2024 Physical Biosciences Research Meeting

Program and Abstracts

DoubleTree by Hilton DC North/Gaithersburg

October 21 – 23, 2024

Chemical Sciences, Geosciences, and Biosciences Divisions

Office of Basic Energy Sciences

Office of Sciences

U.S. Department of Energy

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Forward

This meeting book is a record of the biennial meeting of the principal investigators funded by Physical Biosciences, a program in the Chemical Sciences, Geosciences, and Biosciences (CSGB) Division of the Office of Basic Energy Sciences (BES), U.S. Department of Energy (DOE). CSGB supports basic biochemistry and biophysics research relevant to DOE's mission areas, providing foundational knowledge to advance energy technologies, through 2 core research programs established in 2009: Photosynthetic Systems and Physical Biosciences. These, along with the Solar Photochemistry program, comprise the CSGB Photochemistry and Biochemistry Team, a coordinated group of programs supporting areas of basic research that are central to the science mission of the DOE.

The abstracts in this volume describe research at the leading edge of understanding the natural systems that move, manage, and transform energy in plants, bacteria and archaea. Biology remains capable of complex chemical transformations, electron transfer, and energy management beyond what can be recreated in engineered and synthetic systems currently. These natural processes exhibit enormous structural and chemical diversity and understanding them requires characterization of diverse phenomena including the mechanisms of multielectron catalysis, cofactor and metallocluser biosynthesis, redox tuning of electron transfer within protein and across pathways, and the role of structure, function and conformational change in regulating electron flow. The abstracts in this volume describe research at the forefront of understanding the chemistry, biochemistry, biophysics, and molecular biology that underpin these essential energy relevant processes. The quality and novelty of the research presented here demonstrates the drive, dedication, and talent of the outstanding researchers who make Physical Biosciences an exciting and innovative scientific community

This meeting aims to disseminate recent research accomplishments and foster exchange of scientific knowledge and insights among all participants. Accordingly, it is designed to promote sharing of new results and methodologies; facilitate cooperation and collaboration; challenge old paradigms with new; and provide opportunities to interact with program managers and staff of the DOE. In keeping with this purpose, questions and ideas from meeting participants are welcome.

We thank Teresa Crockett in DOE BES along with Paul Hudson, Kara Lollar, and Kutter Craig of the Oak Ridge Institute for Science and Education (ORISE) for their help with planning and execution of meeting logistics. Thanks also to all participants for sharing their time and their work.

Kate A. Brown, Program Manager, Physical Biosciences, DOE BES

Agenda

2024 Physical Biosciences Principal Investigators Meeting

DoubleTree by Hilton DC North/Gaithersburg, October 21-23, 2024

AGENDA

Monday, October 21

7:30 – 8:45AM	*Continental Breakfast*
08:45 – 09:00AM	Program Overview and Meeting Charge **Kate Brown (**DOE, Physical Biosciences Program Manager)

Section I: *Plants: biosynthesis, structure and growth*
Moderator: *Zhiyang Zhai (Brookhaven National Laboratory)*

09:00 –10:00AM	Cell-type Specific Pectins in Plant Cell Walls: Structure, Interaction and Function **Breeanna Urbanowicz (**University of Georgia) and **Vivek Bhardwaj,** National Renewable Energy Lab
10:00 – 10:30AM	Atomic Resolution of Lignin-Carbohydrate Interactions in Native Plant Tissues from Solid-State NMR **Tuo Wang** (Michigan State University)
10:30 – 11:00AM	*Coffee Break*
11:00 – 11:30AM	Elucidating the Biochemical Mechanisms Controlling Secondary Wall Biosynthesis in Plants **Zheng-Hua Ye,** (University of Georgia)
11:30 – 12:30PM	The Center for Plant and Microbial Complex Carbohydrates at the University of Georgia Complex Carbohydrates Research Center **Parastoo Azadi,** (University of Georgia)
12:30 – 03:00PM	*Working Lunch and Afternoon Break*

Section II: *Enzymes I*
Moderator: *Ekaterina Pletneva (Dartmouth College)*

03:00 – 3:30PM	A Diverse New Family of Electron Bifurcating Enzymes That Play Key Roles in Anaerobic Metabolism **Mike Adams** (University of Georgia)

03:30 – 04:00PM Gating electron transfer in biological energy storage and conversion
Anne-Frances Miller (University of Kentucky)

04:00 –04:30PM Tuning directionality for CO2 reduction in the oxo-acid:ferredoxin superfamily
Sean Elliot (Boston University)

04:30 – 04:45PM *Afternoon Break*

04:45 – 05:15PM Developing a molecular level understanding of carbon monoxide dehydrogenase/acetyl coenzyme A synthase through model metalloenzymes
Hannah Shafaat (University of California, Los Angeles)

05:15 – 05:45PM Elucidating the Catalytic Mechanism of Microbial CO2 Fixation
Ritimukta Sarangi (SLAC National Accelerator Laboratory)

05:45 – 07:30PM *Working Dinner*

07:30 – 10:00PM *Poster Session 1*
(**Odd numbered posters** will be presented. Presenters and titles are listed at the end of the agenda. Refreshments may be purchased at the hotel bar.)

Tuesday, October 22

07:30 – 08:30AM *Continental Breakfast*

Session III: *Enzymes II*
Moderator: *Justin North (Ohio State University)*

09:00 – 10:00AM Mechanism of Photochemical N2 Reduction
Paul King (National Renewable Energy Lab) and **John Peters** (University of Oklahoma)

10:00 – 10:30AM Engineering a Functional Equivalent of Nitrogenase for Mechanistic Investigations of Ammonia Synthesis
Yilin Hu (University of California, Irvine)

10:30 – 11:00AM Light-driven activation of small molecules by nitrogenase hybrids
Markus Ribbe (University of California, Irvine)

11:00 – 11:30AM *Coffee Break*

11:30 – 12:00PM Dissimilatory Nitrite Reduction to Ammonium: Catalyzing Multi-Electron Reductions Using a Pentaheme Scaffold
Eric Hegg (Michigan State University) and **Nicolai Lehnert** (University of Michigan)

12:00 – 12:30PM Production of Ethylene and 3-Hydroxypropionate from the Common Metabolite, 2-Oxoglutarate, by the Ethylene-Forming Enzyme (EFE)
Carsten Krebs (Penn State University)

12:30 – 1:00PM Pathways and Mechanisms in [FeFe]-Hydrogenase Maturation
Joan Broderick (Montana State University)

1:00 – 03:00PM *Working Lunch and Afternoon Break*

Session IV: *Plants II - Biosynthesis and Growth*
Moderator: *Kent Chapman (University of North Texas)*

03:00 – 04:00PM Mechanisms and Regulation of Carbon Allocation and Storage in Plants
John Shanklin (Brookhaven National Laboratory)

04:00 – 04:30PM Molecular Mechanisms of Plant Cell Wall Loosening
Daniel Cosgrove (Penn State University)

04:30 – 05:00PM Understanding Selectivity in Terpene Synthases ¿ Unique Mechanisms to Generate Precursors for Biocrude and Specialty Chemicals
Bernd Lange (Washington State University)

05:00 – 07:30PM *Working Dinner*

07:30 – 10:00PM *Poster Session 2*
(**Even numbered posters** will be presented. Presenters and titles are listed at the end of the agenda. Refreshments may be purchased at the hotel bar.)

Wednesday, October 23

7:30 – 8:30AM *Continental Breakfast*

Section V: *Microbial and Archaeal redox systems*
Moderator: *Kathryn Fixen (University of Minnesota)*

09:00 – 09:30AM Understanding nitrogenase maturation and activity in methanogens
Daniel Lessner (University of Arkansas)

09:30 – 10:00AM Post-translational modifications in archaeal redox biology
Julie Maupin (University of Florida)

10:00 – 10:30AM *Coffee Break*

10:30 – 11:00AM Investigating Extracellular Electron Uptake from Redox Active Solid Substrates: Mechanisms for Gaining Electrons from Minerals, Electrodes, or Other Microbes
Annette Rowe (University of Cincinnati)

11:00 – 11:30AM Transmethylation reaction during methylotrophic methanogenesis in methanogenic Archaea
Joe Krzycki (Ohio State University)

11:30 – 11:40AM *Brief Break*

11:40 – 12:30PM Physical Biosciences and BES Program Update
Kate Brown, Steve Herbert DOE Program Mangers
Gail McClean, CSGB Division Director

12:30PM *Meeting Ends*

Posters

Posters are listed alphabetically by presenter. Odd numbered posters will be presented Monday evening. Even-numbered posters will be presented Tuesday evening.

