we will structurally characterize WT *G. lovleyi* NrfA and various mutant forms of the enzyme.

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

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

Publications supported by this project 2017-2018:

1.

Using Protein Model Approach to Discover the Underlying Principles that Govern Enzymes that Catalyze Complex Multielectron/Multiproton Redox Reactions

Yi Lu, (a,b) Principal Investigator
Simone Raugei(a), Co-PI(s)

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Overall research goals:

The overall goal of our research is to discover the underlying physical and chemical principles that govern enzymes that catalyze complex multielectron and multiproton redox reactions involved in capture, conversion, and storage of energy. Specifically, we are interested in the fundamental understanding of structural features responsible for, and mechanistic insights into, electrochemical properties and catalytic activities of metalloenzymes containing complex metallocofactors at the active sites, such as heme-copper oxidase, heme-[4Fe-4S] sulfite reductase, laccase, nitrogenase, and oxygen evolving complex in Photosystems II (PSII). In addition to learning how these complex metallocofactors are constructed and function under ambient condition, we are elucidating how the redox potentials and the resulting activity of these cofactors can be tuned using ligand coordination and the surrounding environment to reduce overpotential and better enable catalysis using earth-abundant metals.

While much progress has been made in biochemical, biophysical, and computational studies of the native enzymes and their variants, it has been difficult to pinpoint which weak non-covalent interactions in the secondary coordination sphere are important in fine-tuning the activity to the high degree observed in enzymes. Like human beings not being able to learn how to fly by studying birds alone, due to the difficulty in revealing all of the hidden features in birds responsible for flying, designing airplanes, i.e., biomimetic models, is a complimentary approach to elucidate not only individual elements necessary, but also all the elements enough for flying, or in our case, for enzymatic activity. In Richard Feynman’s words: “What I cannot create, I do not understand.” Biomimetic modeling can not only test the principles, but may also reveal new principles that otherwise would be difficult to obtain in studying native enzymes.

Because of the importance of biomimetic modeling, much effort from many labs has been devoted in the past 40 years to synthesizing models using organic ligands. Despite these efforts, most synthetic models focus on simple metallocofactors (e.g., mono- and homonuclear centers), and less complicated reactions (e.g., using 1-2 electrons). Some progress has been made in designing metalloproteins that mimic native ones. However, most synthetic and protein models so far are structural models with no activity; those that display activity do so at levels that are far below those of native enzymes.

To advance the field of using models for better understanding of native enzymes, we strive to design protein models containing much more complex metallocofactors at the active site that catalyze multielectron/multiproton reactions, such as laccase, nitrogenase and PSII to display similarly high activity as native enzymes. Our working hypothesis is that the primary coordination sphere of these metallocofactors, although necessary, is not enough to confer high enzymatic activity; biology has evolved to take advantage of weak non-covalent interactions in the secondary coordination sphere, such as a hydrogen bonding network and fine-tuning electronic structures of the metallocofactors in order to achieve high activity. Most previous studies of synthetic or protein models did not capture enough of these features, due to the difficulty in synthesizing organic molecules or lack of knowledge to incorporate these structural features into the models. We will focus on elucidating the roles of the weak non-covalent interactions in tuning the activity by introducing structural features in the secondary coordination sphere from native enzymes into our protein models, and studying their effects on the activity.

1. The reduction potential \( (E^\circ) \) is a critical parameter in determining the activity of most biological and chemical reactions. Biology employs three classes of metalloproteins (FeS clusters, cytochromes and cupredoxins) to cover the 2 V range of physiological \( E^\circ \)s. An ultimate test of our understanding of how to tune such \( E^\circ \) is to find out the minimal number of proteins and their variants that can cover this entire range and the structural features responsible for the extreme \( E^\circ \). We reported the design of azurin to cover a range from +970 mV to −954 mV (vs. SHE) by mutating only five residues in the secondary coordination sphere that are involved in hydrogen bonding and hydrophobic interactions to the primary coordination sphere and using two metal ions. Spectroscopic methods have revealed geometric parameters important for the high \( E^\circ \). The knowledge gained and the resulting water-soluble redox agents with predictable \( E^\circ \)s in the same scaffold will find wide applications in energy conversion.\[1\]

2. Oxygen reduction reaction (ORR) is much less efficient than the hydrogen oxidation reaction in fuel cells. While much effort has been made to design a catalyst mimicking terminal oxidases to carry out ORR using earth-abundant metals, most biomimetic models have much lower activity than native oxidases. We reported a protein model in myoglobin with an ORR rate similar to that of a cyt \( \text{Cbb}_{3} \) oxidase, by designing more favorable electrostatic interactions between our model and its redox partner, cyt \( \text{b}_{5} \), resulting in a 400-fold ET rate enhancement. Achieving high activity similar to that of native enzymes in a protein model offers deeper insight into the roles of long-range weak interactions in tuning oxidase activity. The principles revealed from this study may help design more efficient ORR catalysts.\[2\]

3. Multi-electron redox reactions often require multi-cofactor metalloenzymes to facilitate coupled electron and proton movement, but it is challenging to design artificial enzymes to catalyze these important reactions due to their structural and functional complexity. We reported a designed heteronuclear heme-[4Fe-4S] cofactor in cytochrome \( \text{c} \) peroxidase as a structural and functional model of sulfite reductase (SiR). The initial model exhibits spectroscopic and ligand-binding properties of the native SiR, and the SiR activity was improved to be close to a native enzyme through rational tuning of the secondary sphere interactions around the [4Fe-4S] and the substrate-binding sites. By offering insight into the requirements for a demanding 6e−/7H+ reaction that has so far eluded synthetic catalysts, this study provides strategies for designing highly functional multi-cofactor artificial enzymes.\[3\]

Future Plan:

We plan to use the approach we have developed in the above systems to test principles revealed from studies of other native enzymes important to this DOE Physical Bioscience program, such as nitrogenase, methane monooxygenase, and photosystem II, and to use our approach to elucidate structural features in the secondary coordination sphere responsible for fine-tuning the activity of these complex metallocofactors.

My scientific area(s) of expertise is/are: Design of metalloproteins that are both structural and functional models of native enzymes, by focusing on roles of weak non-covalent interactions in the secondary coordination spheres, and use of these models to elucidate principles that govern enzyme functions.

To take my project to the next level, my ideal collaborator would have expertise in: a) biochemical/biophysical studies of native metalloenzymes to provide targets and comparison for our protein models; and b) spectroscopic and computational studies of metalloenzymes to help characterize our models.

Selected Publications (2015-2018):


Biocatalytic Functionalization of Alkanes

Qun Liu, Principal Investigator
Gongrui Guo, Postdoctoral Research Associate
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Email: qunliu@bnl.gov; Website: https://www.bnl.gov/biosciences/staff/Qun-Liu.php

Overall research goals:
Our overall objective is to study the mechanism of biocatalytic functionalization of alkanes by the membrane-bound hydroxylase complex AlkBGT. The complex is comprised of three components: the catalytic transmembrane diiron non-heme monoxygenase (AlkB), a soluble iron-sulfur-cluster-containing redox protein (AlkG), and a flavin adenine dinucleotide (FAD)-dependent rubredoxin reductase (AlkT). Alkane biofunctionization requires sequential transfer of two electrons from AlkT to AlkB relayed by AlkG. To understand the underlying mechanisms of electron transfer, the diiron redox reaction, O2 activation, alkane selectivity and hydroxylation, our overall objective is embodied in four specific aims: 1) To assemble and characterize the AlkBGT complex. 2) To determine the structure and activity of the AlkBGT complex. 3) To determine the structural basis of the complex with respect to C-H bond functionalization. 4) To redesign the reaction pathway for improved or novel biocatalysts with respect to C-H bond functionalization.

Science objectives for 2018-2020:
- Determine structures of AlkB and AlkBG complex by X-ray crystallography or single-particle cryo-EM.
- Determine crystal structure of AlkT and its complex with substrate NADH under reducing and oxidative conditions.
- Reconstitute the AlkBGT ternary complex and characterize its activity.

My scientific area(s) of expertise is/are: membrane protein structural biology, X-ray crystallography, single-particle Cryo-EM.

To take my project to the next level, my ideal collaborator would have expertise in: Lipid modification biochemistry, directed evolution of redox enzymes.

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

Carsten Krebs, Principal Investigator
J. Martin Bollinger, Jr.; Amie K. Boal, Co-PIs
Rachelle Copeland, Ph.D. Candidate; Katherine M. Davis, Postdoctoral Research Associate
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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 CO$_2$, 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 (2016-2019):
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 ($^2$H-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. The Asp191 $\rightarrow$ 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 total abolition of ethylene production by this nearly conservative substitution, as well as its much less pronounced effect on ferryl-mediated L-Arg hydroxylation, imply that the unusual primary reaction has more rigid stereoelectronic requirements than the canonical pathway leading to formation of the ferryl complex and two-electron oxidation of the prime substrate (L-Arg).

Science objectives for 2018-19:
- We will solve structures of the enzyme with stable mimics of potential intermediate states in the primary, ethylene-forming pathway, and the secondary, L-Arg-hydroxylating pathway, and of variants with different partition ratios.
- We will continue to probe the mechanism of the primary reaction by rapid-kinetics experiments and use of substrate analogs and isotopologs.

My 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 [Click to Enter Years of Current Grant/FWP, e.g. 2012-2014]:
1. None yet
Session IV
Bacterial microcompartments (BMCs) such as carboxysomes represent biological modularity in the form of a multienzyme-containing proteinaceous organelle. The carboxysome is a self-assembling metabolic module for CO$_2$ fixation found in all cyanobacteria. These large (~100-500 nm) polyhedral bodies sequester Carbonic Anhydrase and RuBisCO within a selectively permeable protein shell, thereby concentrating substrates and presumably protecting RuBisCO from oxygen generated by the light reactions. Bioinformatic analyses have revealed the widespread occurrence of BMCs across the Bacterial Kingdom. The generalized structure of BMCs establishes catalyst proximity and spatial control of local reactant and substrate concentrations, sequesters volatile or reactive intermediates, and controls gas and control metabolite and gas exchange with the surrounding environment. Accordingly, BMCs can be viewed as a biological paradigm for spatially confined chemistry. While resolution of the physical mechanisms underlying biological energy capture and conversion is advancing with remarkable sophistication at the level of individual redox proteins, there is comparatively little known about the mechanisms for 3-D spatial and temporal control that are needed to organize catalysts and direct chemical energy capture and conversion though reaction networks. In addition to fundamental studies of the structure and function of BMCs, recent advances in programming and assembling BMCs in vivo and in vitro poised this biological architecture to become a platform for the study spatially confined chemistry. BMC architectures provide a template for combining synthetic chemistry with synthetic biology to resolve mechanisms for spatial control of reaction networks with unprecedented precision. Relative to lipid-bound compartments, the protein-based boundary of the BMC can be precisely structurally defined and the multiple shell constituents can be individually tuned for electron, substrate, product, and potentially gas transport properties. We have also established methods to spatially control recognition/anchoring sites on the shell’s interior and exterior surfaces. Knowledge of how BMCs self-assemble, circumscribe a private co-factor pool, and how they variously confine radicals, volatiles, and toxic intermediates provides poises this biological architecture to become a platform for exploring the mechanistic properties of catalysis in spatially organized, multi-scale, hierarchical host confinement.
Mechanistic studies of the FdsABG formate dehydrogenase from *Cupriavidus necator*

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**Overall research goals:**  
The overall research goals of this project are to understand in chemical detail the reaction mechanisms of molybdenum-containing enzymes catalyzing energy-relevant reactions. The two enzymes of recent focus have been the molybdenum- and copper-containing CO dehydrogenase from the aerobe *Oligotropha carboxidovorans* and the molybdenum-containing formate dehydrogenase from the aerobe *Cupriavidus necator* (formerly *Ralstonia eutropha*).

**Significant achievements (2016-2018):**  
During the present funding period our most significant achievements have involved the mechanism of action of the above formate dehydrogenase. We have provided critical new insight into the nature of the reaction, with convincing evidence that the enzyme operates via a simple hydride transfer mechanism, in which the Cα hydrogen of formate is transferred to the active site molybdenum center as shown [1]:

We have also been able to clearly identify the EPR signals for four of the iron-sulfur clusters of the enzyme, and find suggestive evidence for two others; we observe a magnetic interaction between the molybdenum center and one of the iron-sulfur centers, permitting assignment of this signal to a specific iron-sulfur cluster in the enzyme.

We have also examined the ability of the enzyme to catalyze the reverse of the physiological reaction, the reduction of CO₂ to formate utilizing NADH as electron donor. Contrary to previous studies, we demonstrate that it is in fact effective in catalyzing the reverse reaction, with a k<sub>cat</sub> of 10 s⁻¹. We also quantify the stoichiometric accumulation of formic acid as the product of the reaction and demonstrate that the observed kinetic parameters for catalysis in the forward and reverse reaction are thermodynamically consistent, complying with the First Law of Thermodynamics.

Finally, and importantly, we have demonstrated the reaction conditions necessary for gauging the ability of a given formate dehydrogenase or other CO₂-utilizing enzyme to catalyze the reverse direction so as to avoid false negative results. We conclude that all molybdenum- and tungsten-containing formate dehydrogenases and related enzymes likely operate via a simple hydride transfer mechanism and are effective in catalyzing the reversible interconversion of CO₂ and formate under the appropriate experimental conditions [2].

**Science objectives for 2018-2020:**
- Determine the X-ray crystal structures of formate dehydrogenases from *E. coli*, *C. necator* and *Pectobacterium atrosepticum*, and characterize the rapid reaction kinetics of the *E. coli* and *P. atrosepticum* enzymes; characterize reaction intermediates in all three enzymes using ENDOR spectroscopy and 13-C-labeled formate.
My scientific area(s) of expertise is/are: enzyme reaction mechanism; rapid-reaction kinetics; EPR spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: ENDOR spectroscopy, the cloning and overexpression of formate dehydrogenases and related enzymes.

Publications supported by this project (2016-2018):
Novel microbial based enzymatic CO₂ fixation/carboxylation mechanisms

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Overall research goals:

The research objectives of this proposal are to study three aspects coordinated to CO₂ fixation in Xanthobacter autotrophicus Py2: (1) the mechanism of a novel carboxylating disulfide oxidoreductase, 2-ketopropyl-CoM oxidoreductase carboxylase (2KPCC), (2) the structure of the acetone carboxylase (AC) enzyme, and (3) the biochemistry of the pathway of CoM biosynthesis. The results obtained in the study will reveal new insights into these novel carboxylation reactions and will provide the basis for comparison to other well-characterized biological mechanisms for carboxylation. Knowledge concerning the mechanism of these enzymes provides increased fundamental understanding of carboxylation chemistry that could contribute to future mechanisms for CO₂ capture and fixation and, in turn, to mitigating the anthropogenic effects of increasing CO₂ concentrations.

Significant achievements on this project DE-SC0018143, 2017-2018:

- **Progress on the mechanism of 2-ketopropyl coenzyme M oxidoreductase carboxylase (2-KPCC):** We have provided new mechanistic insights that clearly differentiate 2-ketopropyl coenzyme M oxidoreductase / carboxylase as a unique member of the disulfide oxidoreductase family of enzymes. We have recently submitted studies to the *Journal of Biological Chemistry* and *Biochemistry* detailing the significant elucidation of the reactive form of 2-KPCC as well as the specific carboxylate-stabilizing role played by a key active site histidine.

- **Mechanism studies on the ATP-Dependent acetone carboxylation:** We have determined the structure on acetone carboxylase from *X. autotrophicus* in nucleotide-bound and nucleotide-free states implicating that during catalysis the enzyme undergoes large scale conformational changes that protect reactive intermediates from exposure to aqueous media. The structure was published during the current funding period in *Nature Scientific Reports*.

Figure 1: Acetone Carboxylase structure and active site. Acetone carboxylase is a (αβγ)₂ heterohexameric enzyme containing a nucleotide binding site and a Mn active site. The enzyme undergoes a large conformational shift allowing reactive intermediates to travel the distance from the nucleotide binding site to the Mn site. At the Mn active site a carboxylation of acetone occurs.
Progress on coenzyme M Biosynthesis: We have made significant progress in elucidating the pathway of CoM biosynthesis in the bacterium *X. autotrophicus* Py2, resulting in the discovery of a phosphosulfolactate synthase that initiates the PEP-dependent CoM biosynthetic pathway (XcbB1) and a new activity for a member of the aspartase / fumarase family of enzymes (XcbC1). The results are highly significant demonstrating definitively that CoM biosynthesis in bacteria is distinct from that observed in methanogens. This work was published during the funding period in the *Journal of Biological Chemistry*.