1. Modifications and Chaperones of Coenzyme F430
   Kylie Allen (Virginia Tech University)

2. Allosteric control of electron transfer in nitrogenase-like enzymes
   Edwin Antony (Saint Louis University)

3. Photosynthetic Energy Capture, Conversion and Storage: From Fundamental Mechanisms to Modular Engineering
   Christoph Benning (Michigan State University)

4. Electron Bifurcation Theory
   David Beratan (Duke University)

5. Structure, Biochemistry, and Physiological Roles of Multiheme Cytochrome Nanowires
   Eric Bond (University of Minnesota), **Allon Hochbaum** (UC Irvine), and **Fengbin (Jerry) Wang** (university of Alabama)

6. Probing novel pathways of iron sulfide acquisition and trafficking from minerals in model biocatalytic systems
   Eric Boyd (Montana State University)

7. Elucidating the Cellular Machinery for Lipid Storage in Plants
   Kent Chapman (University of North Texas)

8. Formate metabolism in hydrogenotrophic methanogens
   Kyle Costa (University of Minnesota)

9. Nitrogenase Reduction of N2
   Dennis Dean (Virginia Tech University) and **Lance Seefeldt** (Utah State University)

10. Structure and function of the methyl-coenzyme M reductase activation complex
    Edwin Duin (Auburn University) and **Barny Whitman** (University of Georigia)

11. Redox-regulation of electron flow in an anaerobe
    Katie Fixen (University of Minnesota)

12. Mechanistic studies of energy-relevant molybdenum enzymes
    Russ Hille (UC Riverside)

13. Towards the Mechanism of N2 Fixation by Nitrogenase
    Brian Hoffman (Northwestern University)

14. Elucidating the control of electron transfer and role in enzymatic reactivity
    Effie Kisgeropoulos (National Renewable Energy Lab)

15. Bioinorganic Chemistry of Nitrification: Structure and Function of Ammonia Monooxygenase
    Kyle Lancaster (Cornell University)

16. Characterizing Plant-Specific Features of Mitochondrial Respiratory Complexes
    James Letts (University of California, Davis)

17. Transformative Biohybrid Diiron Catalysts for C-H Bond Functionalization
    Qun Liu (Brookhaven National Lab)

18. Synchronization of Energy in Electron Bifurcation
    Cara Lubner (National Renewable Energy Lab)

19. Energy conservation, electron transfer and enzymology during methane production by Methanosarcina species
    Bill Metcalf (University of Illinois)

20. Tuning catalytic bias in [FeFe]-hydrogenase
    David Mulder (National Renewable Energy Lab)

21. Structure and function of the nitrogenase-like methylthio-alkane reductase that converts volatile organic sulfur compounds into hydrocarbons
    Justin North (Ohio State University)

22. Novel microbial based enzymatic CO2 fixation mechanisms: Conformational control of enzymatic reactivity
    John Peters (University of Oklahoma)

23. Alterations in Electron- and Proton-Transfer Circuits of cbb3 Oxidases at the Onset of Denitrification
    Ekaterina Pletneva (Dartmouth College)

24. Enzymology of Methanogenesis: Mechanism of Methyl-Coenzyme M Reductase)
    Steven Ragsdale (University of Michigan)

25. Enzymatic Energy Conversion
    Simone Raugei (Pacific Northwest National Laboratory)

26. Regulated Electron Flux Through Archaeal Energy Production Systems
    Tom Santangelo (Colorado State University)

27. Structure and function of the bacterial carbon concentrating machinery
    David Savage (University of California, Berkeley)

28. Understanding Redox Proportioning through Ferredoxins, Low Potential Iron-Sulfur Proteins Acting as Electrical Hubs to Control Metabolism
    Joff Silberg (Rice University)

29. Primary and Secondary Sphere Effects on Iron-Sulfur Cofactors
    Daniel Suess (Massachusetts Institute of Technology)

30. Molecular Mechanism of Energy Transduction by Plant Membrane Proteins
    Micheal Sussman (University of Wisconsin)

31. Exploring the Role of TOR kinase in the Regulation of Central Metabolism and Lipid Synthesis
    Zhiyang Zhai (Brookhaven National Lab)

Abstracts

(in alphabetical order)

Structure and Mechanism of a Diverse New Family of Electron Bifurcating Enzymes That Play Key Roles in Anaerobic Metabolism

Michael W. W. Adams, Principal Investigator
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Email: adamsm@uga.edu

Overall research goals:

Microorganisms utilize electron bifurcating enzymes to carry out thermodynamically in reactions where a single enzyme reversibly couples an endergonic reaction to an exergonic reaction to generate a net reversible reaction with minimal free energy change. In essence, an exergonic chemical reaction is used to drive an endergonic one. Electron bifurcation is now recognized as a major energy coupling system in biology although only a limited number of examples are known. We very recently discovered a diverse family of bifurcating enzymes, termed Bfu, that is surprisingly ubiquitous in the microbial world. They are all predicted to couple the reduction of ferredoxin and NAD to the oxidation a range of substrates, many not previously known to be involved in bifurcation reactions. The overall aims of our research are: 1) to determine the nature of the bifurcating site in Bfu enzymes and elucidate the bifurcation mechanism, 2) to characterize new types of bifurcating Bfu enzymes, including those that potentially utilize unexpected substrates, and 3) to characterize a membrane-bound Bfu family member that couples electron bifurcation to the formation of chemical gradients. Our objectives will be achieved utilizing fermentations of thermophilic microbes, recombinant production and anaerobic purification of cytoplasmic and membrane-bound Bfu complexes, and various biochemical and kinetic techniques. This project is also leveraging on-going collaborations using EPR spectroscopy as well as SAXS and cryo-EM to determine the structures of Bfu enzymes.

Significant achievements (2022-2024):

Our discovery of the diverse Bfu family was based on the characterization an unusual heteropentameric NiFe-hydrogenase (NiFe-BfuABCSL) from a thermophilic microbe that contained subunits (HydABC) of an electron bifurcating (BF) FeFe-hydrogenase. The cryoEM structure of the NiFe-enzyme was determined by Dr. Huilin Li of the Van Andel Institute (MI), the first for any HydABC-type enzyme. The arrangement of its iron-sulfur and flavin cofactors showed that it has a new type of bifurcating site that involves flavin and multiple iron sulfur clusters. We subsequently identified a family of BF-enzymes that all have a BfuABC heterotrimeric core where BfuBC is proposed to reversibly reduce NAD and Fd while BfuA directly or indirectly interacts with the third redox substrate (Figure 1). In the NiFe-Bfu hydrogenase, the site of bifurcation was proposed to be FMN in combination with four FeS clusters (C1, B1, B2 and B5, Figure 1) and Fd was postulated to be reduced at the B4 cluster, but this was based on structure not experimental evidence. In some Bfu enzymes, additional subunits reversibly oxidize a third substrate (such as H_2 or RCHO) and feed electrons to A3 (H_2, NiFe-Bfu, Type 3) or to A4 (RCHO, Wor-Bfu; Type 2), while in others, a third redox substrate, such as NADPH, is reversibly oxidized by BfuA directly (Nfn-Bfu; Type 1). In the current funding period, we have been working with the three classes of Bfu enzyme shown in Figure 2. The cryo-EM structures of Wor-Bfu and Nfn-Bfu (from thermophilic archaeal and bacterial sources) have now been determined by Dr. Huilin Li. We also have a collaboration with Dr. Greg Hura (LBNL) to examine conformational states of Bfu enzymes in solution using a newly developed anaerobic size exclusion-coupled small-angle X-ray scattering (SEC-SAXS) approach and have an on-going collaboration with Drs. Greg Vansuch and Cara Lubner (NREL) who are carrying out EPR and transient absorption spectroscopy on wild-type and site directed mutants of various Bfu enzymes. These studies are providing new insights into the nature of the novel BF-site and the potential mechanism of catalysis by which Fd and NAD reduction can be coupled to the oxidation of a variety of substrates, as shown in Figure 1.

Science objectives for 2024-2025: The goals are 1) to elucidate the mechanism of electron bifurcation in the Wor- and Nfn-Bfu enzymes and 2) to characterize unprecedented types of bifurcating enzymes, including ones that potentially utilize pyruvate and are involved with non-redox cellular processes, such as proteolysis (Por and AAA in Figure 1).

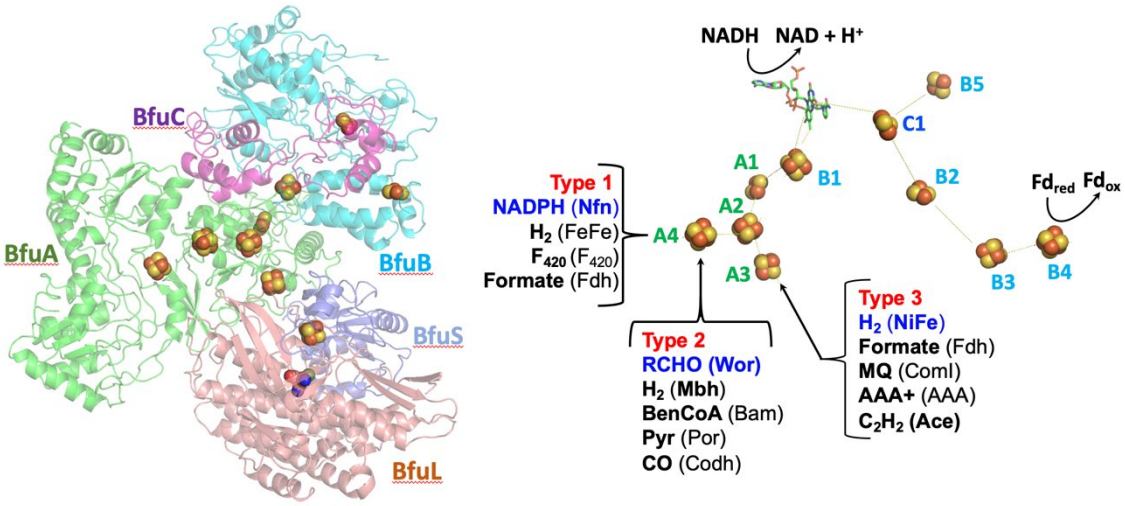

Figure 1. Cluster content and nomenclature of Bfu enzymes based on the NiFe-Bfu structure.