Science objectives for 2018-2019:

- **Mechanism of 2-ketopropyl coenzyme M oxidoreductase carboxylase (2-KPCC):** We will investigate the oxidative half reaction of 2-KPCC, in which the facets of substrate (CO\(_2\) or H\(^+\)) discrimination are highlighted.

- **Mechanistic studies on the ATP-Dependent acetone carboxylation:** We will determine the relationship between metal ion content and the stoichiometry of ATP-dependent acetone carboxylation. We will use EPR spectroscopy to probe the interaction of reactive intermediates with the active site Mn\(^{2+}\) ion. We will use microscale thermophoresis to examine nucleotide interactions and nucleotide dependent conformational change during catalysis.

- **Coenzyme M Biosynthesis:** We will investigate the subsequent steps in the coenzyme M biosynthetic pathway and the specific role of XcbD1, XcbE1, and XcbA2.

My 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: Mass spectrometry to probe protein conformational change during catalysis

Publications supported by this project DE-SC0018143, 2017-2018:


Redox-regulation of electron flow in an anaerobe

Overall research goals:
Many anaerobic bacteria and archaea have metabolisms that are well suited to generate electron-rich compounds because to grow they have evolved diverse ways of managing electron flow that often naturally result in the release of reduced compounds like H$_2$ and CH$_4$. Although methods are available to track electron flux in anaerobes, little is known about how the individual enzymes that comprise metabolic pathways may monitor and control electron flow. We have a microbial system in hand, the phototrophic anaerobe *Rhodopseudomonas palustris* (*Rpal*), for which we can change electron fluxes by changing the intensity of light to which we expose cells. The effect of this is to change the redox state of the cytoplasm. At high light, the cytoplasm is relatively reduced and at low light it is relatively oxidized. In collaboration with Aaron Wright at PNNL, we found that cells contain about 200 redox sensitive proteins, many of which are metabolic enzymes. The goal of this study is to determine the role of redox regulation in controlling electron flow to different metabolic modules in *Rpal*.

Significant achievements (2017-2018):
We finished analysis of a signal transduction pathway that controls expression of light harvesting genes under low light, and we found that redox sensing by a component of this pathway is key for this regulation. In addition, by combining RNA-seq data with proteomics data, we found that redox regulation likely plays a role in electron increasing flux through the tetrapyrrole pathway. We have also started to identify the redox-sensitive cysteine(s) in metabolic enzymes and will determine the role of these cysteines in regulating the activity of these enzymes. In particular, we were interested in the redox-sensitive cysteine(s) in nitrogenase. Using a proteomics approach, we attempted to identify the specific cysteine(s) involved in responding to redox changes under low light. Resolution of the cysteine(s) modified in redox sensitive proteins was poor, but we identified Cys95 in NifK as the cysteine that is bound by the redox probes. However, this cysteine is required for coordinating the P-cluster of nitrogenase, an essential...
cofactor for nitrogenase activity, which will make it difficult to determine the role of redox-sensitive cysteines in modulating nitrogenase activity. We have therefore focused our efforts on determining redox regulation of a protein associated with nitrogen fixation that may be involved in protecting nitrogenase from oxygen. We are also looking at other metabolic enzymes such as Rubisco. Rubisco is a focus for us because it controls electron flux into the Calvin cycle for CO₂ fixation. Finally, we have also begun testing the hypothesis that thioredoxin (Trx) and/or glutaredoxin (Grx) play a role in transmitting a redox signal from light to alter the redox state of proteins in the cytoplasm (Fig. 1). Although we are still investigating the role of Trxs, we found that one Grx in particular seems to play a role in how *RpaI* responds to low light. Finally, in the last year we analyzed the regulon of the redox responsive regulatory system RegRS. we found that in *RpaI* this system seems important for controlling the acquisition of iron, an element that is central to the ability of many enzymes to carry out electron transfer reactions.

**Science objectives for 2018-2019:**

- We will test the hypothesis that *RpaI* may be increasing electron flux through the tetrapyrrole pathway and the lower half of the TCA cycle to generate more succinyl-CoA, a key intermediate of tetrapyrrole biosynthesis, under low light. We will test this hypothesis by determining the amount of △-ketoglutarate and succinyl-CoA in cells grown under high light intensities versus low light intensities. We will also determine how light intensity affects *in vivo* activity of isocitrate lyase and malate synthase, and how the redox state of these proteins affects their *in vitro* activity.

- We have made amino acid substitutions of cysteines in metabolic enzymes such as Rubisco and in proteins associated with nitrogen fixation. We will continue to determine the role of redox on regulating the activity of these proteins by looking at the activity of redox-insensitive variants of these enzymes under high light and low light intensity. For proteins associated with nitrogen fixation, we will determine if expression of redox-insensitive variants of these proteins affects nitrogenase activity.

- We will continue to explore the potential role of Trx and Grx in transmitting this redox redox signal by creating deletion mutants in each Trx, as well as double deletions in Trx and Grx, and determine their role in *RpaI* adaptation to low light.

**My scientific area(s) of expertise is/are:** molecular biology, bacterial genetics.

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

**Publications supported by this project 2017-2018:**

Session V
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 H-cluster assembly in the [FeFe]-hydrogenase. Our approach involves using physical biochemical methods to elucidate the reactions catalyzed by the radical SAM maturases HydE and HydG, to explicitly identify the products of these enzymes, and to determine how these products come together on the scaffold protein HydF. We made major strides in the past funding period towards these goals, by providing new insights into each of the three maturation enzymes while also laying the foundation needed to pursue understanding of the complex biological process of H-cluster maturation. It is clear that these three enzymes interact with one another, although the nature and timing of the interactions have yet to be determined. HydE and HydG synthesize small molecules (dithiomethylamine and the diatomics CO and CN−, respectively) that serve as ligands to the irons in the H-cluster. These ligands are synthesized and then transferred to HydF, where a precursor of the H-cluster is formed (Figure 1). This “loaded” HydF EG then interacts with the hydrogenase protein HydA to transfer the 2Fe subcluster to HydA. Our approach involves first developing an understanding of the structure and function of each of the three proteins in isolation, then studying how each protein impacts the others, and specifically whether cluster assembly intermediates can be formed on HydF when either HydE or HydG are omitted from the process, and finally incorporating studies of protein-protein interactions until a thorough understanding of H-cluster maturation is achieved.


• We have completed a spectroscopic and computational characterization of [2Fe] 2 using modified HydF EG expression/purification methods that yielded protein exhibiting well-resolved vibrational bands attributable to at least four Fe-CO and at least two Fe-CN− stretching modes that are substantially more intense than prior preparations. Our new spectral analysis shows the definitive absence of Fe-CO-Fe species in [2Fe] 2, and a lack of reactivity toward either exogenous CO or H2, indicating coordinatively saturated Fe(I) sites.
• We have shown that HydF binds a redox-active [2Fe-2S] cluster, in addition to a [4Fe-4S] cluster.
• We have shown that the [4Fe-4S] and [2Fe-2S] clusters of HydF are not in close proximity.
• We have used Mössbauer spectroscopy to more fully describe the iron-sulfur cluster speciation in HydF produced in different genetic backgrounds (i.e., in the presence and absence of HydE and HydG).
• We have used spectroscopic and biochemical approaches to examine the structure of the H-cluster assembly intermediates present on HydF when it is expressed in the presence of HydE alone or HydG alone.
• We have shown that HydG generates the organometallic intermediate Ω during the initial stages of catalysis, presumably as a precursor to the 5'-dAdo• that abstracts a hydrogen atom from substrate tyrosine.
• We have developed methods for optimal loading of HydG to include the “dangler” iron on the C-terminal cluster.
• We have shown that dangler-loaded HydG produces significant quantities of free CO, and that free CO production is reduced but not eliminated in the absence of the dangler iron. Our results point to a catalytic role in CO production for the dangler iron.
• The HydG variant H272A produces very little free CO, suggesting that H272, which sits near the C-terminal cluster, plays an important role in CO production.
• We have developed an in vitro assay for hydrogenase maturation, in which purified HydE, HydG, and HydF are used to activate HydA. This assay is being used to address specific questions regarding the functions of each of the three maturation enzymes during H-cluster assembly and hydrogenase activation.

Science objectives for 2018-2019:
• Characterize the cation binding site in HydE and its potential functional relevance.
• Use our in vitro maturation assay to probe potential substrates for HydE.
• Use our in vitro maturation assay to probe the functional state of HydG.
• Probe the source of the iron on HydF and HydA using Mössbauer spectroscopy.
• Characterize the cluster states in HydF loaded using the in vitro maturation assay.

My scientific area(s) of expertise is/are: Bioinorganic chemistry, iron-sulfur clusters in biology, biological radical reactions, radical SAM enzymes, EPR spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: protein-protein complex analysis and mass spectrometry.

Publications supported by this project 2016 – 2019:
Mechanism of Proton-Coupled Electron-Transfer by [FeFe]-hydrogenase in Coupling to Dynamic Photosynthetic Energy Transduction

David W. Mulder, Staff-Scientist, NREL
Paul W. King, Principal Investigator, NREL
Jacob H. Artz, Postdoctoral Research Associate, NREL
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Overall research goals:
The overall goal of this research project is to understand the role of H₂ catalysis and mechanism of proton-coupled electron-transfer (PCET) in mediating the dynamic proton-dependent electron flux of photosynthetic energy transduction. The algal [FeFe]-hydrogenase HydA1 from Chlamydomonas reinhardtii (CrHydA1) couples the reducing potential generated from the photosynthetic water splitting reaction to proton reduction through the reversible \(2e^- + 2H^+ \rightleftharpoons H_2\) reaction during transitions in light and O₂ availability. We have been studying the reaction chemistry of the catalytic H-cluster and how secondary protein interactions enable coordinated movement of electrons and protons to H₂ catalysis. We hope to reveal new insights on fundamental properties related to reaction kinetics, cofactor tuning, modulation of thermodynamics, and molecular interactions that function together to control the movement of electrons and protons over dynamic landscapes. The knowledge gained through these studies will establish a framework for understanding how the kinetics and reactivity of PCET reactions are modulated by environmental factors for efficient energy transformation and conversion.

Significant achievements (2016-2018):
- **Metal-hydride intermediate reveals extended protein interactions for rapid H₂ catalysis.** Using a combination of site-directed mutagenesis, H-D isotope FTIR, EPR, Mössbauer spectroscopy and DFT (collaboration with Alex Guo, Carnegie Mellon University), we determined the electronic state configuration of the H-cluster hydride (H_{hyd}) intermediate of [FeFe]-hydrogenase (Figure 1). The results gave new mechanistic information for how H₂ binding and proton transfer are linked to intramolecular electron-transfer at the catalytic cofactor. Shifted midpoint potentials of reduced and oxidized transitions of the H-cluster due to alteration of a nearby Cys to Ser residue revealed how protein secondary interactions tune H₂ reactivity and catalysis. Further characterization of the hydride species by Nuclear Resonance Vibrational Spectroscopy revealed an extended hydrogen bonding network between the hydride and protein.

![Figure 1. Catalytic H₂ reaction model for [FeFe]-hydrogenase and depiction of the H_{hyd} intermediate (center).](image)
environment, further demonstrating how PCET is actively tuned during catalysis. Together these findings show how the enzyme structure interacts with the catalytic metal site to control PCET reaction steps to enable fast, H₂ activation.

- **Electron-injection mechanism is linked to catalytic proton reduction.** The role of PCET in the initial H-cluster reduction step was investigated using light-driven reduction of [FeFe]-hydrogenase by nanoparticle mediated photo-excited electron transfer (collaboration with Gordana Dukovic, University of Colorado Boulder). Using rapid-scan FTIR, we determined the enthalpy of H_red formation to be 19 kJ mol⁻¹ with a 2.5-fold kinetic isotope effect, values that are consistent with a concerted PCET process. This foundation provides us with a model for investigating the role of PCET under catalysis with natural electron donors such as ferredoxin, with the hypothesis that this process will be tightly coupled to proton transfer.

- **Optimized geometry is critical for efficient PCET.** Several conflicting models have emerged regarding the geometries of reduced H-cluster intermediates from FTIR studies of CrHydA1 and pose significant implications in the mechanism of H₂ formation. From H/D isotope exchange and temperature annealing FTIR, we found that 1-electron (H_redH⁺) and 2-electron (H_red, H_hyd) reduced states maintain bridged μ-CO geometries by virtue of having low frequency μ-CO bands. A bridged H-cluster geometry affords entatic states (i.e., a rotated diiron site and open coordination on FeD) that support fast H₂ activation. More generally these findings show how metal-ligand geometries can favor specific electron- and proton-transfer steps to control substrate reactivity and limit unproductive side reactions.

Science objectives for 2018-2020:

- Establish the electron-transfer kinetics between CrHydA1 [FeFe]-hydrogenase with native redox partners (ferredoxin and flavodoxin) over different pH and electron flux conditions.
- Develop H₂ reaction mechanisms that describe PCET transitions at the H-cluster when coupled to external carriers and explore cofactor energetics of individual PT/ET steps through combined potentiometric, spectroscopic approaches.
- Determine the role of molecular tuning of the H-cluster with external redox donors to understand the underlying factors that control enzyme reactivity in varying electron and proton flux conditions for efficient energy conversion.

My scientific area(s) of expertise are: Biochemistry and biophysics of redox enzymes; EPR and infrared spectroscopy of electron transfer and catalysis mechanisms.

To take my project to the next level, my ideal collaborator would have expertise in: Theoretical and computational biophysics, electron-transfer reaction kinetics.

Publications supported by this project: (2016-1018):

Overall research goals:

This project focuses on biochemical and functional characterization of proteins encoded by the extended particulate methane monooxygenase (pMMO)/ammonia monooxygenase (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 DUF461. These four genes are coregulated with the pMMO genes in a copper-dependent fashion. CopC and DUF461 belong to periplasmic copper chaperone families, and CopD is a putative copper importer. PmoD does not belong to any known protein family and close homologs thereof are only found in methane and ammonia oxidizing bacteria. The research strategy combines genetic and biochemical approaches with spectroscopy, X-ray crystallography, and advanced biophysical tools.

Significant achievements (2016-2018):

- We discovered that the periplasmic domain of PmoD forms a copper center with optical and electron paramagnetic resonance (EPR) spectroscopic features similar to those of the CuA sites in cytochrome c oxidase and nitrous oxide reductase. Using size exclusion chromatography with multi-angle light scattering, we showed that the CuA species is formed by a homodimer.
- With our collaborators, we further characterized the CuA site by EPR, X-ray absorption, and paramagnetic NMR spectroscopies. These data are consistent with a short Cu-Cu interaction of 2.4 Å.
- We determined the 1.9 Å resolution crystal structure of a monomeric form of PmoD that does not contain the CuA site. The canonical CuA ligands and binding loop are not present. Instead, PmoD contains a cluster of cysteine, methionine, and histidine residues near its N-terminus.
- Using site-directed mutagenesis, we identified the ligands to the CuA site (Figure 1). Formation of a CuA site by two protein molecules and by this part of the protein fold differs from all previously characterized CuA sites, which are housed within a single cupredoxin domain.
- We disrupted the pmoD gene in the extended copy of the pmo operon in Methylosinus trichosporium OB3b and observed a significant growth defect under copper-replete conditions (Figure 2). Thus, PmoD is important for cell growth under pMMO-utilizing conditions.
- We conducted time-dependent, copper-responsive qPCR analysis of all the Methylosinus trichosporium OB3b pmoD genes as well as proteomic analysis of PmoD in membrane fractions from several methanotrophs. The combined results suggest that PmoD homologs play distinct roles in vivo.
We performed a comprehensive bioinformatic analysis of PmoD proteins, identifying several core sequence motifs and establishing correlations between these motifs and genomic neighborhoods. We showed that only PmoDs with a Cx7MxHxnC motif can form the CuA site (Figure 3).