Figure 2. The three types of Bfu enzyme currently under study.

My scientific area(s) of expertise is/are: Metalloenzymes and the metabolism of anaerobic microbes

The ideal collaborator for my project would have expertise in: Protein film electrochemistry

Publications supported by this project [2022-2024]:

Feng, X., Schut, G. J., Haja, D. K., Adams, M. W. W. and Li, H. (2022) "Structure and electron transfer pathways of an electron bifurcating NiFe-hydrogenase" *Science Advs.* **8,** eabm7546 (doi: 10.1126/sciadv.abm7546)

Schut, G. J., Haja, D. K., Feng, X., Poole, F. L., Li, H. and Adams, M. W. W. (2022) "An abundant and diverse class of bifurcating enzyme with a non-canonical catalytic mechanism" *Front. Microbiol.* **13,** 946711 (doi: 10.3389/fmicb.2022.946711)

Feng, X., Schut, G. J., Adams, M. W. W. and Li, H. (2024). Structures and electron transport paths in the four families of flavin-based electron bifurcation enzymes. *Subcell. Biochem.* 104, 383-408 (doi:

10.1007/978-3-031-58843-3)

Modifications and Chaperones of Coenzyme F_{430}

Kylie Allen, Principal Investigator
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Overall research goals:

The focus of this project is coenzyme F_{430}, the nickel hydrocorphin prosthetic group of methyl-coenzyme M reductase (MCR). MCR catalyzes the final methane-forming step of methanogenesis in methanogenic archaea as well as the initial methane activation step of anaerobic methane oxidation in anaerobic methanotrophs. This enzyme holds great potential for use in sustainable and efficient biocatalytic approaches to produce valuable energy-rich compounds. In this project, we will uncover key aspects of F_{430} biochemistry, including elucidating the functions and biosynthesis of modified F_{430}s that likely impact the activity of MCR as well as identifying and characterizing chaperone proteins important for delivery of F_{430} to the MCR active site.

Significant achievements: (2021-2024)

- We have identified two proposed thioether F_{430} modifications in methanogens – mercaptopropionate-F_{430} (mp-F_{430}) and the related mercaptopropanamide-F_{430} (mpa-F_{430}). We hypothesize that these modifications have a role in fine-tuning the reactivity of the coenzyme and the dynamics of the MCR active site, thus influencing the catalytic efficiency and/or the directionality of the reaction. Our growth experiments revealed that the production of modified F_{430}s in methanogens varies depending on the organism and the growth conditions. For example, *Methanocaldococcus jannaschii* upregulates the production of mp-F_{430} during late log phase and stationary phase (up to 30% of total F_{430} is modified) and *Methanosarcina acetivorans* produces high levels of mpa-F_{430} (~20% of total F_{430} pool) when grown on acetate, but the modification is absent when grown on methanol or trimethylamine. These results could indicate that the modifications are produced under low energy conditions.
- With Co-PI Lemkul, we performed extensive molecular dynamics (MD) simulations of MCRs from *M. acetivorans* and ANME-1 in the presence of the canonical F_{430} vs. modified F_{430}s. Our goal was to describe the active site conformational dynamics and how differences in the MCRs from each organism could affect catalysis as well as accommodate specific versions of F_{430}. We found that each MCR active site is optimized for their respective F_{430} species; however, the methanogenic MCR can accommodate modified F_{430}s through active site reorganization.
- To investigate the role of the MCR active site electronic environment in driving the methane synthesis reaction, we further performed electric field calculations based on MD simulations (with methane formation substrates) with *M. acetivorans* MCR and ANME-1 MCRs. Such fields acting on the thioether S-CH_3 bond of CH_3-S-CoM are thought to facilitate its homolytic cleavage. Pronounced differences in the effective electric field were observed in the two systems, which suggests that the two MCRs have differences in catalytic capabilities. Interestingly, the ANME-1 MCR active site better optimizes the electric field, indicating that ANME-1 MCR may have an enhanced catalytic efficiency compared to *M. acetivorans* MCR. Further calculations revealed that five conserved aromatic residues, comprising a hydrophobic cage surrounding CoM and the space between CoM and CoB in the MCR active site, are responsible for up to half of the magnitude of the effective electric field; thus, highlighting the key role of these residues in promoting catalysis.

- A major goal of our work has been to identify the enzyme responsible for installing a methylthio group on F_{430} in ANME-1. The enzymes known to catalyze this type of reaction are radical SAM methylthiotransferases (MTTases). We identified a single MTTase in ANME-1 genomes and biochemically characterized this enzyme. We never observed evidence that this enzyme catalyzes the methylthiolation of F_{430}; instead, this MTTase catalyzes methylthiolation of tRNA substrates. Overall, this work was key towards defining the substrate specificities of MTTases in archaea and provided essential information for the future elucidation of the true biosynthetic machinery for 17^2-methylthio-F_{430} and other thioether F_{430}s.
- We have identified new likely F_{430} binding proteins via native fractionation/purification of proteins from methanogen cells. We hypothesize that one or more of these proteins are important for delivering F_{430} to the MCR active site.

Science objectives for the next year: (2024-2025)

- Reactor-scale growth of methanogens and purification of modified F_{430}s for NMR structural determination
- MCR activity assays with modified F_{430}s compared to canonical F_{430}
- MD simulations with a polarizable force field to define how induced polarization influences cofactor coordination and electric fields in the MCR active site
- Isotope feeding studies to gain insight into the biosynthetic origin of modified F_{430}s in methanogens
- Determine the ability of recently identified putative F_{430} chaperones to bind F_{430} and/or apo-MCR

My scientific area(s) of expertise is/are: anaerobic enzymology and microbiology, methanogen culturing and genetic manipulation, cofactor biosynthesis, radical SAM enzymes

The ideal collaborator for my project would have expertise in: structural biology of metalloenzymes and protein complexes, biophysical techniques to assess small molecule-protein interactions and protein-protein interactions, bioinformatics

Publications supported by this project: (2021-present)

1. Dinh, T-A. and Allen, K.D. (2024) Toward the Use of Methyl-Coenzyme M Reductase for Methane Bioconversion Applications. *Accounts of Chemical Research*. In Press. https://doi.org/10.1021/acs.accounts.4c00413
2. Polêto, M. D., Allen, K. D., and Lemkul, J. A. (2024) Structural Dynamics of the Methyl-Coenzyme M Reductase Active Site Are Influenced by Coenzyme F_{430} Modifications. *Biochemistry* **63**(14), 1783–1794. https://doi.org/10.1021/acs.biochem.4c00168
3. Boswinkle, K., Dinh, T.-A., and Allen, K. D. (2023) Biochemical and Genetic Studies Define the Functions of Methylthiotransferases in Methanogenic and Methanotrophic Archaea. *Frontiers in Microbiology* **14**. https://doi.org/10.3389/fmicb.2023.1304671
4. Gendron, A. and Allen, K.D. (2022) Overview of Diverse Methyl/Alkyl-Coenzyme M Reductases and Considerations for Their Potential Heterologous Expression. *Frontiers in Microbiology* **13**. https://doi.org/10.3389/fmicb.2022.867342

Allosteric control of electron transfer in nitrogenase-like enzymes

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Overall research goals:

Enzymes that catalyze multi-electron substrate reduction reactions are found throughout nature including several enzymes important for nitrogen fixation and photosynthesis. The mechanisms by which these enzymes keep track of electrons accumulated at the active site and accurately transfer them to the substrate are subjects of intense investigation. The biological design of these enzymes and how they mediate long-range electron transfer over a series of sophisticated metal centers have served as blueprints for the design of bio-inspired catalysts. Knowledge of how long-range electron transfer reactions are catalyzed will be directly applicable to engineer designer enzymes capable of reducing substrates. Many such enzymes that catalyze electron transfer reactions are structurally arranged as higher order oligomers and use long-range allosteric control between subunits as a mechanism to regulate activity in response to cellular needs. However, the mechanistic basis of such control is poorly understood. Our lab focuses on the mechanism of action of nitrogenase and nitrogenase-like enzymes, which function as oligomeric complexes that utilize ATP to coordinate electron transfer. Our work uncovered that both these structurally symmetric, multi-subunit, ATP-utilizing enzymes, function asymmetrically and use ATP binding/hydrolysis to establish asymmetry. Our current efforts focus on investigating the origins of allostery within nitrogenase-like enzymes using single-molecule and CryoEM approaches.

Significant achievements: [2021-2024]:

Our efforts to investigate the ATPase coupling of electron transfer in the nitrogenase system revealed an asymmetry and intrinsic negative allostery controlling substrate reduction (Duval et. al. PNAS 2013 and Danyal et. al. PNAS 2016). Since this discovery, we have shown that the principles that govern asymmetric electron transfer in nitrogenase are also conserved in the nitrogenase-like enzyme – dark operative protochlorophyllide oxidoreductase (DPOR). DPOR is a key enzyme in the maturation of bacteriochlorophyll and shares structural homology with nitrogenase. However, they differ in the composition of the metal clusters and a large active site that binds the substrate protochlorophyllide (Pchlide) and reduces it to chlorophyllide (Chlide). We uncovered that the electron donor protein (BchL) uses a disordered N-terminus as a regulatory mechanism for electron transfer (Corless et. al. JBC 2021). In addition, we revealed that substrate binding induced asymmetry in the system (Corless et. al. JBC 2020). Along the way, we also developed cell lines and methodologies to better overproduce FeS containing enzymes (Corless et. al. JBac 2020 and Corless et. al. Methods Mol.Bio). One of our main goals during the current funding period was to use CryoEM to capture snapshots of DPOR during substrate reduction with the objective to uncover why these enzymes use two halves and asymmetric electron transfer activities. We solved several structures of DPOR in the absence and presence of substrate, and during substrate reduction in the presence of ATP. Since protochlorophyllide (Pchlide) is a large porphyrin-like substrate molecule, we are able to capture the conformational changes induced upon substrate binding and electron transfer. In addition, substrate (Pchlide) and product (Chlide) differ by a single double-bond. An overarching question in enzymology remains as to how an enzyme can differentiate between such ring-shaped substrate versus product and control binding of substrate versus release of product. We here provide direct evidence of the enzyme physically bending or puckering the porphyrin ring during turnover. This stunning revelation provides an answer to this age-old question! Key findings about long-range allosteric communication are listed below:

Discovery of a new di-copper cluster in DPOR: In addition to the two known [4Fe-4S] clusters in the donor and acceptor component proteins, we uncover an additional bi-Cu cluster at the tetramer interface of the acceptor (NB-protein). The cluster is essential for substrate reduction, undergoes conformational changes during turnover, and is in the center of the allosteric path of communication. The finding raises the possibility that the current models for substrate reduction need to be revisited.

Asymmetry is inherent to the electron acceptor component protein complex and enhanced upon substrate binding. Where does asymmetry in the system arise? In nitrogenase, binding of the Fe-protein to one half was

proposed as the determinant of asymmetry. In DPOR, we here show that asymmetry is intrinsic to the NB-protein (electron acceptor component) and substrate binding amplifies the asymmetry. Thus, we propose that control of asymmetry is driven from within the active site.

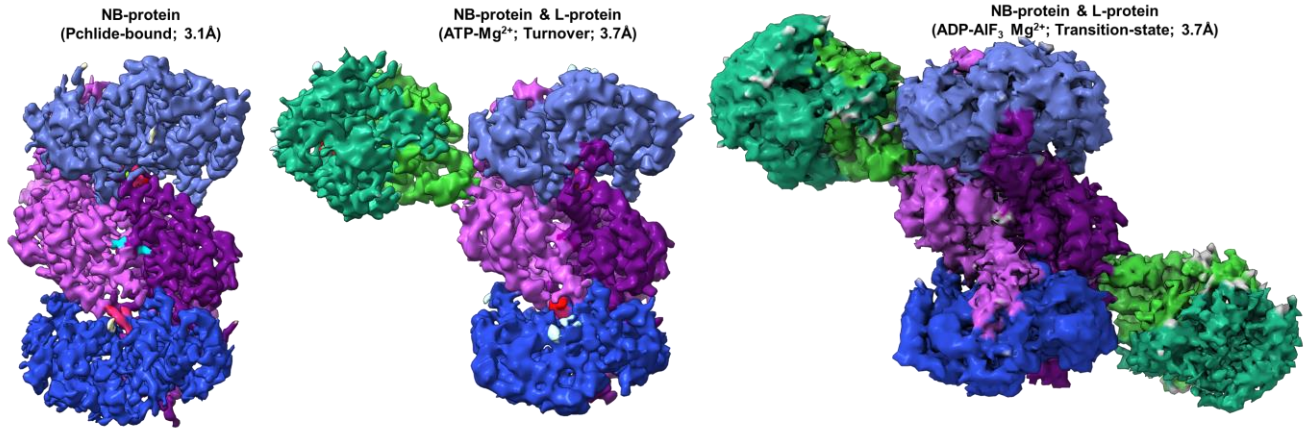
First visualization of an allosteric path of communication emerges from the structures. CryoEM allows us to capture dynamic motions in the protein and thus we can map these changes across the entire complex in the various structures we have solved. Asymmetric movements are captured in all the structures, and they map from one half through to the other and a path of communication is visualized and threads through the bi-Cu cluster in the middle. To our knowledge, this is the first such observation of long-range allosteric and asymmetric communication in large protein complexes.

DPOR differentiates ring-shaped substrate versus product through physical puckering. In enzyme catalyzed reactions, especially for ring-shaped substrates, how are substrate versus product identified within the active sites. We here for the first time directly capture puckering of the porphyrin ring within the active sites.

Substrates are engaged differentially in the two active sites. Interestingly, in the turnover complex, the two substrate molecules are asymmetrically puckered. These data show why asymmetry is needed in this complex. The puckering also enables the enzyme to direct the electron to the specific double bond in the porphyrin ring and to prevent spurious reduction of other double bonds in the substrate.

Binding of donor to one side of the acceptor tetramer prevents engagement of the donor on the other half. In the turnover complex, the electron donor binds to only one half. This binding site is situated 100Å away from the donor binding site on the other half of the electron acceptor component protein. We capture the amino acid changes in both halves and show evidence for how such control is enacted.

A model for asymmetric electron transfer and direct proof for asymmetric electron transfer. Finally, we show that aromatic amino acids serve as a conduit for electron transfer from the donor to the active site of the acceptor. These residues are aligned in one half while misaligned in the other. When aligned, electron transfer occurs and when misaligned, electron transfer is blocked. The ATP-coupled hydrolysis in the donor on one half is used post-electron transfer to misalign the residues to prevent reverse flow of electrons. This energy is used to align the residues in the other half to promote electron transfer.



Science objectives for 2025-2026:

Continue the mechanistic exploration of how electrons are transferred in DPOR. Solve the structure of COR, another nitrogenase-like enzyme that functions subsequent to DPOR.

My scientific area(s) of expertise is/are: Transient-state kinetics, single-molecule fluorescence microscopy, biophysical chemistry, and CryoEM microscopy.

The ideal collaborator for my project would have expertise in: Computational calculations of protein motions. Data collection at DOE cryoEM facility.

Publications supported by this project [2021-2024]:

R. Kashyap, J. Deveryshetty, N. Walsh, M. Tokmina-Lukaszewska, B. Bothner, B. Bennett, and E. Antony. Cryo-EM captures the coordination of long-range allostery and asymmetric electron transfer through a bi-copper cluster in the nitrogenase-like DPOR complex. BioRXiv. (2024). doi.org/10.1101/2024.04.26.590571

E. Corless and E. Antony. Methods for heterologous overproduction of Fe-S proteins. Methods Mol Biol 2353; 69-78 (2021). doi: 10.1007/978-1-0716-1605-5_4.

E. Corless, S. M. Saad Imran, M.B. Watkins, J.P. Bacik, J.R. Mattice, A. Patterson, K. Danyal, M. Soffe, R. Kitelinger, L.C. Seefeldt, S. Origanti, B. Bennett, B. Bothner, N. Ando, and E. Antony. The flexible N-terminus of BchL autoinhibits activity through interaction with its [4Fe-4S] cluster and released upon ATP binding. J. Biol. Chem. 296:100107 (2021). doi: 10.1074/jbc.RA120.016278.

J. Deveryshetty, and E. Antony. Electrons and Protons: Nitrogenase. EBC. 2:586-595. (2021). doi.org/10.1016/B978-0-12-819460-7.00246-2.

J. Deveryshetty, G.R. Sorg, and E. Antony. Dark-operative protochlorophyllide oxidoreductase. EiBC. (2022). doi.org/10.1002/9781119951438.eibc2820

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

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Overall research goals:

The overall goal of our research is to enhance our understanding of rhamnogalacturonan I (RGI), an important and extremely complex pectin polysaccharide present in plants. We are approaching the goal of this research on two separate but interconnected tracks: (1) through the development of analytical methods to determine the structure(s) of RGI and (2) through elucidating the biosynthesis of RGI. Structural studies focus on using enzymatic and chemical derivatization strategies, alone or in combination with ionic liquids, to solubilize insoluble RGI fractions for composition and structure analysis by mass spectrometry and NMR. The goals of the RGI biosynthesis studies are to identify the enzymes that add the glycosyl and non-glycosyl substituents during the synthesis of the RGI backbone and side chains. The enabling knowledge and technology developed will be applied towards the structural characterization of other insoluble or sparingly soluble plant and microbial polysaccharides through our many collaborative efforts of the DOE Center. In addition, we also disseminate our methods and protocols through hands-on and virtual training courses/workshops.