Science objectives for 2018-2019:

- We will obtain a crystal structure of the CuA form of PmoD. We will complete ongoing studies of CuA site formation.
- We will determine whether the CuA site is directly linked to the growth defect in the ΔpmoD strain.
- We will characterize the wildtype and ΔpmoD strains to fully understand the ΔpmoD phenotype.
- We will prepare full-length PmoD, including the transmembrane helix, and address the question of its oligomerization state and ability to form the CuA site.
- We will study the other operon proteins.
- We will conduct protein-protein interaction, copper transfer, and activity studies to probe the function of PmoD.

My 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, microbial imaging/tomography

Publications supported by this project 2016-2018:

Session VI
**Nitrogenase Reduction of N$_2$ and CO$_2$**

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**Overall research goals:**  
The research objectives of this project are to reveal molecular level insights into the mechanism for how the enzyme nitrogenase catalyzes the reduction of N$_2$ to NH$_3$ and the reduction of CO$_2$ to formate, CO, and methane. 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 should provide foundational information about the assembly and reactivity of the complex metal clusters of nitrogenase, giving guidance to the design of next generation N$_2$ and CO$_2$ reduction catalysts.

**Significant achievements (2017-2018):**  
Progress over the last year has resulted in 10 publications (see below). We have made significant progress in understanding how the active site of nitrogenase, called FeMo-co, accumulates electrons and protons as Fe-hydrides (Fe-H'). Using cryo-photoactivation, we have gained critical new insights into the reactivity of these hydrides. These experimental studies have been complemented by high level calculations in collaboration with the Raugei group at PNNL, with this work recently accepted for publication in the *Proc. Natl. Acad. Sci. USA*. These studies reveal, for the first time, the energetics of H$_2$ release and N$_2$ binding steps at the active site.

In another advance, we discovered a way to connect the nitrogenase MoFe protein to an electrode for delivery of electrons. This is important because normally electrons must be delivered by the Fe protein at the cost of hydrolysis of ATP at a fixed potential and with the rate limiting step for the overall reaction in the Fe protein cycle. Using the new approach, we explored the mechanism of H$_2$ formation at the active site FeMo-cofactor. Complimented by DFT calculations with the PNNL group, we were able to propose a reaction pathway for H$_2$ formation by nitrogenase.

Over the last year, we have successfully purified and characterized the Fe-only nitrogenase. This nitrogenase is significant because it does not contain Mo at the active site, and thus provides a way to explore how Mo and the protein surrounding the active site control the reactivity toward substrate reduction. We demonstrate that the Fe-nitrogenase utilizes a reductive elimination mechanism for N$_2$ reduction, just like the Mo-nitrogenase, thus solidifying this mechanism across the two enzymes. Electrochemistry studies, however, reveal fundamental differences between the nitrogenases for CO$_2$ reduction, setting the stage for detailed mechanistic studies to understand what controls CO$_2$ reduction reaction pathways.

Finally, we have made significant progress in understanding key steps in the maturation and insertion of the active site metal clusters in Mo-nitrogenase. These studies are enabled by development of a Streptag purification system for nitrogenase that allows rapid purification of proteins over hours rather than days. We are now in a position to gain insights into the activation pathway and the roles of key protein partners.

**Science objectives for 2018-2019:**
- Develop kinetic models that describe the reactivity of the activated E₄ state of nitrogenase toward N₂, proton, and CO₂ reduction reactions.
- Trap intermediates during reduction of substrates on the Mo- and Fe-nitrogenases.
- Develop energy diagrams for substrate reduction reactions using high level QM calculations.
- Develop electrochemical methods that will provide a tool to directly measure electron transfer in nitrogenase and to use this tool to advance mechanistic understanding.
- Further characterize the Fe-only nitrogenase at the mechanistic level and initiate studies on the maturation of nitrogenase components in this system.
- Explore the protein factors involved in the delivery of Mo during the maturation of the MoFe protein.

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

To take my project to the next level, my ideal collaborator would have expertise in: Theory bounded by experimental observations.

Publications supported by this project 2017-2018:

Uncovering Novel Energy Paradigms in Sulfate-Reducing Bacteria and Methanogenic Archaea

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Overall research goals:
In 1978, discovery of sulfate reduction in oxic niches challenged the classical view that sulfate-reducing bacteria (SRB) are obligate anaerobes. Subsequently, SRB were shown to respire O₂ (1990) and grow aerobically with O₂ as the terminal electron acceptor (2016). We have discovered a novel cytochrome oxidase (Cox) in SRB and one of our goals is to determine its function. Independent of SRB, a longstanding dogma posits that O₂ is toxic to methanogenic archaea. However, it is at odds with the fact that methanogens thrive in O₂-rich environments. We have found that some methanogens encode Cox genes. Our second goal is to probe whether they are involved in energy conservation.

Significant achievements (2017-2018):

**SRB:** We have isolated and characterized a previously unknown membrane-integral metalloprotein supercomplex, which is comprised of formate dehydrogenase (FDH), undecaheme cytochrome c (uhc), and an unusual c-heme-dependent cytochrome bd oxidase (cbd). Using cytochrome oxidase-deficient SRB strains generated in Judy Wall’s laboratory, we have validated the biochemical function of FDH-uhc-cbd supercomplex (Fdh-uhc-cbd). Similarly, we have probed the native expression of this supercomplex via chromosomal gene tagging. At a minimum, the heteropentameric FDH-uhc-cbd retains 18 transmembrane helices, four [4Fe-4S] clusters, molybdopterin, selenocysteine, twelve c-hemes, and one each of b- and d-hemes. Through a combination of heterologous and homologous expression, electronic spectroscopy, assay development, oxygen electrode measurements, and inhibitor studies, we have established that the supercomplex is highly active under aerobic conditions. It is also self-sufficient in utilizing the electrons derived from formate oxidation (HCOO⁻ ↔ CO₂ + 2e⁻ + H⁺; $E_m,7 = -420$ mV) towards O₂ reduction (O₂ + 4H⁺ + 4e⁻ → 2H₂O; $E_m,7 = +860$ mV). Neither superoxide nor hydrogen peroxide are generated during this process, indicating a four-electron reduction of O₂ to water. In the absence of FDH and uhc, the cbd heterodimer fails to reduce O₂. Surrogate electron donors (ascorbate / N,N,N′,N′-tetramethyl-p-phenylenediamine) are also inefficient in transferring electrons to cbd when FDH and uhc are unavailable. Notably, our physiological data demonstrate that the FDH investigated here is incapable of supplying electrons for sulfate reduction. Phylogenetic comparison of the FDH-uhc-cbd systems reveals new ways of evolving redox biochemistry in the context of disparate macromolecular assemblies. Implications: (a) Unlike eukaryotes, bacteria harbor respiratory chains that facilitate the utilization of alternative terminal electron acceptors, such as nitrate, nitrite, trimethylamino-N-oxide, and fumarate when O₂ is limiting. Bacterial energy conservation mechanisms effectively link the reduction of these respiratory substrates to the oxidation of electron donors, such as formate and H₂. The FDH-nitrate reductase respiratory chain of *E. coli* serves as a paradigm for proton motive force (PMF) redox loop in which electrons derived from formate oxidation are transferred to the nitrate reductase via the menaquinone pool. We advance a new paradigm for energy conservation in SRB, where formate oxidation is coupled to O₂ reduction by an unprecedented FDH-uhc-cbd supercomplex, poised to generate a transmembrane PMF without involving the quinone pool or soluble cytochromes c; (b) Given that aerobic formate metabolism is enigmatic even in well-studied model systems, such as *Escherichia coli*, our discovery of an oxygen-insensitive selenocysteine FDH in a “strict” anaerobe has broad implications for understanding O₂ respiration, as well as for the development of innovative biocatalysts; (c) Prevailing wisdom posits the existence of functionally redundant FDHs in SRB with a proclivity for H₂ generation from formate (via hydrogenases; in the absence of sulfate) or channeling reducing
equivalents for sulfate reduction (via soluble periplasmic cytochromes c and the menaquinone pool). However, our data supports neofunctionalization of at least one of the FDHs to achieve direct O2 reduction; (d) Poor abundance of the supercomplex during SRB growth on various electron acceptors argues against a function in O2 detoxification.

**Methanosarcinales:** We have identified conditions under which cytochrome bd oxidase (CydAB) is naturally expressed in different *M. barkeri* strains and the aerotolerant obligately H2-dependent methylotroph *Methanimonococcus blatticola*. CydAB is highly abundant in the latter, but not during methanogenic growth. CydAB-enriched *Methanosarcina* membranes display characteristic spectral signatures of b and d hemes, analogous to those previously reported for CydAB from Gram-positive *Bacilli*. We have also homologously expressed and characterized *M. acetivorans* CydAB (expression strain generated in the laboratory of Arpita Bose), which when purified aerobically yields a heterodimeric d-heme-containing integral membrane protein (17 transmembrane helices). More importantly, it couples menaquinone oxidation to O2 reduction. Its spectroscopic properties are virtually indistinguishable from detergent-solubilized native *M. barkeri* or *M. blatticola* CydAB. Heterologously expressed (in *E. coli* strain lacking native CydAB) *M. barkeri* CydAB purifies with an oxyferrous d-heme, underscoring its high affinity for O2. The redox potential of the b and d hemes of *Methanosarcina* CydAB are on par with bacterial counterparts. Implications: (a) This is the first investigation of an archaeal CydAB. 40 years ago, Gottschalk’s laboratory searched for d-heme signal in *M. barkeri*, but did not find it. Using alternative strategies, we have successfully identified d-heme-containing CydAB in both *M. barkeri* and *M. blatticola* membranes; (b) Our physiological findings suggest that *Methanosarcina* CydAB does not function in O2 detoxification; (c) It is widely held that *Methanosarcina* will cease to grow via methanogenesis unless the redox potential of the anoxic growth medium is below -300 mV. Moreover, cytochrome-containing methanogenic archaea lack quinones – instead, they utilize methanophenazine (MP/MPH2, Em,7 = -165 mV) as redox cofactor. Consequently, it is surprising that a menaquinol-oxidizing (Em,7 = -80 mV) O2 reductase is expressed in *Methanosarcina*. However, Conrad’s observations that methane production by *M. barkeri* remained unaffected when redox potential of the anoxic medium was varied between -80 mV and +420 mV raise the possibility that exogenous quinones could potentially substitute for MP under certain conditions. Because very little is known about how extracellular electron acceptors modulate *Methanosarcina* physiology (Bond and Lovley), our work has the potential to shed light on the versatility of cytochrome-containing methanogens vis-à-vis oxygen metabolism; (d) Based on these and other observations, we propose a provocative new hypothesis for *Methanosarcina* CydAB function, which has broad relevance to other energy-starved bacterial and archaeal systems.

**Science objectives for 2018-2019:**
(a) Study the biochemistry and physiology of novel non-CydAB systems in Methanosarcinales; (b) Investigate structure-function relationships of *Methanosarcina* CydAB; (c) Explore the potential role of quinones and O2 in Methanosarcinales; and (d) Assess the bioenergetics of SRB FDH-uhc-cbd

My scientific areas of expertise are: redox biochemistry, membrane-integral metalloprotein complexes, structural biology

To take my project to the next level, my ideal collaborator would have expertise in: anaerobic methane oxidation and ANME, syntrophic consortia, interspecies electron transfer, genetics/physiology of sulfate-reducing bacteria.

**Publications supported by this project:** Manuscript in submission; New award funded September 1, 2017.

Electron Transport in Methanogenesis and Reverse Methanogenesis by *Methanosarcina acetivorans*

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**Overall research goals:**  
*Methanosarcina acetivorans* is a marine methanogen capable of acetotrophic methanogenesis and also the anaerobic oxidation of methane by reversal of acetotrophic and carbon dioxide reducing methanogenic pathways. The overall objective is to advance the understanding of electron transport pathways and mechanisms of electron transport proteins that impact energy conservation, response to oxidative stress and interactions with partner microorganisms.

**Significant achievements (2017-18):**  
An electron bifurcating heterodisulfide reductase (HdrA₂B₂C₂), up regulated in acetate- vs. methanol-grown cells, oxidizes reduced coenzyme F₄₂₀ (F₄₂₀H₂) (\(E_m = -380\) mV) and reduces ferredoxin (Fdx) (\(E_m = -520\) mV). The endothermic electron transfer is driven by reduction of the heterodisulfide of coenzyme M and coenzyme B (CoMS-SCoB) (\(E_m = -140\) mV). Physiological roles for HdrA₂B₂C₂ in pathways of methanogenesis and reverse methanogenesis are proposed. Collaborations have been initiated to decipher intramolecular electron transfer in HdrA₂B₂C₂. Fe(III)-dependent respiration plays roles in acetotrophic methanogenesis and reverse methanogenesis for which the understanding of electron transport and energy conservation has been advanced. Specific achievements follow:

1. Established a role for cytochrome c and a flavodoxin in respiratory metabolism of *M. acetivorans*.
2. Completed characterization of an unusual flavodoxin from *M. acetivorans* stabilizing an anionic semiquinone and documented a role for the flavodoxin in reduction of the ferredoxin:disulfide reductase that contributes to the oxidative stress response.
3. In collaboration with Paul King at NREL, further characterized transient absorption (TAS) spectra of the flavin in the HdrA₂ subunit of HdrA₂B₂C₂. The result lays a foundation for further characterization to elucidate the mechanism.
4. Entered collaborations with Yisong (Alex) Guo at Carnegie-Mellon University and Paul King at NREL investigating the electronic structure of the catalytic HdrB₂ subunit of HdrA₂B₂C₂. \(^{57}\)Fe-enriched enzyme was generated and now in the hands of the collaborators. Preliminary results will be presented.
5. Continued collaborations with Evert Duin and John Golbeck to characterize the two Fe/S clusters in HdrB₂ addressed by EPR spectroscopy of wild-type and variants. Preliminary results indicate that the clusters are non-identical.
6. Plasmids have been constructed for heterologous production of formate dehydrogenase and hydrogenase from *Methanosaeta thermophila*.

**Science objectives for 2018-19:**  
Continue collaborations to investigate intramolecular electron transport and the mechanism of electron bifurcation in HdrA₂B₂C₂. Further, we will express and characterize formate
dehydrogenase and hydrogenase from *M. thermophila* to provide the foundation to determine the physiological role in co-culture with *Geobacter* spp.

To take my project to the next level, my ideal collaborator would have expertise in:
EPR, Mössbauer and transient absorption spectroscopies.

Publications supported by this project:


Doing great science in the lab and publishing it in professional journals is only one side of the science communications coin – albeit a very important one. But the other side to this coin – and an increasingly important one – is the need to more effectively communicate your science to non-specialists as well.

In our talk we will introduce you to the numerous channels that the DOE Office Of Science Communications has available to help publicize the important work you do, why communicating science to non-specialists matters, tools and resources that are available to help you communicate your science, and we’ll close by pointing out some of the important ingredients that go into a good communications piece.
Poster Session II
Engineering a Functional Equivalent of Nitrogenase for Mechanistic Investigations of Ammonia Synthesis

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Overall research goals:
The overarching goals of this project are (a) to generate an active nitrogenase equivalent on a NifEN template via an ‘add-on approach’ and (b) to construct VnfDGK- and NifDK-based hybrid systems with modified protein domains or cofactor components via a ‘domain/cofactor swapping approach’. We hope that a combination of these approaches will (1) enable identification of the functional determinants for the catalytic activity of nitrogenase; (2) provide a proof-of-concept for minimizing/simplifying the essential nif gene set for engineering a metabolic pathway of nitrogenase assembly and function; and (3) facilitate capture of substrates/intermediates or permit identification of candidates with desired product profiles of nitrogenase reactions. Success along this line of efforts will contribute to a better understanding of the mechanism of ammonia synthesis by nitrogenase.

Significant achievements (2016-2018):
NifEN, a scaffold protein that hosts the biosynthesis of the M-cluster (the cofactor of Mo-nitrogenase), can be viewed as a simplified, functional homolog of NifDK (the catalytic component of Mo-nitrogenase). Our project aims at generating a functional MoFe protein equivalent by sequentially reconstructing a P-cluster site, duplicating an M-cluster site and re-establishing proton gating residues in NifEN. Additionally, functional variants of nitrogenase with altered activities and/or product profiles will be generated by mixing-and-matching the reconstructions of the key catalytic features in NifEN. Using the Azotobacter vinelandii NifEN protein (designated NifENAv) as a mutational template, we started out with the systematic reconstruction of a functional MoFe protein homolog. In parallel, we screened through a wide range of NifEN homologs from other nitrogen-fixing organisms that already has crucial structural/catalytic elements, such as the P- and/or M-cluster ligands, in place to minimize the amount of genetic handling. Excitingly, we have identified NifEN from Methanosarcina acetivorans (designated NifENMa) and Desulfovibrio vulgaris (NifENDv) as two additional promising candidates for our proposed work. Contrary to NifENAv, which contains an incomplete set of P-cluster ligands that only allows coordination of one [Fe4S4] cluster, both NifENMa and NifENDv has a complete set of P-cluster ligands for the ligation of two [Fe4S4] clusters (i.e., a P-cluster precursor) at the P-cluster site. Moreover, both proteins expressed well heterologously, which allowed purification of these proteins at high yields. Currently, we are focusing on restoring the P-cluster (i.e., via fusion of the [Fe4S4] cluster pair) in NifENMa and NifENDv and attaching the M-cluster to these proteins in order to tailor the NifEN template further toward a functional equivalent of MoFe protein.

Other than the NifEN-based work, recently, we generated MoFe and VFe proteins containing citrate-substituted cofactors (designated NifDKCit and VnfDGKCit) by combining the mutational approach with strategic variations of growth conditions. Compared to their respective native counterparts (NifDK and VnfDK), both citrate-substituted variants show (a) a decrease of the cofactor-specific S=3/2 EPR feature that is indicative of the presence of the citrate-substituted cofactors in a
higher oxidation state than the native, homocitrate-containing cofactors; and (b) an increase of the P-cluster-specific $S=1/2$ EPR feature that is indicative of a further reduction of the P-clusters in these proteins relative to their respective native counterparts. Surprisingly, when N$_2$ is supplied as a substrate, VnfDGK$^{\text{Cit}}$ generates NH$_3$ and H$_2$ at a substantially increased ratio of NH$_3$/H$_2$, pointing to a significant shift of electrons towards the formation of ammonia. To our knowledge, this is the first and only example for such a shift towards ammonia formation by any nitrogenase variant reported so far. In this light, it is interesting to note the subtle variations of the cofactor ligand and homocitrate anchor in VnfDGK as compared to those in NifDK, which could account for the unique catalytic behavior of VnfDGK$^{\text{Cit}}$. This exciting finding opens a new avenue for us to target the key features of the cofactor site that contribute to the N$_2$-reducing activity.

Science objectives for 2018-2019:

- Restoring the P-cluster in the available NifEN homologs, as well as attaching the M-cluster to these proteins. We expect to see a stepwise increases of electron flux and, possibly, a restoration of some N$_2$-reducing activity in one of these modified systems.
- Performing computational analysis in order to account for the observed shift towards ammonia formation by VnfDGK$^{\text{Cit}}$.

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

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

Publication related to this project:

Tuning Hydrocarbon Formation by Vanadium Nitrogenase via a Hybrid Approach

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Yilin Hu, Co-PI(s)
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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. The project aims at tuning 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 (2016-2018):
Compared to the Mo-nitrogenase, the V-nitrogenase is only ~50% active in N₂ reduction; however, it is ~700-fold more active than its Mo-counterpart in the in vitro reaction of CO reduction. Consistent with this observation, an A. vinelandii strain that expresses the V-nitrogenase can reduce CO to up to C₃ hydrocarbons under in vivo growth conditions, a trait that is absent from the A. vinelandii strain that expresses the Mo-nitrogenase. The fact that CO is a very poor substrate (and conversely, a good inhibitor) of Mo-nitrogenase has facilitated capture of a CO-inhibited conformation of Mo-nitrogenase, in which a ‘belt’ sulfide of the M-cluster is displaced by a CO moiety that is bridged between a pair of Fe atoms. By analogy, CO could be bound to the V-cluster in a similar configuration; only in this case, CO is activated and can be subsequently turned over into hydrocarbon products. Apart from allowing structural analogy to be drawn on CO binding, the observed similarity and discrepancy between the reactivities of Mo- and V-nitrogenases toward CO can be used to assess the impact of different components on CO reduction via mixing-and-matching of features of the two nitrogenases or introduction of new features into these systems. Taking advantage of this approach, our work in the last funding period has led to the identification of the minimum requirement for the CO reactivity of nitrogenase, as well as the characterization of nitrogenase hybrids with altered electron and/or proton fluxes that could be used to tune product profiles of CO reduction and provide mechanistic insights into the reaction pathway.

Science objectives for 2018-2020:
In the current funding period, we plan to use a hybrid approach to tune the formation of hydrocarbons from CO reduction by V-nitrogenase. Specifically, we will generate nitrogenase hybrids that either contain cofactors with altered heterometals and organic compounds or comprise a mismatched electron donor to regulate electron and proton flows through the enzyme and push the CO reduction toward the release or trapping of oxygenated intermediates. The identities of oxygenated intermediates of this process have remained elusive so far. Information in this regard will provide important insights into the reaction mechanism of nitrogenase-catalyzed CO reduction, which could prove instrumental in future development of strategies to recycle carbon wastes (e.g., CO and CO₂).
into useful carbon fuels. In particular, knowledge of the identities and distributions of the oxygenates and how they are altered upon changes in electron and/or proton flows will not only enable us to piece together a reaction pathway of CO reduction, but also shed light on selective productions of oxygenates, which are valuable chemical commodities for fuel industries. In light of our discovery in the last funding period that whole cells of \textit{A. vinelandii} can release hydrocarbon products during cell growth at reasonable yields, success in developing strategies for whole-cell production of oxygenates seems to be within reachable distances.

My 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

Publication related to this project (2016-2018):


Hyperthermophilic Multiprotein Complexes and Pathways for Energy Conservation and Catalysis

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Overall research goals: This research specifically addresses several of the overall goals of the Physical Biosciences Program. 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. Moreover, 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. This is particularly relevant to the DOE mission since a fundamental problem in all photosynthetic reaction systems is the conversion of low potential reductant to a useable form of energy such as an ion motive force.

Significant achievements [2017-2018]: 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 (S°) to H₂S gas. It also has a respiratory metabolism in which it couples H₂ production by a ferredoxin-dependent, 14-subunit membrane-bound hydrogenase (MBH) to ion translocation and formation of a membrane potential that Pfu utilizes to synthesize ATP. Addition of S° to Pfu prevents the synthesis of MBH and induces the synthesis of a highly homologous 13-subunit membrane-bound complex termed MBX. MBX is proposed to oxidize ferredoxin but how it is involved in S° reduction was not known. A significant achievement in the prior funding period was the purification of MBX and the demonstration that it is a sulfane sulfur reductase. It catalyzes the reduction of internal S-S bonds in polysulfides (S₂⁻, n = 4-9) but does not itself generate H₂S directly. In addition, we determined the structure of MBX to 4.4 Å and of MBH to 3.3 Å by cryo-EM (with Huilin Li, Vandel Research Institute). For MBH a detailed structure-based mechanism was proposed for how electron transfer and catalysis lead to the conservation of energy by the formation of a sodium ion gradient. MBX retains several, although not all, of the structural features thought to be involved in energy transduction in MBH. MBX contains an additional ion pumping module not present in MBH that is thought to enable polysulfide reduction by MBX to conserve more energy than proton reduction by MBH. In addition, MBH and MBX are close relatives of modern day respiratory complexes such as Complex I of the ubiquitous aerobic respiratory chain of mitochondria. The structure of Complex I is available but how it couples electron transfer to energy conservation is controversial. Understanding how these “ancestral” MBH and MBX complexes of Pfu conserve energy is therefore of fundamental significance.

In the prior funding period we also extensively characterized the Pfu redox regulator SurR and showed that it is the master control for the primary electron flow pathways leading to H₂ and H₂S production by Pfu. In addition, we showed that the gene in Pfu that is the most up-regulated by S° encodes a 19 kDa protein termed IssA for iron-sulfur storage protein A. IssA has sequence similarity to proteins involved in the biosynthesis of the nitrogenase MoFe cluster. Pfu stores iron and sulfur in IssA as thioferrate, an inorganic anionic polymer previously unknown in biology. IssA forms nanoparticles reaching 300 nm in diameter and is the largest natural metalloprotein complex known. Purified nanoparticles appear to be generated from 20 nm units containing ~6,400 Fe atoms and ~170 IssA monomers. In support of a role in FeS storage, IssA reconstituted the 4Fe-cluster in Pfu Fd in vitro.
Science objectives [2019-2020]: The overall goal of our research is to elucidate the mechanisms of energy conservation by MBH and MBX at the atomic level. The fundamental question is, how does electron transfer and catalysis lead to ion pumping? In addition, we will characterize related energy-conserving respiratory complexes that oxidize carbon monoxide and formate and evolve H₂. We will also identify novel genes essential for the assembly and function of MBH and MBX. These goals will be achieved utilizing gene and site-directed mutations of these respiratory systems, up to 600-liter fermentations of *Pfu*, anaerobic purification of cytoplasmic and membrane sub-complexes, and various physiological, biochemical, kinetic and spectroscopic analyses. This project also leverages both on-going and new collaborations involving metabolic reconstruction modeling, sulfur-based metabolomics, and random barcoding transposon-site sequencing (RB-TnSeq) mutagenesis.

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

To take my project to the next level, my ideal collaborator would have expertise in: biochemistry of complex I and its mechanism of energy transduction

Publications supported by this project 2017-2018:


Overall research goals:
The long-term vision of the PNNL Enzymatic Energy Conversion program is to provide 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 substrate for selective conversions and allosteric regulation. Over the past few years, we focused on understanding the energetics of electron and proton delivery in the metalloenzyme nitrogenase (Figure 1).

Significant achievements (2016-2018):
We carried out an extensive quantum chemical study of N2 activation by nitrogenase. Binding and activation of N2 takes place at the E4 state, which is activated by the accumulation of 4e-/H+, with the concomitant reductive elimination of H2. Our results suggest that at ambient temperature, various low-lying E4 isomers coexist (Figure 2, left). In particular, the lowest in energy, E4(4H), has two [Fe-H-Fe] bridging hydrides consistent with low-temperature ENDOR measurements by Hoffman (Northwestern University). A second relevant low-lying E4 state is the dihydrogen species E4(2H;H2), which features a strongly activated di-hydrogen located on Fe2, and illuminates our recent finding that an H2 complex forms during reductive elimination (re) of the hydrides of E4(4H). Notably, we found a nearly isoergic equilibrium, in which the loss of H2 is coupled to N2 binding and the possible formation of a diazene-level intermediate, in close agreement with the recent direct measurement of this equilibrium. In this process, E4(4H) undergoes reductive formation of H2 with formation

![Figure 1](image1.png)

**Figure 1.** Nitrogenase proteins and cofactors. Shown are the Fe proteins and the MoFe protein along with two ATP molecules, the [4Fe–4S] cluster of the Fe protein, the P-cluster, and the FeMo-co of the MoFe protein.

![Figure 2](image2.png)

**Figure 2.** Relative free energy of various E4 species (left) potentially involved in FeMo-co reductive activation (re) of H2 and the breaking of the N2 triple bond (right). Protic and hydridic hydrogens are indicated in green and red, respectively.
of the di-hydrogen adduct $E_6(2H;H_2)$; this binds $N_2$ to form $E_6(2H;N_2)$ with the concomitant elimination of $H_2$, and subsequently to the diazene $E_6(NHNH)$ intermediate (Figure 2, right hand side of the equilibrium arrows).

It is possible to achieve a profound understanding of the thermodynamic necessity of coupling $H_2$ formation via $r_e$ to cleavage of the $N≡N$ triple bond by looking at the overall process for the formation of any of the diazene-level intermediates from $E_6$. Focusing just on the formation of $E_6(NHNH)$, which requires the consumption of four ATP, the overall process is exergonic by $\Delta G_{E_6} = -103$ kJ/mol at pH = 7. What makes this process so favorable can be understood by decomposing it into the two parts: (1) the endergonic formation of $E_6(NHNH)$ from $E_0$, $N_2$, $2H^+$, and $2e^-$, provided by the Fe protein and the hydrolysis of four ATP, with $\Delta G_{E_0} = +67$ kJ/mol, and (2) the exergonic formation of $H_2$ from $2H^+$, and $2e^-$, again provided by the Fe protein with ATP hydrolysis, with $\Delta G_{H_2} = -170$ kJ/mol. Thus, although direct formation of $E_6(NHNH)$ without generation of $H_2$ is thermodynamically unfavorable, nitrogenase couples this unfavorable reaction of $N_2$ with a highly favorable one, generation of $H_2$, resulting in an overall exergonic process to drive $N_2$ fixation.

Science objectives for 2018-2020:
In the next funding period, we will expand our focus to other enzymes, leveraging and enhancing the current state of knowledge collected within the DOE Physical Biosciences experimental portfolio. As before, nitrogenase will represent our prime test bed for investigating various critical biological activities. At the same time, we will study the interplay between electrochemical and mechanical energy in the hydrogen gas-evolving membrane-bound hydrogenase complex, how methyl-coenzyme M reductase and monoterpene synthase are able to selectively manipulate extremely reactive substrates (a methyl radical and carbocations, respectively) and how desaturase selectively dehydrogenate hydrocarbon chains.

My scientific area(s) of expertise is/are: Theoretical and computational biophysics.

To take my project to the next level, my ideal collaborators would have expertise in: experimental characterization of enzymatic mechanisms and protein redesign. In this regard, we are already fruitfully collaborating with John Schanklin (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 2016-2018:
Tuning Directionality for CO$_2$ Reduction in the Oxo-acid:ferredoxin oxidoreductase superfamily

Sean J Elliott, Principal Investigator

Madeline López-Muñoz, Postdoctoral Research Associate

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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 CO$_2$. 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 CO$_2$ 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 (2016-2019):
• Over the past two-year period we have focused on studies of the pairing of Fd proteins with OFOR enzymes of varying reactivity. In doing so, we have characterized the reactivity of the a reverse TCA-cycle bearing 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. Our structural studies show how domain rearrangements and second-layer binding interactions are conserved across the OFOR family, allowing for future studies of the tuning/biasing of an OFOR to participate in reductive versus oxidative catalysis.

• We have examined the reactivity of Mm OGOR with the potential native Fd proteins as well, demonstrating that the three different Mm Fds that are potential candidates for supporting the rTCA cycle are not equivalent in their reactivity, and kinetic support of CO$_2$ reduction and the measured redox potentials of the three 2x[4Fe-4S]-bearing Fds suggest that Mm Fd1 is the native redox partner for KorAB.

• Concerning the reactivity of Fd proteins as a variable unit, we have completed our study of the influence of redox potential of a consistent Fd upon the ability to support electrocatalytic oxidative and reductive chemistry of a given OFOR. In this study, we have shown that a single Fd can possess a range of redox potentials spanning 0.5 V through generation of single or double site-directed mutagenesis. The resulting small library of Fd from either Hydrogenobacter thermophilum (Ht) or Desulfovibrio africanus (Da) organisms resulting a range of redox potentials where the structural elements of each Fd is the same aside from singular or double mutations. This has allowed us to minimize the contribution of protein-protein interactions that might contribute to OFOR catalysis, and probe how CO$_2$ reduction reaches a maximal bias not at infinite driving forces, but at a specified range of reductive redox potentials.
Direct visualization of redox potentials of the three Fe-S clusters of *Chlorobium tepidum* and *Moorella thermoaceticum* PFOR enzymes for the first time by direct protein-film electrochemistry. We are now in a position to finally address specific molecular questions about the protein environment controls the redox potentials of the [4Fe-4S] clusters of canonical OFOR enzymes. As will be presented, thus far we still cannot engage *Mm* or *Ht* KorAB at an electrode, which we can now rationalize on the basis of steric occlusion that occurs when a “single-cluster” OFOR has evolutionarily lost the typical Fd-domain that houses the two additional FeS clusters found in canonical OFORs.

Finally, we have been successful in our collaboration with Prof. Gordana Dukovic at University of Colorado, Boulder, in docking *Mm* KorAB with nanoparticles to develop a light-induced process for capture of CO₂ and the generation of products.