Significant achievements: 2023-2024:

- We applied our enzymatic toolbox to elucidate the most detailed structure of RGI to date of the pectins in the walls of *Arabidopsis thaliana* by NMR, ESI-MS, and MALDI-TOF MS. We found HG in Arabidopsis was substituted with acetyl and pentosyl residues and methyl-esterified, especially adjacent to RGI. Arabidopsis RGI had galactan, arabinan, and arabinogalactan side chains and was acetylated on both Rha and GalA. Rha was acetylated at O-3 with acetyl moieties, and galactan sidechains were decorated with GlcA or MeGlcA.
- We completed studies using acetylation in ionic liquid for accurate glycosyl composition and linkage analysis of insoluble and acidic polysaccharides. We optimized our methylation protocol for larger quantities of samples needed for NMR analysis and minimized the amount of β-elimination during permethylation. NMR analysis of permethylated cress mucilage enabled detailed structural characterization of its glycans and led to the discovery of novel RGI and non-pectin structures. Furthermore, we developed a method to determine the location of *O*-acetyl substituents in RGI.
- We identified duckweed RGI and apiogalacturonan containing pectic AGPs that form borate diesters with each other and with the pectin RGII, offering an explanation for plant resistance to breakdown in high pH water.
- We investigated the activities of two TBLs. We have made transgenic Arabidopsis plants that overexpress these two genes and plants that express GUS and GFP, whose expression is driven by the TBL promoters, to study the expression patterns of these two genes. In addition, we have used CRISPR to knock out four RRT genes to investigate the biological function of RGI *in planta*.
- The first galacturonosyl transferase biochemically shown to catalyze the addition of galacturonic acid into the RGI backbone, RGGAT1, was identified and shown to catalyze the synthesis of polymeric RGI backbone *in vitro*. RGGAT1 is the founding member of GT family GT116, has a predicted GT-A fold structure, and employs a metal-independent mechanism which is rare among GTs with a GT-A fold.
- We used molecular dynamics models to study the bifunctional activity of GAUT13 and GAUT14, two pectin HG biosynthetic enzymes that have both acceptor dependent and *de novo* synthesis activity. This led to the identification of nine single site amino acid mutations in GAUT14 that affected pectic oligosaccharide acceptor binding.
- We have collaborated with over 145 groups all over U.S and internationally applying the methods we have developed at the DOE Center to number of plant and microbial glycoconjugates. To make our tools and methodologies available to investigators outside of the Center, we have offered 4 hands-on and 2 virtual training courses in glycoscience attended by over 119 and 973 course participants respectively.

Science objectives for 2024-2025:

- We plan to validate a linkage method using NMR of partially methylated monosaccharides and develop a simpler method for quantifying cellulose/hemicellulose from insoluble polysaccharides. We will explore ionic liquids as NMR solvents for plant cell wall polysaccharides, including RGI, and continue investigating acetyl location by MS and NMR of permethylated polysaccharides, particularly RGI.
- We will further study the RGGAT family, collaborating with Takeshi Ishimizu to characterize new RGGAT members with RGI activity. We will scale up HEK293 protein cultures to purify proteins for enzyme characterization, substrate specificity, and complex pectin structure analysis.

My scientific area(s) of expertise is/are: Carbohydrate structural characterization of plant and microbial polysaccharides by MS, HPLC, and NMR. Carbohydrate chemistry; plant cell wall pectins and glycoproteins. Pectin and hemicellulose biosynthesis and structure. Characterization of carbohydrate active enzymes. Pectin function. Purification of glycosyltransferases and polysaccharides. Biochemical characterization of glycosyltransferase enzyme function.

The ideal collaborator for my project would have expertise in: Mutagenesis of biomass, targeted enzymatic degradation of plant polysaccharides. Atomic force microscopy, biomechanics and protein crystallography, biological function of carbohydrates. .

Selected publications supported by this project 2023-2024 (from a total of 54):

1. Delmer, D., Dixon, R.A., Keegstra, K., Mohnen, D. "The plant cell wall - dynamic, strong and adaptable – is a natural shapeshifter." Invited review for *The Plant Cell* 36(5):1257–1311 (2024).
2. Shahin, L., Zhang, L., Mohnen, D., Urbanowicz, B.R. "Insights into Pectin O-acetylation in plant cell wall: structure, synthesis, and modification." The Cell Surface 9: 100099 (2023). DOI: 10.1016/j.tcsw.2023.100099
3. Mohnen, D. Atmodjo, M. A., Jayawardhane, P. "Reconsidering pectin structure: A historical perspective informed by identity and function of the biosynthetic enzymes." In The Plant Cell Wall – Research Milestones and Conceptual Insights. Ed. Anja Geitmann, CRC Press/Taylor & Francis Group, LLC. Chapter 5, pages 94-126 (2024).
4. Poulhazan, A., A.A. Arnold, F. Mentink-Vigier, A. Muszyński, P. Azadi, A. Halim, S.Y. Vakhrushev, H.J. Joshi, et al. "Molecular-level architecture of chlamydomonas reinhardtii's glycoprotein-rich cell wall." Nature Communications **15,** 986 (2024). DOI: 10.1038/s41467-024-45246-7
5 Backman, T., S.M. Latorre, E. Symeonidi, A. Muszyński, E. Bleak, L. Eads, P.I. Martinez-Koury, S. Som, et al. "A phage tail–like bacteriocin suppresses competitors in metapopulations of pathogenic bacteria." Science **384,** eado0713 (2024). DOI: 10.1126/science.ado0713.
6. Bruni, G.O., Y. Qi, E. Terrell, R.A. Dupre, C.P. Mattison "Characterization of levan fructan produced by a gluconobacter japonicus strain isolated from a sugarcane processing facility." Microorganisms **12,** 107 (2024). DOI: 10.3390/microorganisms12010107.
7. Cyran, M.R., K.K. Snochowska, M.J. Potrzebowski, S. Kaźmierski, P. Azadi, C. Heiss, L. Tan, I. Ndukwe, et al. "Xylan-cellulose core structure of oat water-extractable β-glucan macromolecule: Insight into interactions and organization of the cell wall complex." Carbohydr. Polym. **324,** 121522 (2024). DOI: 10.1016/j.carbpol.2023.121522.
8. Ndukwe, I.E., I. Black, C.A. Castro, J. Vlach, C. Heiss, C. Roper, P. Azadi "Permethylation as a strategy for high-molecular-weight polysaccharide structure analysis by nuclear magnetic resonance—case study of xylella fastidiosa extracellular polysaccharide." Magn. Reson. Chem. **62,** 370-377 (2024). DOI: 10.1002/mrc.5413.
9. Tan, L., J. Cheng, L. Zhang, J. Backe, B. Urbanowicz, C. Heiss, P. Azadi "Pectic-agp is a major form of arabidopsis agps." Carbohydr. Polym. **330,** 121838 (2024). DOI: 10.1016/j.carbpol.2024.121838.
10. Black, I.M., I.E. Ndukwe, J. Vlach, J. Backe, B.R. Urbanowicz, C. Heiss, P. Azadi "Acetylation in ionic liquids dramatically increases yield in the glycosyl composition and linkage analysis of insoluble and acidic polysaccharides." Anal. Chem. **95,** 12851-12858 (2023). DOI: 10.1021/acs.analchem.3c02056.
11. Tan, L., M. Ishihara, I. Black, J. Glushka, C. Heiss, P. Azadi "Duckweed pectic-arabinogalactan-proteins can crosslink through borate diester bonds." Carbohydr. Polym. **319,** 121202 (2023). DOI: 10.1016/j.carbpol.2023.121202.

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

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Overall research goals:

An interdisciplinary team of 11 investigators and talented scientists with complementary expertise has come together with the long-term goal of exploring basic processes of light capture and conversion into products sustaining the growth of cyanobacteria, algae, and plants. Collectively, we strive to gain an understanding of the natural dynamic aspects of photosynthesis that ensure the robustness of photosynthetic processes in nature under environmental conditions ranging from extreme temperatures to biotic insults. Furthermore, the need of photosynthetic organisms to cope with varying conditions may limit their productivity in agricultural settings. We are developing strategies to overcome these limitations through the engineering or recombination of photosynthetic modules to meet current and future challenges. We expect that gaining a multiscale mechanistic photosynthetic knowledge will allow us to improve photosynthetic efficiency and, therefore, plant productivity, and to expand the production of photosynthesis-based bioproducts.

The project transects different scales of time and space (Fig. 1) and is organized accordingly into three Subprojects ranging from photon/electron processes, to the molecular, cellular and to the organismal scales. Processes covered include the interaction of photons with pigment protein complexes, redox reactions and chemical conversions, which are carried out by mesoscale photosynthetic modules such as bacterial microcompartments and large protein complexes including phycobilisomes. Chloroplasts harbor the photosynthetic machinery in plants and algae, and in turn are functionally connected to other organelles, requiring an analysis of photosynthesis in the context of cells and ultimately the organism in its environment for a comprehensive, multiscale understanding of photosynthetic processes. Representing these three different scales, the research is grouped into three themes: The first addresses photosynthetic responses to changing environmental conditions, particularly those that generate reactive oxygen species. The second theme emphasizes the concept of modularity within photosynthesis, either by mesoscale studies of carbon concentrating mechanisms or antenna complexes, or through genetic engineering of new components into chloroplasts. The third theme integrates photosynthetic processes into a cellular context, such as managing photorespiration and carbon fluxes throughout metabolism.