Science objectives for 2018-2019:

- Pre-steady-state kinetic analyses of the KorAB enzyme electron transfer reactions, in the presence of oxidative and reductive substrates.
- Mapping of the observed redox potentials of PFORs onto the three [4Fe-4S] cluster
- Deploying the mutated Fds into a synthetic biology format to examine the impact upon a model system (such as *E. coli*)
- Complete biochemical and biophysical characterization of the second rTCA cycle enzyme, PorAB, which is presumed to act as a pyruvate synthetase, but unlike KorAB, possesses all 3 of the typical Fe-S clusters.

My scientific area(s) of expertise is/are: Metallobiochemistry and Electrochemistry.

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 microorganisms.

Publications supported by this project [2016-2019]:

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, Camelina sativa) 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.

To improve our understanding of genetic control and the structure of biochemical pathways in oil synthesis, a bioinformatics approach was developed for identification of cis-regulatory elements in gene promoters across multiple plant genomes (Phylogenetic Footprinting). This allowed to identify conserved putative binding sites for seed specific transcription factors.

Science objectives for 2018–2020:

- We have the capability to assess metabolic flux in central metabolism during seed development based on stable isotope tracer experiments. Several transgenic oil crop lines affecting oil level and quality will be biochemically characterized in order to reveal the impact of modifications in central metabolism genes on carbon partitioning and regulation of the associated biochemical processes.

- Our previously published work indicated an allosteric feedback mechanism to be involved in the control of metabolic flux through glycolytic steps during oil synthesis in developing seeds. To further study this mechanism, we have identified glycolytic enzymes from non-plant organisms with minimal allosteric control and well characterized biochemical properties. Synthetic genes have been generated and will be expressed oilseed crops under control of chemical induction.

- The control of single enzyme steps over flux through the oil synthesis pathway will be assessed by use of genetic constructs that allow gradual increase of gene expression based on chemical inducible promoters. For this purpose, the dose response behavior and potential side effects of the chemical inducers have to be carefully characterized.

- We will establish protocols for experimental Metabolic Control Analysis. Single enzymes are manipulated in a controlled manner by chemical induction. Then the response in enzyme activities, metabolic flux and metabolite levels is to be quantitatively assessed.
My 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: [Click to Enter fields, areas of study, knowledge of an individual or group of individuals to enhance your current grant/FWP.].

Publications supported by this project 2016-2018:
Mechanism of Photochemical N2 Reduction

Paul W. King, Principal Investigator
Gordana Dukovic, Yisong Guo, John W. Peters, and Lance Seefeldt, Co-PI(s)
Katherine A. Brown, and David W. Mulder, Staff Scientists

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Overall research goals:
The activation and reduction of dinitrogen (N2) to ammonia (NH3) catalyzed by nitrogenase is one of the most energy demanding and difficult chemical reactions. Nitrogenase catalyzes this chemical transformation by coupling ATP hydrolysis by the Fe protein to drive the sequential delivery of electrons to the MoFe protein. We recently demonstrated that light-harvesting and photoexcited electron transfer by semiconducting nanocrystals can be coupled to nitrogenase to accomplish light-driven N2 reduction. This molecular system enables innovative research on the mechanisms of biological nitrogen reduction not previously possible. In this work, nanocrystalline materials will be used to photochemically activate electron transfer into MoFe protein, in lieu of ATP-dependent electron transfer by the nitrogenase Fe protein. The extraordinary light-harvesting properties and tunability 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 photochemistry of nanocrystals with nitrogenase will further enable the development and use of light-driven biophysical techniques to further resolve and address key structural and functional questions of the MoFe protein catalytic mechanism. The ability to synchronize electron injection combined with tuning of photochemical potential will enable profound new insights into the mechanisms that control electron transfer and catalysis and a deeper understanding of the energetic and kinetic requirements for achieving difficult, energy demanding, multi-electron chemical transformations.

Significant achievements (2018-2020):
• Defining how nanocrystal dimensions affect the control of molecular complex formation and electron flow into nitrogenase. The photoexcited state properties of nanocrystals are sensitive to the nanocrystal dimensions (physical), surface properties and chemical compositions. Through subtle changes in the synthesis, the photochemical properties and surface chemistry of nanocrystals can be adjusted to measure the specific effects of driving force (Figure on the left), donor-acceptor distances, and molecular orientations of nanocrystal-nitrogenase complexes on the intermolecular electron transfer kinetics. We have observed that the chemistry of the reaction mixtures that effect surfaces and the binding interface also effect electron transfer kinetics,
binding interactions between nitrogenase and nanocrystals, and the photochemical reaction
chemistry. These results are providing insights on the requirements for nitrogenase photochemistry
and how the electron transfer kinetics and quantum yields are influenced by nanocrystal dimensions.

- **Establishing the mechanism of intermolecular and intramolecular electron transfer from nanocrystals into nitrogenase MoFe protein.** We are addressing how nanocrystal-nitrogenase complexes accomplish photoexcited electron transfer (see Figure) using a combination of physical and kinetic measurements to establish the nature of the complexes, the physical interface, and the kinetics of electron transfer. Biological electron transfer complex involves Fe protein binding and ATP hydrolysis is coupled to N_2 activation and reduction by MoFe protein (On the left, top Figure). The mechanistic models that detail this process implicate a role for structural changes in the P cluster that arise during formation of an Fe-protein-nitrogenase complex summarized as a “deficit spending model” of electron transfer. Initial EPR studies of nanocrystal-nitrogenase complexes suggest that certain aspects of the biological process are “mimicked” by nanocrystals to drive electron injection (On the left, bottom Figure). One way we are testing this model is by using nitrogenase P cluster variants that poise the P cluster in a pre-formed P\(^+\) oxidation state, which is competent for photoreduction by the nanocrystal, and following redox transitions using EPR under illumination.

**Science objectives for 2019-2020:**

- **Measuring the effect of photochemical potential (driving force) and flux on the kinetics of electron transfer into nitrogenase MoFe protein.** We are initiating studies on the use of quantum dots to vary the driving force for electron transfer in nitrogenase complexes in combination with transient absorption measurements to quantify the effect on both the rate constants and yields of electron transfer.

- **Light pulse-controlled of electron transfer to capture MoFe protein catalytic intermediates for EPR analysis.** We will continue to evolve biophysical studies on the process of intermolecular electron transfer to address the mechanism of photoexcited electron transfer, and how this influences the intramolecular events for electron transfer to the Fe-Moco site, and catalytic reactivity.

- **Photochemical flux control of catalytic product formation by nanocrystal:nitrogenase complexes.** Using the insights from the driving force and EPR studies of electron transfer in nanocrystal:nitrogenase complexes we will measure the rates, and yields of reaction products (ammonia, H\(_2\), and other reaction substrates) to address questions on the effects of photochemical flux and potential of the reaction mechanism of nitrogenase.

My scientific area(s) of expertise is/are: Redox biochemistry; physical biochemistry; photochemistry of redox enzymes.

To take my project to the next level, my ideal collaborator would have expertise in: Theory of redox enzyme catalysis and electron transfer.
Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosystem II

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Overall research goals:
Photosystem II (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. This project focuses on key deficits in our current understanding of the PSII reaction center (PSII RC). The project involves the development of new spectroscopic methods, and their application to address the following open questions:

1) What is the electronic structure of the PSII RC?
2) What are the charge separation pathways in the PSII RC?
3) Do key pigment and/or protein dynamics enhance energy transfer and charge separation in the PSII RC?

Significant achievements (2016-2018):
In this grant period we have used a combined approach of developing new spectroscopic measurements and using the new approaches to build on our extensive dataset to address the open questions that we have identified in the PSII RC. The new methods include 2D electronic spectroscopy (2DES) experiments with a continuum probe, 2D electronic Stark spectroscopy\(^1\), and 2D electronic vibrational (2DEV) spectroscopy. We have continued to study the isolated D1D2 PSII RC and have also examined the larger PSII core complex. To uncover the charge-transfer states involved in the charge-separation in the PSII RC we developed 2D electronic Stark spectroscopy (2DESS) and transient-grating Stark spectroscopy (TGSS)\(^1\). We expect these methods to be widely applicable to studies of charge separation in other systems. We have collected high quality 2DESS data on the PSII RC and are currently working on modelling the data. To better understand the information obtained in the new spectroscopic methods we have also performed theoretical simulations of a dimer model, using similar parameters to the most strongly coupled pair of chlorophyll molecules in the PSII RC\(^2\).

We have published an improved excitonic model of the PSII RC based on simultaneous fitting to a wide range of linear spectra\(^3\) and are currently using the model to fit our 2D spectroscopy and 2DESS data for the PSII RC. We also used polarization-dependent 2DES to study the PSII core complex (PSII CC)\(^4\). We observe unexpectedly rapid (sub 100 fs) energy transfer, which was surprising given the reportedly small electronic coupling in this system as determined from crystal structures. We attribute the rapid energy transfer as arising from exciton-polaron formation, implicating the importance of vibrational degrees of freedom in shaping the energy landscape\(^4\).

We have continued our experiments to probe the functional relevance of coherence, which has been a subject of broad interest for a wide range of systems\(^5\). This has led us to perform extensive comparisons between the frequencies and 2D spectral signatures of coherence in chlorophyll a and the PSII RC. We see very similar coherent modes in both systems, as shown in Figure 1A. In an effort to better understand the chlorophyll a signals we have performed a polarization-dependent study of the electronic structure of the Q_x and Q_y bands, and have compared it with that of bacteriochlorophyll a. Interestingly, we find significant differences between the two monomers, despite their similar structures. In chlorophyll a we find evidence of significant vibronic coupling between Q_x and Q_y, which may assist in the rapid internal conversion we observe in chlorophyll a compared to bacteriochlorophyll a.

We have also developed 2D electronic-vibrational (2DEV) spectroscopy and have demonstrated the method on a dye system. Within the next few months we will progress to studying the PSII RC using a mid-IR probe to detect charge separation. In combination with our previous 2DES and 2DESS data from the Q_y region, and broadband 2D data across the visible region (shown in Figure 1B) we will test different models of charge separation.
Figure 1 A: Comparison of coherent modes reflecting ground state (red) and excited state (blue) coherence in chlorophyll a (top) and the D1D2 PSII RC (bottom). B: 2D Electronic Spectra of the D1D2 PSII RC complexes at 77K recorded at different t2 waiting times as labeled. Above the dashed line the signal has been scaled by 1000x to reveal weak features outside of the Qy region. After 1700 fs the anion band signal arising from charge separation is evident near 22000 cm⁻¹ (455 nm).

Science objectives for 2018-2019:

- Perform the first 2DEV studies of the PSII RC
- Use the full multispectral 2D dataset to improve the excitonic model and test models of the charge separation mechanism
- Perform polarization-dependent comparison studies of coherence in the PSII RC and chlorophyll

My scientific area(s) of expertise is/are: Multidimensional spectroscopy and microscopy of photosynthetic systems

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: (2016-2018)
Enzymatic Energy Conversion - Molecular Dynamics and Enhanced Sampling

Simone Raugei, Principal Investigator

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The long-term vision of the PNNL Enzymatic Energy Conversion program is to provide a better understanding of the core principles employed by enzymes that control the flow of energy and matter that lead to specificity and efficiency in catalytic processes. Our program integrates state-of-the-art theory, computation, and 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 substrate for selective conversions and allosteric regulation.

In this context, my efforts are focused on joining experiment and theory in an iterative hypothesis generation/refinement process. Specifically, my aim is the calculation of experimental observables, spectroscopic signatures (e.g., infrared spectra, X-ray absorption), and experimentally accessible properties, such as $pK_a$ of non-standard residues and cofactors, binding free-energies of substrates.

Any realistic theoretical and modeling approach must account for the intrinsic conformational flexibility of proteins, leading to a plethora of conformations that proteins sample from thermal fluctuations. Biological systems are characterized by a nearly flat free-energy surface with many, almost equi-energetic minima, separated by only small barriers. To characterize this complexity, appropriate sampling is crucial. Additionally, many motions and processes are anharmonic in nature, which leads to non-trivial large entropic contributions to the free-energy. As a consequence, Experiments inherently measure observables that are averages over many conformations and often long periods of time.

Molecular dynamic is an effective method to tackle this problem effectively. However, to describe the occurring multiple length- and time-scale of process of interest require use of a multi-scale modeling and advanced statistical mechanics approaches. Indeed, treating systems with quantum mechanics or hybrid quantum mechanics/molecular mechanics (QM/MM) methods, as needed to describe bond breaking and forming, we are able to simulate systems sizes of the order of 100s of atoms for up to 1 ns. Decreasing the complexity in the description of interactions by using molecular mechanics or force fields, we can simulate systems with up to $10^6$ atoms.

Figure 1 Enhanced sampling capabilities allow accessing diverse time scales to address outstanding scientific questions. All these questions are addressed by a variety of enhanced sampling methods like Thermodynamic Integration, Umbrella Sampling, Steering Dynamics and Metadynamics. All center around the concept of collective variables that allow to describe the process or property in compact form. Finding and discriminating the “correct” collective variables is key.
atoms and time scales of $1 \mu$s. This is long enough to obtain insights into slow loop motions or fast domain dynamics. The time scale accessible by direct molecular dynamics will often not be long enough to answer many scientific questions at hand. Most enzymatic processes are rare events and involve large conformational changes. Therefore, we make use of our extensive capabilities in using enhanced sampling techniques (see Figure 1).

Using Molecular dynamics in conjunction with enhanced sampling techniques to generate ensembles of observables allows for direct comparison between theory and experiment. Advantages and capabilities of this approach will be highlighted for two types of spectroscopy often used to study conformational changes, oxidation states of metal centers and follow reactions, namely IR and EXAFS, and the calculation of binding energies (see Figure 2.a). Even though the theoretical study of molecular vibrations in the gas phase can be considered one of the hallmarks of quantum chemistry, these treatments are not well-suited for the condensed phase. Two main theoretical frameworks for the calculation of the line IR shape based on MD have emerged that account for the dynamics of the chromophore and its environment. Either using the classical dipole approximation to calculate spectra as it relates to the Fourier transform of the dipole autocorrelation, [1,2] which can be obtained from QM/MM simulations (see Figure b), or using empirical relationships connecting instantaneous vibrational frequencies to the local structure, which is usually applied for theoretical 2D-IR spectroscopy for proteins [3].

X-ray absorption spectroscopies, such as XANES, to probe the oxidation state of a metal centers in complex environments, which are often part of active sites, and EXAFS to probe the fluctuations of the local environment, are effective. The spectra are sensitive to the structure and dynamics of the first shell, usually up to 5 Å. Using a combination of MD to generate an ensemble of structures in conjunction with either scattering codes or electronic structure methods these complex spectra can be calculated [4].

My scientific area(s) of expertise is/are: Theoretical and computational chemistry.

To take my project to the next level, my ideal collaborators would have expertise in: experimental characterization of enzymatic mechanisms on the molecular scale and interest in structural and dynamical properties.

The molecular basis of dirigent proteins in plant metabolism:
Lignans/neolignans, aromatic terpenoids, stilbenoids and pterocarpans

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Overall research goals:

Proposed Research Plan: Dirigent Proteins in Diverse Metabolic Pathways. Biochemical redox mechanisms of how various metabolically diverse plant phenol-derived C-C and C-O bond forming processes stipulate distinct stereo-selective, stereo-specific, and/or regio-specific reactions were poorly understood at electron transfer and protein (active site) levels. We correctly deduced that a key component had not been detected or considered, and which enabled control of stereo-selective, stereo-specific, and/or regio-specific plant phenol derived C-C and C-O redox-mediated bond-forming processes in vitro. The key “missing” component was our discovery of dirigent proteins, DPs (Latin: dirigere, to guide or align) in probing how stereo-selective inter-molecular coupling occurred in 8–8’ lignan biosynthesis.

DP metabolic diversity known thus far stipulating highly diverse metabolic pathway plant phenol derived redox mediated C-C and C-O bond-forming outcomes is summarized in Figure 1, i.e. to lignan, aromatic terpenoid, pterocarpan, and lignin formation, as well as being implicated in stilbenoid and allyl/propenyl phenol based coupling control. These advances have been extended DP functions to both inter- and intra-molecular biochemical processes in diverse branches of plant metabolism.

Significant achievements (2018):

Our proposed studies are significantly leveraged (resource-wise) through 3 peer-reviewed, recently awarded Science Theme projects at PNNL EMSL and Stanford SSRL, i.e. to collaboratively access state-of-art instrumentation not available at WSU. Our investigation addresses resolving urgent fundamental mechanistic questions in pterocarpan, lignan, neolignan, aromatic terpenoid and stilbenoid biosynthesis. Specifically, we have completed crystallization of pterocarpan synthases from Glycyrrhiza echinata (GePTS1) and Pisum sativum (PsPTS1). The crystal structure of GePTS1 has been completed at 2.6 Å resolution, and is a hexamer (Figure 2, manuscript in finalization). Work is currently underway to obtain GePTS1 as its ternary complex with bound substrate and/or product, or analogs thereof. We wish to develop a general DP biochemical mechanism, as regards here how binding of substrate, generation of

Figure 1. Dirigent protein metabolic pathway diversification known thus far in plants.
corresponding (putative) quinone methide, and subsequent intramolecular cyclization, occurs. Computational DP substrate and DP product modeling occurs of interactions at the DP active site are also being studied.

Science objectives for 2018-2019:

**Objective 1:** With DP X-ray crystallography, molecular modeling and site-directed mutant approaches, establish biochemical mechanisms of DP substrate binding, (stereo-selective, stereo- and/or regio-specific) electron transfer in plant-phenol derived C-C and C-O bond forming processes, and determine basis for substrate diversity observed in various DPs.

1.1. **Probing DP substrate binding/coupling mechanisms and putative DP active sites:** Intra-molecular and inter-molecular bond-forming processes. Investigate (compare/contrast) DP engendered biochemical systems in pterocarpan, lignan, aromatic terpenoid and stilbenoid metabolism.

1.2. **Apply and evaluate IMS-MS approaches to characterize DP active sites for inter-molecular C-C coupling or phenol-based intra-molecular C-O bond formation and product formation.**

**Objective 2.** Establish precise nature of putative DP supramolecular proteinaceous complexes (including interacting proteins with DP’s, those providing 1e oxidation/co-factors and others with potential scaffold roles) in inter-molecular coupling. Investigate whether a general biochemical model controls inter-molecular phenoxy radical coupling and involves similarly organized supramolecular DP containing proteinaceous complexes, but differing in DPs specific for distinct metabolic pathways, i.e. differentially stipulating outcome in lignan, stilbenoid, terpenoid, and pterocarpan pathways. Our inter-molecular phenoxy radical coupling DP (DRR206 and AtDIR6) X-ray structures, as well as homology modeled GhDIR4 and viniferin-forming DP, provide tantalizing evidence in the form of conserved residues and structures on the DP’s exterior surface that are strongly suggestive of protein interaction sites for an oxidase (preferred), or other proteins, such as in scaffolds. To address DP-interacting proteins and whether there are general supramolecular protein complex biochemical mechanisms and organizations tightly controlling the redox mediated electron transfer reactions, we will use Arabidopsis, P. sativum, and V. vinifera, with various interlocking approaches, including cryo-EM.

Publications supported by this project 2017 – 2018:


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 structural organization, self-assembly, and function of redox-active enzymes and biological energy transduction systems localized at biological (cellular membranes) as well as manmade bio-nano interfaces. Specific aims for the current grant period include development of (1) nanopore surfaces for electrochemical access to redox active enzymes and (2) experimental protocols for loading of redox active enzymes into nanopores and taking advantage of self-assembly for forming layer(s) of redox active enzymes inside the nanochannels formed in anodic aluminum oxide (AAO) substrates.

Significant achievements (2017-2018):
EPR spectroscopy is a highly sensitive, informative, and the most direct tool to characterize free radicals and transition metal ions – with many of these paramagnetic species playing the key roles in electron transfer and redox biological reactions. One of the most common approaches for generating such species is based on electrode electrochemistry but the latter method was proven to be notoriously difficult to adopt for high-Q EPR resonators which separate electrical and magnetic microwave components for the maximum EPR sensitivity. This year we introduced a radically new line of high Q-factor resonators for W-band (95 GHz) EPR that are based on one-dimensional photonic band-gap (PBG) structures. A resonator is formed by creating a defect in all-dielectric 1D photonic crystal split by a metal mirror in the middle (Figure 1, ref. [1]). A sample (either liquid or solid) up to ca. 5 μl in volume is located on the top of the metallic mirror, corresponding to the $E=0$ node, and the position of the metal mirror is adjusted for the frequency tuning. Nanoporous ceramic discs of 50 μm in thickness developed in the course of this DOE project were employed as an aqueous sample holder that provides for a macroscopic alignment of membrane protein samples with concentration sensitivity exceeding conventional EPR cavities by at least 7-10 fold. The “open” structure of the new PBG resonator with a metallic mirror, which could serve as one of the electrode, and the nanoporous holder for immobilizing redox active enzymes, provide a base for building efficient electrochemical high Q-factor resonator for studying redox-active enzymes during the next phase of this project.

Other significant achievements included development of ALD methods for uniform coating the inner surfaces of AAO nanopores with TiO$_2$ for consequent deposition of catalytic molecules (in collaboration with of Drs. David Tiede and Alex Martinson (ANL).
Furthermore, we have developed two new experimental chemical protocols to obtain a reliable and reproducible high density ligand coating on the inner surfaces of AAO pores. These methods open a broad avenue for designing hybrid nanostructures based on covalently attached lipid arrays, proteins, and oriented lipid-protein complexes.
Science objectives for 2018-2019:

- Complete development of an electrochemical cell based on photonic band gap (PBG) resonator structure and nanoporous sample holders for aqueous and membrane protein samples.
- Carry out benchmark electrochemical EPR studies using electrochemical PBG resonator at W-band (95 GHz) EPR frequency.
- Expand the electrochemical EPR studies to redox-active enzymes attached to the nanopore surfaces to take advantages of an exceptionally high degree of macroscopic alignment we have achieved for transmembrane proteins.

My scientific area(s) of expertise is/are: electron paramagnetic resonance, double resonance and pulsed methods such as DEER and HYSCORE, 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 2017-2018:

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

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Overall research goals:
The overarching goal of this project 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. In this project period, we will focus on three unsolved questions that lie at the center of methanogenic metabolism, using the genetically tractable *Methanosarcina acetivorans* as a model organism. The first question addresses the installation, essentiality and mechanistic function of the extensive post-translational modifications found in methyl-coenzyme M reductase (MCR), an enzyme that plays a critical role in both methane production and anaerobic methane oxidation. The second addresses the role of “methanogenesis marker proteins” in MCR maturation and activation, while the third 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 reduction and assimilation, which are central research goals 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 methanogenesis marker proteins will involve determining the viability of mutants lacking marker protein genes and characterizing their physiology, characterizing the modification state and biochemical activity of MCR in viable mutants, and identifying proteins that co-purify with affinity-tagged versions of the marker proteins. Finally, 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 or solution NMR.

Significant achievements (2018-21):
- 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 at 1.6 angstrom resolution (in collaboration with Dr. Satish Nair, Department of Biochemistry, University of Illinois).
- We have used a Cas9-based genetic approach to show that mutants lacking genes encoding the *M. acetivorans* methanogenesis marker proteins are viable, paving the way for planned phenotypic and biochemical studies.

Science objectives for 2018-2021:
Studies on the post-translational modification of methyl-coenzyme M reductase (MCR).
• Identify the genes required for the installation of the remaining post-translational modifications using a comparative genomic approach.
• Characterize the physiology of all viable mutants, including multiply mutated strains and ones that express MCR-modifying enzymes not normally found in Methanosarcina.
• Purify and biochemically characterize affinity-tagged MCR derivatives with various combinations of post-translational modifications.
• Determine the crystal structures of various MCR derivatives.

Studies of methanogenesis marker proteins.
• Determining the viability of mutants lacking marker protein genes and characterizing the physiology of all viable mutants.
• Characterizing the modification state and biochemical activity of MCR in viable mutants.
• Identifying proteins that co-purify with affinity-tagged versions of the marker proteins.

Studies of the ferredoxin-dependent electron-transfer networks in Methanosarcina.
• Determine the viability of mutants lacking each of the thirteen Methanosarcina Fd-encoding genes, including ones lacking Fd genes in various combinations, and characterize the physiology of all viable mutants.
• Establish the protein interaction network for each ferredoxin by identifying proteins that co-purify with affinity-tagged derivatives.
• Characterize the metal content and redox properties for each ferredoxin.
• Determine the structures for each ferredoxin via protein crystallography or solution NMR.

My 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 2018-2021:
BETCy: Biological Electron Transfer and Catalysis

John W. Peters, Principal Investigator
Brian Bothner, Co-PI
Monika Tokmina Lukaszewska, Postdoctoral Research Associate
Department of Chemistry and Biochemistry Montana State University
Email: bbothner@montana.edu; Website: http://betcy-efrc.org/; http://www.montana.edu/bothnerlab/

Overall research goals:
The mission of the BETCy EFRC is to define the molecular mechanisms controlling electron flow in coupling electrochemical potential energy to chemical bond formation. BETCy examines the mechanisms of Electron Bifurcation (Combining exergonic and endergonic electron transfer reactions for the efficient coupling of electrochemical potential to chemical bond formation), Nucleotide Driven Electron Transfer (Combining energy stored in chemical bonds with electrochemical potential in electron transfer reactions to efficiently drive difficult chemical bond forming reactions) and Catalytic Bias (Mechanisms for controlling directional catalytic rates in proton coupled electron transfer reactions).

Significant achievements:
To characterize the structural and dynamic properties of these sophisticated multi-subunit metaloenzyme complexes, we have developed a complementary set of chemical and biophysical approaches that take advantage of cutting edge mass spectrometry and computational tools. These include: (1) non-covalent mass spectrometry to establish mass of the entire complex in its native form, confirm presence of cofactors, and finally determine cofactors and protein component stoichiometry; (2) chemical cross-linking (XL-MS) to ascertain protein-protein interaction networks; (3) surface labeling (SL-MS) to supplement and validate results obtained from cross-linking; (4) interactive modeling to construct a low-resolution model of TmH\textsubscript{2}ase complex; (5) computational methods (molecular dynamics simulations, hydrogen bond analysis and water pathway and water density maps) to establish potential proton pathways in order to validate obtained complex model.

Science objectives for 2018-2019:
- Produce mechanistic models for allosteric regulation and cooperatively in enzyme complexes that take advantage of electron bifurcation.
- Develop models defining the connection between electron / proton transfer and protein dynamics.

My scientific area of expertise are: protein dynamics, systems biology, and mass spectrometry.

To take my project to the next level, my ideal collaborator would have expertise in: Structural biology, molecular dynamics, electron transfer.

Selected publications supported by this project:


Transmethylation reactions during methylotrophic methanogenesis in methanogenic Archaea

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Overall research goals:
Methanogenic archaea and anaerobically respiring bacteria employ multicomponent corrinoid-dependent methyltransferase systems to methylate cellular cofactors such as CoM (methanogens) or THF (bacteria) during growth with methylated compounds. Such systems have a small corrinoid binding protein that interacts with two methyltransferases that either demethylate the growth substrate to methylate the corrinoid protein or demethylate the corrinoid protein to methylate the cellular cofactor. During the catalytic cycle, the corrinoid protein alternates between Co(I) and methyl-Co(III) states, adventitious oxidation to the Co(II) state necessitates reactivation by another component of the system, an iron-sulfur protein that couples ATP hydrolysis to the endergonic reduction of Co(II) to the Co(I) form. Our project has focused on understanding multicomponent methyltransferase systems using the methylamine methyltransferases of methanogens as model systems. The methylamine-dependent methyltransferases are non-homologous, yet each of their encoding genes contains an in-frame UAG codon. Metabolism of methylamines obligately depends on translation of an amber codon as the 22nd amino acid, pyrrolysine (Pyl). In previous periods, we have made inroads into understanding how pyrrolysine is biosynthesized, and how it is genetically encoded. Most recently we focused on active site analysis of members of the TMA methyltransferase superfamily and comparing active sites of proteins that have or do not have pyrrolysine. In the coming budget period, we are expanding our studies to include the mechanism by which RamA, the archaeal corrinoid protein reductase achieves the low potential reduction of Co(II) to Co(I) corrinoid protein.

Significant achievements (2016-2018):

**Active site of MttB, the methanogen trimethylamine methyltransferase.** We have made recombinant MttB, MttC (the cognate corrinoid protein of MttB), and RamA (the corrinoid activation protein). MttB and MttC are produced in a methanogen host, whereas RamA is produced in *E. coli* under anaerobic conditions. Dimethylamine is an inhibitor of MttB activity and we are using this to establish the order of binding of substrates to the protein. Guided by a structure produced by our collaborator Michael Chan, we have now completed analysis of pyrrolysine substitutions with other residues and found that all but a trace amount of activity is lost. We have also established that a residue supporting protonation of the pyrrolysine imine nitrogen strongly affects the rate of the reaction, supporting the hypothesis that the pyrolysyl residue forms a complex with the TMA substrate.

**Active site of MtgB, the bacterial glycine betaine methyltransferase.** A structural analysis of the putative active site of glycine betaine allowed identification of four residues that were candidates for binding glycine betaine. These same residues were further shown to interact with glycine betaine in a recent structure obtained by our collaboration with the Hao laboratory. We constructed a series of substitutions of the substrate binding residues and the resultant variants displayed \( K_m \) and \( k_{cat} \) effects indicating residue roles in binding and/or catalysis. Comparison of the active sites of MttB and MtgB suggests that a pyrolysine-TMA adduct would orient a methyl group of TMA into a position close to that of a methyl group from glycine betaine on MtgB with reference to the aligned TIM barrel structures. 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.
The ATP-dependent corrinoid reductase, RamA. In collaboration with the Ragsdale lab, we are establishing relevant redox potentials of the RamA protein. The two tetranuclear iron-sulfur clusters of RamA average -390 mV. Thus far, we have been able to establish the redox potential of the MttC Co(II)/(I) couple must be below -720 mV. In keeping with this large redox span, we have found consistently that the stoichiometry of ATP hydrolyzed to Co(II) reduced is well above unity whether monitored by formation of Co(I) directly, or indirectly using coupled assays in which Co(I) is captured in the more stable methyl-Co(III) form. Unlike bacterial homologs of RamA, which form dimeric complexes with two corrinoid protein substrates, we have found that RamA forms a 1:1 complex with Co(II)-MttC. We have now established a collaboration with the Drennan lab, and they have recently obtained the first crystals of RamA.

Science objectives for 2018-2019

- Determination of the redox potential for the Co(II)/(I) couple of MttC.
- 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 scientific area(s) of expertise is/are: Metabolism and biochemistry of anaerobic Archaea and Bacteria. Bioinformatic and empirical enzyme identification, 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: redox chemistry and structural biology.

Publications citing DOE support [2016-2019]:
3. Picking, J., Li, Y., Ferguson, D.J., Hao, B., and Krzycki, J.A. Binding site of the quaternary amine on MtgB, the glycine betaine methyltransferase. For submission to J. Biol. Chem.
Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase

Stephen W. Ragsdale, Principal Investigator
Anjali Patwardhan, Lauren Hoff, Thanyaporn Wongnate, Panu Pimviriyakul
Department of Biological Chemistry, Univ. of Michigan Medical School, Ann Arbor, MI 48109-0606
Email: ragsdal@umich.edu, Web: http://www.biochem.med.umich.edu/?q=ragsdale

Overall research goals: Methyl-coenzyme M reductase (MCR) from methanogenic archaea catalyzes the terminal step in the biological synthesis of methane and the first step in methane oxidation. As stated in the goals of our project, we will trap and characterize the CoBS(dot) thyl radical formed during the first step in methanogenesis and the nickel-bound CoBSSCoM anion radical, in the catalytic mechanism of MCR in the forward and reverse directions. We will define the mechanism of methane inhibition of methane synthesis by MCR. This work will reveal the methane-binding site on MCR. Finally, we will test our hypothesis that catalytic bias of the methanogenic MCR is poised for methane synthesis, while that of the methanotrophic ANME MCR favors methane activation. Comparison of the kinetic parameters for the substrates and products of the forward and reverse reaction for the ANME versus the methanogenic MCR will support or negate that hypothesis. We are well poised to move to the next stage in studies of methane synthesis and activation by focusing on three major aims.

Fig. 1. Mechanism of CH₄ formation and transition state for first step, based on (1).

Significant achievements 2017-2018: Our goals were to (a) Trap and characterize the key intermediates in the first and second steps of reverse methanogenesis (anaerobic methane oxidation), (b) Characterize the mechanism and high-affinity binding site involved in methane inhibition of methanogenesis, and (c) Trap and characterize the thyl radical intermediate (CoBS(dot)), see Figs. 1 and 2, involved in the first step of methane synthesis.