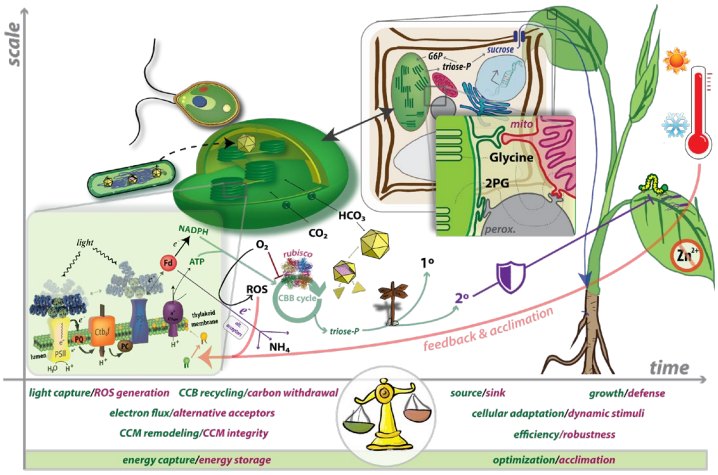

Figure 1. Studying photosynthesis across all scales

Significant achievements: Award Years 2023-2024:

In the following, selected Research Highlights of work published in 2023/2024 and funded by the award are summarized. They are organized approximately according to the three Subprojects. Collaborators are indicated, often exemplifying cases of synergy between the research teams; DOI numbers are given:

• We related the size and shape of cytochrome nanowires to their potential for electron transfer, highlighting that molecular motions on the nanoscale can interfere with electron hopping within protein crystals. Kulke M, Olson DM, Huang J, Kramer DM, Vermaas JV; doi:10.1002/smll.202304013:e2304013

• Using results from a novel platform that can carry electrons over large distances through biomolecules in protein crystal lattices, we tested the applicability of electron transfer (ET) theories developed for short distance over mesoscopic to macroscopic scales. The results show that commonly used theory fails to explain the temperature dependence for ET over long distances, pointing to a major gap in our understanding of this critical process. Parson WW, Huang J, Kulke M, Vermaas JV, Kramer DM; doi: 10.1063/5.0186958

• We characterized a novel aromatic substrate-processing microcompartment in Actinobacteria. Doron L, Sutter M, Kerfeld; doi: 10.1128/mbio.01216-23

• We introduced bacterial micro compartment-derived protein scaffolds into chloroplasts as a first step to engineering chloroplast nanofactories. Dwyer ME, Froehlich JE, Raba DA, Borrusch M, Danhof L, Sharma N, Young EJ, Brandizzi F, Benning C, Kerfeld CA; doi:10.1111/pbi.14462

• We compared pore structure and dynamics for bacterial microcompartment shell protein assemblies in sheets or shells. Raza S, Sarkar D, Chan LJG, Mae J, Sutter M, Petzold CJ, Kerfeld CA, Ralston CY, Gupta S, Vermaas JV; doi: 10.1021/acsomega.4c02406

• Using a new genetic approach, we identified regulatory proteins that repress the biosynthesis of energy-rich defense compounds. Johnson LYD, Major IT, Chen Y, Yang C, Venegas-Cano LJ, Howe GA; doi:10.1111/nph.19114

• We discovered the Coordination of carbon partitioning and photosynthesis by a two-component signaling network in *Synechococcus elongatus* PCC 7942. Santos-Merino M, Sakkos JK, Singh AK, Ducat DC; doi: 10.1016/j.ymben.2023.11.001

• Two chloroplast-localized lipid phosphate phosphatases were shown to be involved in the ER pathway of galactolipid biosynthesis in Arabidopsis. Their activity was also determined to be important for plant growth. Cook R, Froehlich JE, Yang Y, Korkmaz I, Kramer DM, Benning C.; doi:10.1093/plphys/kiae100

• We provided evidence for the nature of ER-chloroplast contact sites. Two ER membrane-associated VAP27 proteins, VAP27-1 and VAP27-3, interact with ORP2A at ER subregions interfacing with chloroplasts, where they preferentially accumulate and physically associate with the chloroplast outer envelope membrane and affect sterol metabolism. Renna, L., Stefano, G., Puggioni, M.P., Kim S-J, Lavell A, Froehlich JE, Burkart G, Mancuso S, Benning C, Brandizzi F; doi: 10.1038/s41467-024-50425-7

• We investigated the responses of growth, photosynthesis, leaf metabolism, partitioning, and metabolic fluxes to daylength variation. Xu Y, Koroma AA, Weise SE, Fu X, Sharkey TD, Shachar-Hill Y; doi:10.1093/plphys/kiad507)

• Because photosynthetic CO_2 response curves are fundamental for the understanding of leaf physiology. we tested the precision and accuracy of a newly developed Dynamic Assimilation Technique (DAT). Tejera-Nieves M, Seong DY, Reist L, Walker BJ; doi: 10.1093/jxb/erae057

• The source of carbon for the process called respiration in the light, carbon dioxide loss during photosynthesis, was tested. All carbon originated in the cytosol of cells. Xu Y, Schmiege SC and Sharkey TD; doi:10.1111/nph.19730

Science objectives for 2025-2026:

We will continue working towards the goals in the three thematic areas outlined above, and we will develop new concepts towards the renewal of the award, building on current accomplishments and taking into account new expertise and talent joining the Plant Research Laboratory during the coming year.

Electron Bifurcation Theory

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Overall research goals:

We are using theory, modeling and simulation to address the following questions about the molecular mechanisms that underpin electron bifurcation reactions in living systems: How does domain motion influence electron bifurcation mechanisms? How does the protein microenvironment determine cofactor reduction potentials, and are the specific reduction states of one cofactor communicated to the other sites using QM/MM? What is the influence of correlated multi-electron motion and of the finite number of electron-reservoir species in the cell on electron bifurcation mechanisms?

Significant achievements (2023 -2024):

In the last year, we have: (1) improved our electron-transport model for electron bifurcation (EB), taking into account finite reservoir size effects and redox pathway thermodynamics, (2) performed bioinformatics and electrostatics analysis to explore correlations between protein structure and inverted potentials at the bifurcating cofactor site, and (3) developed cofactor forcefield parameters for atomistic QM/MM studies to understand the relationship between protein structure and cofactor redox potentials in EB proteins.

An open-system many-particle electron-transport and bifurcation model was developed earlier by our group to understand the physical principles that eliminate short-circuiting electron transport in electron bifurcating networks. Our model relied on bifurcating networks in contact with infinite reservoirs. The infinite reservoir model describes steady-state electron fluxes well, but it fails to describe free energy changes for electron bifurcation since the free energy of microstate transitions is path dependent. In the last year, we developed a model for bifurcation in redox networks that are coupled to finite pools of redox species. This approach resolves the energy path-dependence issue and successfully describes bifurcation kinetics. Using finite pools is also advantageous for modeling biological systems in contact with intrinsically limited redox substrate pools.

Biology recruits a small number of redox cofactors, and tunes their potentials and cofactor-cofactor tunneling interactions to carry out the required redox chemistry. Flavins can perform one- or two-electron chemistry, and the potential ordering can be normal or inverted. The gap between the two potentials can be as large as one Volt. Using structural bioinformatics and computational (continuum) electrostatics, we found that introducing negative charges near the flavin increases the redox potential inversion by increasing the free energy of the second electron-transfer step; adding positive charges near the flavin reinforces the normal ordering of the redox potentials by decreasing the free energy of the second-electron step in flavodoxins and bifurcating flavin structures.

An atomistic-level understanding is lacking for how electron bifurcating protein structures tune the cofactor reduction potentials and promote potential inversion. Using a combination of long-timescale molecular dynamics, electron-transfer theory, and QM/MM simulations of structural and energetic

fluctuations, we are working toward understand the control of electron flow in Nfn1, which is among the simplest EB systems known. We have developed potential models for flavins, NADPH, and FeS clusters (the five charge carrying cofactors in Nfn1) to be used in molecular dynamics simulations. We now have a working forcefield compatible with AMBER14 for all Nfn1 cofactor redox states, which will be used to probe redox state dependent conformations of Nfn1. These studies are being carried out in collaboration with researchers at NREL (Lubner) and PNNL (Raugei, Ginovska, Baer). Our Duke graduate student was awarded a 2025 DOE SCGSR Fellowship in support of these studies.

Science objectives for 2024-2025:

Over the next year, we will use our finite reservoir model to analyze HydABC in order to understand how EB function is influenced by conformational change. We will use our electrostatics framework to determine how the changes in the flavin protein environment may switch flavodoxin reduction potentials from normal to inverted. We will analyze FldA, an unusual flavodoxin with a stable anionic semiquinone, and will use QM/MM analysis to calculate redox potentials for the first and second electron-transfer steps for wild-type and mutant species. With the new classical force-field parameters we have developed for the redox cofactors in Nfn1, we will study how the reduction potentials of the cofactors in Nfn1 in the accessible redox states, and will explore how they are tuned by the local protein environment. We will calculate the reduction potentials of the five redox cofactors using classical molecular dynamics redox-state dependent conformational sampling with vertical energy calculations derived from QM/MM methods.

My scientific areas of expertise are: Theory, modeling and simulation of biological electron transport structures, including those that bifurcate electrons.

The ideal collaborator for my project would have expertise in: Experimental experts in biochemistry, biophysics, and structural biology of electron bifurcating proteins. Experts in hybrid QM/MM methods for treating multi-cofactor proteins.

Publications supported by this project (2023-2024):

K. Terai, K. Parker, A. J. Smith, D. N. Beratan, "Simulating electron bifurcation with finite electron reservoir models." in preparation (2024).