With respect to Aim (a), having trapped the key catalytic intermediate and determined the transition state for the first step in MCR-catalyzed methane synthesis (Fig. 1), we employed transient kinetic and spectroscopic (EPR, UV-VIS-NIR, MCD, XAS) approaches to characterize the first steps in methane oxidation (Fig. 2). Characterizing this reaction will also reveal the last intermediate in methane synthesis.

We reacted the active Ni(I) enzyme with substrate, CoBSSCoM and the analog, CoB₆SSCoM (Fig. 2). By stopped flow and EPR methods, we observed formation of a Ni(I)-CoBSSCoM intermediate, the enzyme-substrate complex. This intermediate exhibits pronounced
changes in the intensity of the hyperfine lines of the Ni(I) EPR spectrum and in the 600-800 nm NIR range, shifting from 710 to 750 nm. Then the EPR spectra of the first intermediate emerge with a Ni(III) EPR spectrum resembling that of MCRox1, and a shift in the UV-vis-NIR 650 nm. Over time, we observe conversion of the ox1 to the ox1-silent Ni(II) species.

We plan to complete XAS, MCD, NIR and to perform temperature-dependent transient kinetic experiments and density functional theory calculations of these proposed intermediates.

With respect to Aim b, we recently made the surprising discovery that methane, at concentrations (0.1 atm) well below the \( K_m \) value (>10 atm) for reverse methanogenesis, inhibits 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 (EPR, ENDOR), kinetic and computational methods.

Science objectives for 2018-2019:

- Isolate and characterize intermediates in the MCR reverse reaction using pre steady-state kinetic and spectroscopic methods
- Characterize the methane binding site in MCR
- Perform and compare computational and experimental studies with wild-type and site-directed variants of the “methanogenic” and “methanotrophic/ANME” MCRs in the forward and reverse directions. We are collaborating with Gunter Wegener (Univ of Bremen, Germany) to grow ANME2 and purify the MCR. This enzyme has previously been purified and the crystal structure is known.

References to work supported by this project 2017-2018:


And finally, to take my project to the next level my ideal collaborators would have expertise in:

**Protein expression and mutagenesis in methanogens.**

Other current collaborators have expertise in crystallography (Cathy Drennan, MIT); X-ray absorption (Ritimukta Sarangi, SSRL), MCD (Thomas Brunold & Nicolai Lehnert, U. Michigan) and ENDOR (Brian Hoffman, Northwestern U.) spectroscopies; as well as quantum mechanical computations of reaction pathways (Simone Raugei).

REFERENCES in write-up:

Mission Statement: To develop a nano- to meso-scale understanding of cellulosic cell walls, the energy-rich structural material in plants, and the physical mechanisms of wall assembly, forming the foundation for new technologies in sustainable energy and novel biomaterials.

Plant cell walls - also known as cellulosic biomass or lignocellulose - are among the most complex, diverse and useful materials on Earth. These hierarchical structures represent an abundant and renewable source of valuable biomaterials and bioenergy, presenting untapped transformative opportunities for engineering them with new properties while simultaneously providing lessons on how to mimic the nano-scale structure and means of assembly of these complex living materials for synthesis of man-made materials with specific, tunable properties.

CLSF’s research is at the nexus of physics, chemistry and biology and draws on expertise from diverse fields, diagrammed at right. Insights from our research will form the foundation for future efforts to optimize the structures and utility of plant cell walls, which are essential to plant life and comprise a large-scale source of renewable biomaterials and bioenergy.

CLSF goals in the current phase will build upon advances made in the previous funding period to:

1. Combine multiple state-of-the-art methods of electron microscopy with neutron and X-ray scattering, computational modeling and biochemistry to solve the structure and catalytic mechanism of plant cellulose synthases (CesAs) and native cellulose synthesis complexes (CSCs).

2. Manipulate active CesA assemblies in vitro and in vivo to learn how artificial and native CSCs are assembled and how cellulose microfibril structure depends on CSC structure. We will use these new experimental platforms to test computational models of CSC and cellulose microfibril assembly.

3. Develop new experimental and quantitative methods for assessing cellulose microfibril organization in cell walls and use them to uncover the physical mechanism(s) of microfibril bundling.

4. Extend newly-developed methods and results by CLSF to analyze the physical basis of microfibril-matrix interactions in cell walls with different matrix polymers and study the structural, physical and mechanical consequences of altering these interactions in primary and secondary cell walls.
5. Develop new biological systems (such as the growing Arabidopsis inflorescence stem and xylem-transdifferentiation in transgenic seedlings) to study the processes of microfibril bundling, primary cell wall assembly and maturation, and secondary cell wall formation.

These topics are linked to one another as illustrated graphically below:

Above: Research questions addressed by CLSF include the structure and kinetics of cellulose synthase (CesA); the structure and activity of the cellulose synthesis complex (CSC); cellulose microfibril (CMF) structure and CMF interactions with water, matrix polysaccharides and lignin.

These goals involve new teaming arrangements and development of novel approaches, experimental platforms and advanced instrumentation. The five goals will synergistically produce new insights for potential means to achieve control of man-made materials and for ways to tune cell walls for specific properties in the materials and energy fields. Overall success with even a subset of these goals will enable a quantum leap in understanding how plants assemble these complex hierarchical structures.

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Session VII
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 (2016-2019) are:

(1) Measure long-range electron transport in multiheme cytochrome networks in situ, by performing electrochemical gating and conductance measurements of both living cellular layers (Shewanella) and cell-free systems where the outer-membrane cytochromes are measured in lipid bilayer environments.

(2) Develop algorithms to perform stochastic and mean-field calculations of charge hopping in cytochrome networks lining cellular membranes and bacterial nanowires. These calculations inform, and are informed by, the measurements in Task 1 above.

(3) Expand the knowledge-base of EET from outward mechanisms (e.g. metal reducing microbes) to the reverse process of inward EET by electrochemically characterizing microbial strains that can acquire energy for biosynthesis via electron uptake from electrodes.

Significant achievements (2016-2018):

Goal 1. We successfully completed, and reported, temperature-dependent measurements of micrometer-scale conduction through living Shewanella oneidensis MR-1 cells (Xu et al. JACS, 2018). Specifically, we reported electrochemical gating measurements of conduction through S. oneidensis cells bridging electrodes, but not through mutant cells lacking specific multiheme cytochromes, including the entire Mtr-Omc pathway. The conduction current peaks at a gate potential corresponding to the formal potential of Mtr proteins, in support of our proposed cytochrome-mediated multistep redox conduction mechanism similar to redox polymers. In addition, we find that this electron transport process is thermally activated, with an activation energy (0.29 ± 0.03 eV) that is in remarkable agreement with our kinetic Monte Carlo calculations of the overall thermal activation barrier for transport through the decaheme chains of MtrF and MtrC. This is both the first measurement in Shewanella and the first time that a specific molecular pathway is implicated in biofilm conduction in any organism.

Goal 2. On the simulations front, QM/MM calculations of the 10-heme redox potentials in the decaheme cytochrome MtrC allowed us to perform stochastic calculations of electron hopping through the decaheme chain (Xu et al. JACS, 2018), which revealed activation barriers (0.26-0.32 eV) in excellent agreement with the measured 0.29 ± 0.03 eV of transport across whole cells. For transport

Fig. 1. Electrochemical gating of living S. oneidensis cells bridging interdigitated source-drain electrodes (pictured in inset AFM image). (A) Dependence of source-drain current on gate potential (average of source and drain potentials), for different temperatures, showing the characteristic peak-shaped signature of redox conduction at the formal potential of the Mtr/Omc cytochromes. (B) Arrhenius-style plot yields an activation energy $E_a = 0.29\pm0.03$ eV. From Xu et al. JACS, 2018.
over larger length scales, we performed mean-field calculations of the limits of transport through bacterial nanowires – the cytochrome-containing membrane extensions of *S. oneidensis*. Electron cryo-tomography provided input (i.e. observed cytochrome density) for these calculations. On the basis of these calculations, we proposed that EET along whole nanowires likely requires a combination of direct electron hopping and physical molecular diffusion (Subramanian et al. PNAS 2018).

**Goal 3.** We demonstrated that cathodic electrons enter the electron transport chain of *S. oneidensis* under aerobic conditions (Rowe et al. mBio 2018). While no growth was observed under these conditions, this process correlated with higher ATP levels of cathode-respiring cells relative to controls, and an increase in the cellular redox pool (NADH/FMNH₂). Expanding to other model systems where cathode oxidation is expected to result in higher energy yield, we demonstrated that the sulfur-oxidizing bacterium *Thioclava electrotrophica* Elox9 can perform electron uptake from cathodes (onset potential ~100 mV vs. SHE) under autotrophic conditions and that this ability is likely linked to a direct mechanism, rather than via soluble molecules. In addition, we have confirmed the ability of *Methanosarcina barkeri* – an important model system for methanogenesis – to enhance cathodic current linked to increases in methane production. One underlying pathway relies on cell-derived free extracellular enzymes e.g. hydrogenases that catalyze H₂ formation on cathode surfaces. However, after minimizing the contributions of extracellular enzymes and using a mutant lacking hydrogenases (courtesy of Prof. Bill Metcalf), we still observe a lower potential hydrogen-independent pathway for extracellular electron uptake in *M. barkeri* (Rowe et al. bioRxiv 2018).

Science objectives for 2018-2019 (and renewal directions):
- Continue development of cell-free systems, by studying multiheme cytochromes in natural outer-membrane vesicles isolated from *S. oneidensis*.
- Determine the role of protein dynamics in facilitating micrometer-scale electron transport, by quantifying the mobility of cytochromes on cellular membranes and bacterial nanowires.
- Characterize specific redox conduits for inward extracellular electron transport
- Examine mechanisms of direct inter-species electron transfer.

My 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 2016-2018:**
Flavin-Based Electron Bifurcation

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Overall research goals:
The Biological Electron Transfer and Catalysis (BETCy) EFRC research is focused on elucidating the mechanisms of conversion of electrochemical potential into chemical bond energy. In particular, BETCy is investigating the challenging chemical transformations of flavin-based electron bifurcation, nitrogenase, and hydrogenase enzymes.

Flavin-based electron bifurcation is a recently accepted third mechanism of biological energy conservation. Bifurcation is fundamental to the biochemistry that drives microbial life at the thermodynamic limits observed for global anaerobic processes, including methanogenesis, acetogenesis and hydrogen metabolism. A key feature common to all bifurcating enzymes is the ability to use the free energy generated by an exergonic oxidation-reduction reaction to drive a coupled endergonic reaction. We have elucidated the mechanistic first principles in the bifurcating enzyme, NADH-dependent ferredoxin-NADP+ oxidoreductase I (Nfn) by employing a variety of biochemical, spectroscopic and electrochemical approaches. Transient absorption spectroscopy techniques were developed to probe the unique bifurcating flavin site, and we have observed the formation of an unstable flavin anionic semiquinone species with a lifetime of 10 picoseconds. It is now known that flavin-based bifurcation exploits specific redox properties of flavin cofactors, whereby the semiquinone species is highly energetic, and combined with the short lifetime drives the high barrier reaction while preventing unproductive or short circuit electron transfer events. Determination of the energetic landscape of the electron transferring cofactors within Nfn now allows us to ask detailed mechanistic questions regarding protein control and tuning of the unique bifurcating flavin cofactor in Nfn that coordinates the key catalytic transformations. Specifically, the extensive H-bonding interactions surrounding the bifurcating flavin N5 position and the site-differentiated coordination of the iron-sulfur clusters that flank the bifurcating flavin site are hypothesized to impart control over the initial bifurcating events.

Biophysical techniques, including light-driven spectroscopy, are employed to probe how the protein environment controls the energetics and thermodynamics of electron bifurcation by exploiting proton
transfer events, cofactor geometry and coupling, and properties of the flavin catalytic intermediates to establish the molecular mechanisms of complex enzymatic catalysts.

**Science objectives for 2018-2019:**

- Identify how the protein environment tunes the properties of the bifurcating flavin site to destabilize the semiquinone species.
- Determine how the unique coordination of iron-sulfur clusters in Nfn contribute to their extreme reduction-oxidation potentials and the underlying biophysical properties that control electron transfer.
- Elucidate the role(s) and mechanistic implications of proton coupled electron transfer events in Nfn.

**My 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: Our expertise spans from structural biology to biophysical spectroscopies such as transient absorption and electron paramagnetic spectroscopies and electrochemistry, which allows us to access the unique properties and features of bifurcating enzymes.

**Publications supported by this project 2015-2018:**

Regulated reductive flow through archaeal respiratory and energy production systems

Thomas J. Santangelo, Principal Investigator
Department of Biochemistry and Molecular Biology, Colorado State Univ., Fort Collins, CO, 80523
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Overall research goals:
The energy production strategies supporting growth of hyperthermophilic Archaea push the known limits of energy conversation mechanisms. 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. The central metabolism of Thermococcus kodakarensis utilizes a modified Embden-Meyerhof (EM) pathway wherein the early steps of glycolysis do not result in substrate-level phosphorylation, but rather result in the reduction of ferredoxins. The reduced ferredoxins (Fd\textsubscript{red}) act as temporary carriers of high-energy cargo and typically deliver electrons to membrane-bound complexes that couple the exergonic transfer of electrons to protons or elemental sulfur (S\textsuperscript{0}) with the simultaneous translocation of ions across the cellular membrane. Electron transfer to a terminal acceptor oxidizes and recycles the ferredoxins (Fd\textsubscript{ox}) and the resulting ion-gradient drives ATP synthesis. Many studies have focused on the individual enzymes that transfer electrons to and from Fds, however very little is known about the regulation and roles of the distinct Fds that are critical to the flux of electrons from glycolysis to energy conserving systems.

Here we establish the interplay, reactivity, and distinct role(s) of the three genetically encoded ferredoxin proteins in T. kodakarensis.

Significant achievements (2017-2018):
We demonstrate that the three loci encoding known Fds in T. kodakarensis (TK1087, TK1694, and TK2012, encoding Fd-2, -1, and -3, respectively) are subject to distinct regulatory mechanisms and that specific Fds are utilized to shuttle electrons to distinct respiratory complexes during different physiological states. Details will be provided at the meeting.

We have also established additional genetic and molecular tools to aid in vivo studies of hyperthermophiles.

Science objectives for 2019:
1 – Crystallographic resolve the structure of TK2012 and establish the redox potential of this ferredoxin.
2 – Establish the mechanisms employed by the redox-regulated transcription factor SurR to control expression of nearly all loci associated with energy transactions and electron flux in vivo.

My 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: Redox potentials; Sulfur metabolism (bioavailability of S\textsuperscript{0}); redox sensitive transcription factors.

Publications supported by this project (current funding period is 2017-2019):
Understanding redox proportioning through ferredoxins, low potential iron-sulfur proteins acting as electrical hubs to control metabolism

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Overall research goals: Iron-sulfur (Fe-S) cluster containing ferredoxin (Fd) proteins 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) metalloproteins behave as central energy-conserving redox hubs, serving as conduits between multiple redox donors and acceptors. While it is clear that many microbes use multiple Fd protein electron carriers to control electron flow, we do not yet fully understand what controls the proportion of electrons relayed by individual Fds (2Fe2S and 4Fe4S types) among different oxidoreductases within cells. Our goal is to elucidate the chemical and physical 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 individual Fds deliver to their various natural redox partners within organisms. Additionally, we seek to understand how the cellular roles of Fd paralogs relate to those from the flavodoxin (Fld) family of electron carriers, which use flavin mononucleotide as cofactors.