N. Singh, P. Zhang, D. N. Beratan, "The influence of protein electrostatics on potential inversion in flavoproteins." in preparation (2024).

Probing novel pathways of metal sulfide acquisition and trafficking from minerals in model biocatalytic systems

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Devon Payne, Manjinder Kour, Michael Dulay, Gahinger Alam, James Larson, Postdoctoral Research Associates
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Overall research goals:

Pyrite (FeS_2) is the most abundant iron sulfide in Earth's crust and is a reservoir of trace elements of national strategic importance including nickel (Ni), cobalt (Co), and molybdenum (Mo). Traditional methods to extract trace elements from pyritic ores employ oxidative leaching that generates acid. In our initial DOE supported project, we showed that methanogens can reductively dissolve FeS_2 through a process that does not generate acid. During reduction of FeS_2, methanogens assimilate dissolution products to meet their iron (Fe) and sulfur (S) demands, including for biosynthesis of simple and complex metalloclusters. Our DOE renewal seeks to forge new understanding of the mechanisms used by methanogens to reductively dissolve FeS_2 and assimilate, traffic, and bioconcentrate Fe. Further, our data indicate that methanogens can acquire and bioconcentrate Ni, Co, and Mo from FeS_2. Given the growing need for economic and domestic Ni, Co, and Mo sources, this motivates additional experiments to characterize the mechanisms used by methanogens to acquire, traffic and bioconcentrate these metals from FeS_2. A suite of physiological, biochemical, -omic, and spectroscopic approaches are being combined to address these research objectives. Further, state of the art imaging and computational approaches are being used to address the reaction mechanisms, kinetics, and chemical transformations that take place at the surface of FeS_2 during reduction, focusing on molecular interactions at the mineral-water-cell interface. Through this integrated approach, we aim to identify the enzymes and pathways that allow methanogens to bio-mine Fe, Ni, Mo, Co, and S from FeS_2 ore to enable the cost-effective recovery and/or conversion of raw substrate into catalysts for electrochemical bioenergy generation.

Significant achievements: 2022-present:

• ***Physiology***. Sulfide is commonly used as the sulfur source for anaerobes, yet sulfide markedly decreases the solubility/availability of trace metals for methanogen, a phenomenon that was shown to drastically alter the metalloproteome of cells. Further, data indicate that methanogens can synthesize all nitrogenase metallocofactors from FeS_2 and molybdate ion. Growth was enhanced in this condition relative to cells grown with soluble Fe(II), sulfide, and molybdate which was attributed to the higher bioavailability of molybdate when grown with FeS_2. This was due to thiolation of molybdate ion to form tetrathiomolybdate and ultimately molybdenum sulfide, the latter of which is less bioavailable.

• ***Computational Chemistry and Geochemistry.*** Atomic scale models of FeS_2 nanoparticles have been built, validated, and were used to model surface transformations and reactivity during reduction. The model for FeS_2 reduction suggests initial release of sulfide into solution is concomitant with the precipitation of pyrrhotite ($Fe_{1-x}S$) on the mineral surface. Dissolved Fe, but not S, from $Fe_{1-x}S$ then combines with sulfide in solution to form aqueous iron sulfur clusters (FeS_{aq}) that are neutrally charged and likely passively diffuse into the cell. To examine the kinetics of FeS_{aq} cluster growth, dynamic light scattering was used to reveal the influence of temperature, Fe(II)/HS^- concentration, pH, and ligand environment on FeS_{aq} growth kinetics; such information provides constraints on FeS_{aq} utilization by cells. FeS_2 was synthesized that contains Co-, Ni-, and Co/Ni impurities for additional growth studies.

• ***Spectroscopy.*** Experiments (EPR, Mössbauer, UV-Vis, XAS) on purified IssA homologs or on whole cells heterologously expressing these proteins highlight their role in metal bioaccumulation. Comparison of spectroscopic data from laboratory synthesized minerals to that coordinated by IssA is narrowing the

candidates for identification of the biomineralization product. Expression of IssA homologs in E. coli affects growth kinetics and metal accumulation in those cells.
• ***Biochemistry***. Several conserved and differentially expressed proteins identified in FeS_2 versus ferrous iron/sulfide-grown cells are being heterologously expressed (*E. coli*) and functionally characterized. This includes DUF2193 and two novel radical SAM domain proteins, including one with what is termed a SPASM domain likely involved in post-translational modification of cysteine-rich proteins..

Science objectives for 2024-2025:

- Determine the effect of $NiFeS_2$, $CoFeS_2$, and $Ni/CoFeS_2$ on growth of methanogens, metal bioaccumulation, and chemotaxis behavior
- Document growth of methanogens on geochemically relevant sources of Mo
- Evaluate the influence of heterometals, light, and organic ligands on the reactivity of FeS_2
- Expand the FeS_2 reduction and FeS_{aq} utilization phenomenon to other microbial groups.
- Quantify the effect of protein ligands on FeS_{aq} coordination

My project addresses BES cross-cutting priority areas by:
Our research combines physiological, molecular, biochemical, computational, and geochemical approaches with a suite of cutting-edge imaging and spectroscopic physical science techniques to characterize the mechanisms of biological FeS_2 reduction and the proteins involved in Fe/Ni/Co/Mo/S acquisition, trafficking, and storage. Since metal sulfides form key components of metalloenzyme active sites that function in the conversion of light or electrical energy to potential energy in the form of chemical bonds, this work has direct relevance to understanding the underlying physical and chemical principles that govern how microbes capture, convert, and store energy via metalloenzymes (electrocatalysts). Further, our work aims to improve mechanistic understanding at the interface of the physical bio- and geo-sciences (project co-funded by physical biosciences and geosciences) by focusing on how Earth abundant pyrite mineral can be transformed into highly tuned electrocatalysts.

My scientific area(s) of expertise is/are: Physiology, geomicrobiology, bioinformatics, evolution.

The ideal collaborator for my project would have expertise in: Electrochemistry, XAS/XANES

Publications supported by this project 2022 to present :

1. R. Spietz, D. Payne, R. Szilagyi, E. Boyd. "Reductive biomining of pyrite by methanogens." Trends in Microbiology. **30**, 1072-1083. (2022) [10.1016/j.tim.2022.05.005]
2. K. Steward, D. Payne, W. Kincannon, C. Johnson, M. Lensing, H. Fausset, B. Németh, E. Shepard, W. Broderick, J. Broderick, J. Dubois, B. Bothner. "Proteomic analysis of *Methanococcus voltae* grown in the presence of mineral and nonmineral sources of iron and sulfur. Microbiology Spectrum. **10**, e0189322. (2022) [10.1128/spectrum.01893-22]
3. R. Spietz, D. Payne, E. Roden, G. Kulkarni, W. Metcalf, E. Boyd. "Investigating abiotic and biotic mechanisms of pyrite reduction." Frontiers in Microbiology. **13**, 878387. (2022) [10.3389/fmicb.2022.878387]
4. D. Payne, R.L. Spietz, D.L. Newell, P.J. Dykstra, and E.S. Boyd. 2023. "Influence of sulfide on diazotrophic growth of a methanogen and its implications for the origin of nitrogenase." Communications Biology. (2023) **6**, 799. [10.1038/s42003-023-05163-9]
5. R.L. Spietz, D. Payne, and E.S. Boyd. "Methanogens acquire and bioaccumulate Ni during reductive dissolution of nickelian pyrite." Applied and Environmental Microbiology. **89**, e00991-23. (2023) [10.1128/aem.00991-23]
6. M. Kour, A. Táborosi, E.S. Boyd, and R.K. Szilagyi. "Development of molecular cluster models to probe pyrite surface reactivity." Journal of Computational Chemistry. **44**, 2486-2500. (2023) 10.1002/jcc.27213.

   Larson, J., M. Tokmina-Lukaszewska, R.L. Spietz, D. Payne, H. Fausset, M.D. Alam, B. Brekke, J. Pauley, E. Hasenöhrl, E.M. Shepard, E.S. Boyd, and B. Bothner. "Impact of mineral and non-mineral sources of iron and sulfur on the metalloproteome of *Methanosarcina barkeri.*" Applied and Environmental Microbiology. **90**, e00516-24. (2024) [10.1128/aem.00516-24].

Mechanisms and Pathways 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 biogenesis of the active site metal cluster of [FeFe]-hydrogenase, an efficient catalyst for hydrogen production. We use biochemical and biophysical approaches to elucidate the reactions catalyzed by the three specific hydrogenase maturase enzymes that are common to all organisms that harbor the [FeFe]-hydrogenase. This research promotes our knowledge of structure/function relationships in complex biological pathways for metal cluster assembly, while additionally contributing to our understanding of how microbes build the protein cofactors that enable them to capture and convert energy. Moreover, as the steps associated with complex metallocofactor biosynthesis are better understood, the knowledge gained will inspire and influence the design of new biomimetic catalysts with applications in biohydrogen technologies.