Significant achievements (2016-2018):
1. Mapped the evolutionary relationships between low potential Fd and Fld electron carriers. Fds and Flds function as electrical hubs in cells, mediating electron transfer between overlapping sets of oxidoreductases. To understand protein electron carrier (PEC) utilization across the domains of life, we evaluated the distribution of [2Fe-2S] Fds, [4Fe-4S] Fds, and Flds genes in 7,079 genomes. The average number of PEC genes per Archaea (~13), Bacteria (~8), and Eukarya (~3) varied. In addition, organisms fell into three groups, including those lacking PECs (3%), specialists with one PEC type (20%), and generalists with two or more PEC family members (77%). Specialists contain between 1 and 37 PEC paralogs (µ=2.3), while generalist have 2 to 54 PECs (µ=9.8). Air sensitive [4Fe-4S] Fds are most abundant in anaerobes (µ=7.0), although they are found in aerobes (µ=3.4) at a similar level as [2Fe-2S] Fds (µ=3.1). Size analysis revealed that [4Fe-4S] Fds in aerobes have more amino acid residues than those in anaerobes, suggesting the evolution of structures that stabilize the cofactor. Organisms utilizing multiple PEC families encode larger proteins than organisms with one PEC, suggesting that the evolution of larger proteins may support specificity in electron transfer. This inquiry provides insight into the ways that PEC classes diverged from their ancestral structures and highlights unstudied PECs whose structure, oxygen stability, and partner specificity should be further characterized.
2. Showed that Flds can transfer electrons from Fd/NADP reductase to a Fd-dependent sulfite reductase using a cellular selection. Fld can function as central energy-conserving redox hubs, distributing electrons to various metabolic pathways within cells through protein-protein interactions. While it is clear that these proteins use sequence changes in their polypeptide to regulate their ability to bind and transfer electrons to other oxidoreductases, it remains challenging to monitor and compare the electron transfer of Fld homologs with defined partner proteins within living cells. We demonstrated that an *Escherichia coli* sulfide auxotroph can be used to report on Fld electron transfer from corn ferredoxin/NADP reductase (FNR) to corn Fd-dependent sulfite reductase (SIR). We showed that Flds from different cyanobacteria can support electron transfer between these partner proteins. Our results provide the first direct evidence that Flds can support electron transfer to an assimilatory SIR.

3. Compared the cellular cycling efficiencies of Fd and Fld in cells. We have found that both Fds and Flds retain the ability to transfer electrons from FNR to SIR when fused to red fluorescent protein (RFP). By using the red fluorescence as a proxy for RFP-Fld concentration in cells, we have begun to measure how the cellular concentrations of Fd and Fld relate to requirements for complementation. Our initial measurements have shown that total biomass is proportional to electron flux through the pathway when electron transfer is rate limiting. In addition, we have found we can measure the cellular concentrations of Fd and Fld using flow cytometry under the conditions where their electron transfer is rate limiting for growth. This synthetic selection represents a simple high throughput assay for comparing the electron transfer efficiency of natural and engineered Flds with defined partner proteins.

Science objectives for 2018-2019:

- Use a bacterial selection to analyze how Fds from diverse organisms vary in their electron cycling between 1 donor (FNR) and 1 acceptor (SIR) protein. Use biophysical and biochemical analysis to develop a model that relates Fd sequence to cycling efficiency.
- Evaluate Fd specificity in cells by measuring how electron transfer from FNR to SIR changes when a second acceptor (GOGAT) is expressed. Use as electron donors *Clostridium* proteins (OGOR and HYD) to enable analysis of linear pathway efficiency in the selection.
- Examine how *Clostridium acetobutylicum* Fds discriminate between their many partner proteins by analyzing the metabolic consequences of deleting or overexpressing Fds. Create a strain that will allow us to analyze consequences of expressing a single native or non-native Fd.

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

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

Publications supported by this project [2016-2018]:


Session VIII
Redox Control of Ubiquitin-Like Protein Modification in Archaea

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Overall research goals:
Redox reactions are central to bioenergy but can also overwhelm antioxidant mechanisms of a cell causing widespread damage to proteins, lipids, carbohydrates, nucleic acids and other biomolecules. Protein oxidation is particularly disruptive, as it leads to the aggregation and misfolding of proteins, breaks in the protein backbone, modifications to amino acid side chains, loss of enzymatic function, and bottlenecks in metabolism. Proteins mildly damaged by redox compounds can be repaired, as exemplified by the methionine sulfoxide (MetO) reductases that reduce MetO in free and protein form back to Met. The ubiquitin proteasome system (UPS) plays a pivotal role in the recognition and destruction of proteins damaged by redox-active species. How these systems may interconnect is poorly understood.

We find in archaea that the MsrA-type MetO reductase is needed for the ubiquitin-like (Ubl) modification of proteins during mild oxidative stress. The MsrA/Ubl system is critical for the archaeal cell survival during severe oxidative stress. The overall goal of this project is to determine the molecular mechanisms of the MsrA/Ubl system in overcoming oxidative stress in archaea. Extending study of MsrA to its role in triggering Ubl modifications that form during mild oxidative stress is expected to reveal novel insights into redox controlled Ubl modifications. This project has implications in improving the viability and recovery of cells from redox balance disturbances and maintaining the function of biocatalysts in extreme conditions.

Significant achievements (2016-2018):
- Ubl modification is demonstrated to trigger proteasome-mediated degradation of select target proteins in archaea. Ser phosphorylation of the target protein is an apparent signal for the Ubl tagging mechanism. A JAMM/MPN+ DUB-like metalloprotease is required for the process and the Ubl tag is stable suggesting that Ubl recycling is used to conserve energy during the destruction of the target protein.
- Archaeal Ubl protein ligation is reconstituted by incubating MsrA with the E1 and Ubl proteins in cell lysate devoid of these factors but supplemented with DMSO (a mild oxidant). The mechanism occurs in the presence or absence of DTT and requires DMSO which inhibits the MetO reductase activity of MsrA. Thus, MsrA has an apparent moonlighting function in Ubl ligation that is independent of its MetO reductase and Met oxidase activities.
- Stable isotope labeling in cell culture (SILAC) and cobalamin activity-based probes were used to advance the analysis of archaean proteomes by LC-MS/MS. By this work, new vitamin-protein interactions and oxidative stress responses are defined at the proteome level in archaea.
- Archaeal transposon mutants that are hypertolerant to oxidative stress are isolated, mapped and correlated with the SILAC-based proteomic results and Ubl-proteasome system components.

Science objectives for 2018-2019:
- The structure and function of archaean MetO reductases is a significant focus for the upcoming year. Putative flavin-based and molydopterin-dependent MetO reductases of archaea are also under investigation. The connection of these different MetO reductase systems to Ubl modification and oxidative stress will be examined. Quantitative multiplex SILAC-based proteomics will be used to define protein-protein interactions of the MsrA/Ubl system.
My scientific area(s) of expertise is/are: microbial biochemistry, genetics and proteomics with emphasis on archaea.

To take my project to the next level, my ideal collaborator would have expertise in: systems biology networks, structural biology and redox biochemistry.

Publications supported by this project 2016-2018:
Elucidating the Cellular Machinery for Lipid Storage in Plants

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Overall Research Goals:
Of the cellular processes that participate in energy conversion and storage, perhaps the least well understood are the mechanisms for packaging reduced lipids into structures compatible with the aqueous environment of the cell. In fact, even the inventory of proteins that participate in the compartmentalization of storage lipids in plant tissues is far from complete. Our overarching goal is to understand the biochemical and cellular processes important for compartmentalization of storage lipids in plant tissues, ultimately to manipulate the energy storage capacity of plants. Our near-term efforts are focused on elucidating the composition and function of proteins involved in the biogenesis and stability of cytoplasmic lipid droplets in plant tissues, particularly in vegetative tissues, such as leaves, through three specific aims: 1) Functionally characterize Lipid Droplet Associated Proteins (LDAPs) and their interacting proteins in the regulation of LD dynamics, 2) Functionally evaluate SEIPINs and their interacting proteins to produce lipid droplets at the ER, and 3) Assess the involvement of additional candidate lipodystrophy-related genes in lipid storage in plants. A detailed understanding of the molecular mechanisms by which photosynthetic organisms store reduced carbon could provide important insights necessary to develop renewable, bio-based forms of usable energy.

Significant achievements 2016-2018:
We have identified and characterized a new protein interactor for the LDAPs, designated LDAP-interacting protein, or LDIP. This is a protein with no known function, but was annotated to contain a domain with similarity to a mycobacterium membrane protein involved in lipid transport. We have shown that LDIP interacts directly with both LDAPs and SEIPINS, and in loss-of-function studies results in aberrant LD ontogeny and increases in total neutral lipid content, possibly due to LDIP’s role in controlling the proper phospholipid composition of the LD monolayer. We have further identified a novel physical and functional interaction between SEIPIN and a member of the Vesicle-Associated membrane Protein family, VAP 27-1. This protein is known to function in tethering different organelle membranes together in plant cells and in supporting vesicle formation. Here we have now identified a new function for VAP27-1 in the formation of LDs, perhaps by interacting with SEIPINS to tether LDs to the endoplasmic reticulum surface while neutral lipids are deposited into this cytoplasmic storage compartment. Finally, we have explored how several mammalian lipogenic proteins function in a plant cell context, and have demonstrated a mechanistic conservation of LD biogenesis, LD elaboration, and LD fusion in plant tissues that is mediated by mouse Fat-Inducing Transmembrane protein 2 (FIT2), mouse Diacylglycerol Acyltransferase 2 (DGAT2), and mouse Fat-Specific Protein 27 (FSP27), respectively. Notably, in several cases, overexpression of these mammalian proteins in plants resulted in significant increases in energy density (i.e., TAG and LDs) of plant vegetative tissues.
Science objectives for 2018-2019:
Our efforts continue to uncover the complex protein networks that function to support the formation and stability of LDs in plant tissues, and better understand how LD formation influences neutral lipid homeostasis, overall. Surprisingly, many of our studies are showing that modulation of LD biogenetic processes increase neutral lipid content in plant cells, revealing previously unappreciated roles for LD formation in limiting neutral lipid accumulation. Assessments of how the LD-biogenetic protein machinery is integrated to influence LD size, number, composition, and rate of LD formation in cell-free systems and in various plant tissues will be used to further elucidate the mechanisms that regulate the biogenesis, dynamics and storage of hydrophobic, energy-dense lipids. We anticipate that our results will help define the mechanistic basis for variation and/or conservation of lipid storage across plant tissue types and in a wide range of organisms, and provide novel tools for increasing the energy density of biofuel crop plants.

References to work supported by this project 2016-2018:


6. USPTO Application Serial No. 15/624,495 - METHODS FOR INCREASING OIL CONTENT IN PLANT TISSUES BY SUPPRESSING HYDROPHOBIC LIPID DROPLET PROTEIN. Chapman, Kent (Denton, TX); Mullen Robert (Guelph, Ontario, Canada); Pyc Michel (Guelph, Ontario, Canada); Dyer, John (Maricopa, AZ). June 15, 2017 (converted from US Provisional Patent Application 62/350,843). Pending

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

And finally, to take my project to the next level my ideal collaborator would have expertise in: Time-lapse Imaging, Structural Biology, Reconstitution of Cellular Systems in vitro
Modification of Plant Lipids

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Overall research goals:
To understand the mechanism(s) of regulation of lipid metabolism in plants, specifically how cells decide when internal 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:
We have investigated the posttranslational regulation of WRINKLED1, the master transcriptional regulator of fatty acid synthesis. During these studies we have identified the molecular events that lead to the degradation of WRI1, which involves first its phosphorylation and subsequent recognition by the ubiquitin-degradation pathway. Specifically, WRI1 is phosphorylated at two specific sites by SUGAR-DEPENDENT NONFERMENTING KINASE 1 (SnRK1), which marks it for recognition by the ubiquitin system which polyubiquitinates it close to its N-terminus. The degradation WRI1-ubiquitin conjugates is mediated via the proteasome (Zhai, Z., H. Liu, J. Shanklin. 2017). In subsequent work we manipulated the sugar export from Arabidopsis leaves and showed that increased sugar content led to large increases in leaf TAG accumulation. Under high-sugar levels, WRI1 levels were increased by stabilization of WRI1, consistent with its known effect as an inhibitor of SnRK1 kinase (Zhai, Z., H. Liu, C. Xu, J. Shanklin. 2017). Sugar is a relatively poor inhibitor, but it is known that the levels of a phosphorylated disaccharide, trehalose 6-phosphate (T6P) are correlated with increased sugar and that it is a much more potent inhibitor of SnRK1 than sugars. We therefore tested the effect of incubating a model Brassica cell culture with T6P, and showed that it indeed stabilizes WRI1, leading to increased fatty acid and oil accumulation. Using a new technique called microscale thermophoresis we evaluated the binding of T6P to a series of proteins known to constitute or associate with SnRK1. We demonstrated that T6P binds to KIN10, the catalytic subunit of SnRK1 at physiological concentrations. We showed that KIN10 also tightly associates with its activating kinase GRIK1. However, when T6P binds to KIN10 it reduces the Kd between KIN10 and GRIK1, reducing its activation by phosphorylation (Zhai Z, Keereetaweep J, Liu H, Feil, R., Lunn, J.E., Shanklin J. 2018). This provides the first mechanistic information as to how T6P inhibits SnRK1, the major carbon sensor/metabolic effector kinase in plants. This work identified a major homeostatic mechanism by which plants coordinate their carbon status.

Model of WRI1 (W) regulation. KIN10 (K), GRIK1 (G); phosphoryl group (P); degradation (DEG)

Microscale thermophoresis data. Trace shows the interaction of KIN10 and GRIK in the presence of T6P
Science objectives for 2018-2019:

- Use BNL’s advanced beamlines at NSLS-II to probe the structural interactions between KIN10 and T6P, that lead to an increased $K_d$ between KIN10 and GRIK1.
- Create KIN10 mutants that have altered affinity for T6P to create a range of sensitivities of KIN10 to enable us to “tune the sugar sensing homeostatic sensitivity” and divert more carbon to lipid production.
- To understand the mechanism of biotin attachment domain-containing protein feedback control of acetyl-coA carboxylase under conditions of excess fatty acid.
- To identify details of the catalytic mechanism of soluble plant desaturases using a combination of theoretical computation and experimentation.

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 have expertise in: atomic-scale cryo-EM.

Publications supported by this project 2016-2018:


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 that regulate lipid homeostasis and fatty acid flux between membrane lipids and storage triacylglycerol. The specific objectives are to: (1) understand how fatty acid synthesis is regulated in plants; 2) identify the genes affected in lipid-trafficking mutants isolated in a forward genetic screen and determine the functional role of the encoded proteins; (3) investigate the factors regulating carbon partitioning and storage lipid accumulation in vegetative tissues of plants. The results from this study should create the knowledge base to enhance our ability to manipulate the processes that regulate photosynthetic membrane biogenesis and carbon partitioning and storage in plants.

Significant achievements (2016-2018):
We carried out a biochemical and genetic analysis of a new mutant designated reduced lipid transport 3 (rlt3). Similar to other lipid transport mutants we previously characterized, both the rate of fatty acid synthesis and triacylglycerol level are significantly increased in rlt3 mutants. Positional cloning in combination of the whole genomic sequence identified a missense mutation in a gene encoding an enzyme involved in phospholipid biosynthesis.

Additional work related to this project focused on analyzing the pathway of triacylglycerol metabolic pathways and the physiological significance of triacylglycerol in plants. Taking advantage of Arabidopsis mutants defective in starch synthesis or fatty acid turnover, we found that triacylglycerol accumulation plays an important role in protecting against oxidative stress. Starch deficiency enhances the synthesis and turnover of fatty acids, membrane lipids and triacylglycerol that contributes to carbon and energy homeostasis and plant growth. These biochemical genetic results support the role of triacylglycerol as an intermediate in the fatty acid degradation pathway. Analysis of mutants defective in starch synthesis also indicates that the increased fatty acid synthesis is attributable to the posttranslational activation of plastidic acetyl-CoA carboxylase.

Science objectives for 2018-2019:

- The rlt3 mutant exhibits a major increase in the rate of fatty acid synthesis, a substantial decrease in lipid transport from the endoplasmic reticulum to the plastid. Detailed genetic and biochemical experiments will be performed to dissect the mechanistic basis for these phenotypes.
• Our recently published work suggests an involvement of autophagy in regulating triacylglycerol accumulation in plants. Further genetic, biochemical and cell biological studies will be carried out to examine the role of this important catabolic process in lipid metabolism and storage in plants.

• Potential mechanisms underlying acetyl-CoA carboxylase regulation include protein phosphorylation, redox modulation, feedback inhibition and regulation by biotin/lipoyl attachment domain containing proteins. Their roles in regulating plastidic acetyl-CoA carboxylase will be systematically tested to determine the mechanistic basis for the regulation of fatty acid synthesis in leaves.

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: membrane protein crystallography

Publications supported by this project 2016-2018:
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