The active site metal cluster of [FeFe]-hydrogenase, referred to as the H-cluster, is a specialized iron-sulfur cluster consisting of a [4Fe-4S] cubane bridged to a 2Fe subcluster that has three carbon monoxide, two cyanide, and a bridging dithiomethylamine group as ligands. Biogenesis of this specialized cluster requires the combined actions of three specific maturation enzymes, denoted HydE, HydF, and HydG. Two of these enzymes (HydE and HydG) are radical-SAM enzymes and use *S*-adenosylmethionine (SAM) and a redox active [4Fe-4S] cluster to initiate radical chemistry. The third protein, HydF, is a GTPase that functions as a scaffold/carrier during H-cluster assembly. Research supported by this DOE project has allowed us to gain new insights into the biochemical and spectroscopic properties of the maturase enzymes and this has in turn informed our understanding of hydrogenase maturation. Recently, we have made a major advance by developing the first fully-defined system that supports in vitro maturation of the [FeFe]-hydrogenase to an active enzyme, a development that has enabled significant new insights into this complex process.

The aim of this project is to develop a molecular-level understanding of the reactions catalyzed by HydE, HydF, and HydG as well as the interactions between the different maturases, in order to clearly delineate the mechanistic chemistry, the order of events, and the protein-protein interactions involved during H-cluster biosynthesis. *Specifically, the current project objectives are 1) to determine the role of iron and HydG during maturation, 2) to provide functional and mechanistic insight into HydE and the other maturases by employing our fully-defined maturation system, and 3) to probe the biological pathway of maturation.* This work stands to reveal fundamentally unique biochemical transformations and will help define new paradigms for complex metal cluster assembly in biology. Further, the work will provide understanding and insight needed to develop biohydrogen catalysts employing the [FeFe]-hydrogenase.

Significant achievements: 2022-2024:

- We have developed a fully-defined in vitro maturation system for the [FeFe]-hydrogenase. By including components of the glycine cleavage system (H-protein and T-protein) as well as serine hydroxymethyltransferase, serine, and ammonium, we were able to eliminate cell lysate while still achieving high levels of maturation. We used this system to demonstrate that the C of the DTMA ligand of the H-cluster arises from serine, while the bridgehead N arises from ammonium under these conditions. These results provide new insights into the missing components previously provided by the absolutely essential cell lysate during maturation.

- We demonstrated that by using the synthetic compound $[Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$ and our defined maturation system, we could bypass the maturases HydE and HydG, and achieve [FeFe]-hydrogenase maturation using only HydF, demonstrating that the radical SAM enzymes HydE and HydG are not needed to synthesize DTMA, and that the glycine cleavage system components are sufficient to synthesize DTMA on HydF.
- We used bioinformatics as well as biochemical and spectroscopic approaches to show that amino acid ammonia lyases such as aspartate ammonia lyase and serine ammonia lyase are important sources of the ammonia needed for DTMA biosynthesis.
- We have shown that Fe/S carrier proteins can replace the high concentrations of Fe(II) previously used in defined in vitro maturations, and that these proteins are able to reconstitute maturase iron-sulfur clusters and the dangler iron of HydG.
- We have shown that the high-CO-affinity H64L variant of Mb significantly enhances in vitro maturation by binding free CO to prevent formation of the CO-inhibited state of the [FeFe]-hydrogenase.
- By including Fe/S carrier proteins and Mb H64L in maturation reactions, we are able to achieve wild-type levels of [FeFe]-hydrogenase activity, indicating we are achieving full maturation.
- We have demonstrated that the [4Fe-4S] cluster of HydF is essential during semisynthetic maturation of HydA using $[Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$, however coordination of the synthetic cluster to the [4Fe-4S] cluster is not essential.

Science objectives for 2025-2026:

- Trap and characterize biosynthetic intermediates formed during maturation on each of the maturases, HydG, HydE, HydF.
- Evaluate whether synthetic compounds are on-pathway: generate and characterize enzymatically- and semisynthetically-loaded HydF and characterize to evaluate any differences.
- Characterize protein-protein interactions important to maturation.
- Pursue structures of protein complexes relevant in maturation.

My scientific area(s) of expertise is/are: bioinorganic chemistry, iron-sulfur clusters, biological radical catalysis and mechanisms, EPR spectroscopy.

The ideal collaborator for my project would have expertise in: protein-protein interactions and biophysical approaches to examine them, cryo-EM and other structural approaches.

Publications supported by this project 2022-2024:

1. Adrien Pagnier, Batuhan Balci, Eric M. Shepard, Hao Yang, Douglas M. Warui, Stella Impano, Squire J. Booker, Brian M. Hoffman, William E. Broderick, and Joan B. Broderick, "[FeFe]-Hydrogenase: Defined Lysate-Free Maturation Reveals a Key Role for Lipoyl-H-Protein in DTMA Ligand Biosynthesis," *Angew. Chem. Int. Ed.* **2022**, *61*, e202203413. [DOI 10.1002/anie.202203413]
2. Adrien Pagnier, Batuhan Balci, Eric M. Shepard, William E. Broderick, and Joan B. Broderick, "[FeFe]-Hydrogenase In Vitro Maturation," *Angew. Chem. Int. Ed.* **2022**, *61(49), e202212074*. [DOI 10.1002/anie.202212074]
3. Batuhan Balci, Roark D. O'Neill, Eric M. Shepard, Adrien Pagnier, Alexander Marlott, Michael T. Mock, William E. Broderick, and Joan B. Broderick, "Semisynthetic Maturation of [FeFe]-hydrogenase using $[Fe_2(\mu\text{-}SH)_2(CN)_2(CO)_4]^{2-}$: Key Roles for HydF and GTP," *Chem. Commun.* **2023**, *59*, 8929-8932. [DOI: 10.1039/D3CC02169F]
4. Adrien Pagnier, Batuhan Balci, Eric M. Shepard, Hao Yang, Alex Drena, Gemma L. Holliday, Brian M. Hoffman, William E. Broderick, and Joan B. Broderick, "Role of Ammonia Lyases in the Synthesis of the Dithiomethylamine Ligand During [FeFe]-Hydrogenase Maturation," *J. Biol. Chem.* **2024**, *in press*. [DOI: 10.1016/j.jbc.2024.107760]

Elucidating the Cellular Machinery for Lipid Storage in Plants

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Overall research goals:

Lipids are among the most energy-dense organic molecules on the planet, and the "fossil" deposits of these plant- and microbial-derived lipids make up the basis of our global energy enterprise. A thorough understanding of the molecular mechanisms by which photosynthetic organisms capture, convert and store reduced carbon could provide important insights necessary to develop renewable, bio-based forms of usable energy. Among cellular processes involved in the conversion and storage of reduced carbon, perhaps the least well understood are the mechanisms for packaging neutral lipids into subcellular compartments called lipid droplets (LDs) for stable deposition within the aqueous environment of plant cells. **Our overarching goal is to understand the fundamental biochemical and cellular processes important for compartmentalization of storage lipids in plant tissues, ultimately to enable dramatic increases in the energy storage capacity of plants.** Building on research advances with prior support, we have identified a core of interacting LD biogenesis proteins, but the information on how these proteins interact and function to efficiently package lipids into LDs remains to be elucidated. With renewed funding in September 2023, we are investigating their interaction and participation in cellular lipid storage with the following research objectives: **1) Determine the structural and functional organization of SEIPIN isoforms and the core LD biogenesis machinery; 2) Determine the functional interactions of additional subcellular factors required for LD formation *in vitro* and *in vivo*; and 3) Identify the mechanistic similarities and differences for the compartmentalization of other energy-dense lipids beyond common triacylglycerols.**

Significant achievements: to date for project supported from 2023-2026

1. Demonstrated physical interactions among the same and different isoforms of Arabidopsis SEIPIN proteins that form an oligomeric complex at the endoplasmic reticulum (ER)-LD junction, suggesting that plant SEIPINs may form both homomeric and heteromeric complexes. Two Arabidopsis SEIPIN isoforms have been successfully expressed in a heterologous yeast expression system, which will be used for subsequent determination of the stoichiometry and structural organization of plant SEIPIN complexes. A proposal for access to the cryo-electron microscopy (Cryo-EM) facilities at Brookhaven National Laboratory was approved recently with training and user-time resources allocated for our project from July 2024 to June 2026.

2. Identified a physical and functional interaction between the LD biogenetic protein SEIPIN1 and the microtubule-localized DRP1A, a member of the dynamin-related protein family. To the best of our knowledge, this is the first time a functional role for DRP1A in lipid droplet biogenesis has been demonstrated in any organism. We are now working with our collaborators to complete and prepare this exciting finding for publication. Dr. Yingqi Cai was invited to present this work at the International Symposium on Plant Lipids in July 2024.

3. Identified a novel LD-related protein, Lipid Droplet Protein in Seeds (LDPS), and characterized its functional role in the dynamics of LD fusion/fission. A manuscript describing these findings is in final stages of edits and will be submitted for publication soon.

4. Determined the structural features of an LDAP1 (Lipid Droplet Associated Protein) isoform from jojoba (a desert shrub that accumulates wax esters instead of TAGs in seeds) that enables the selective partitioning of wax esters from the ER into cytoplasmic LDs. These findings shed light on the mechanistic similarities and differences for the compartmentalization of various hydrophobic compounds in cells. A manuscript describing these results was submitted in early August to *The Plant Cell* for consideration. Payton Whitehead, a PhD student supported by this grant, was invited to present his work on this topic at the International Symposium on Plant Lipids in July 2024.

5. In addition to our research achievements, our group has also contributed three reviews that describe the latest advances in lipid deposition at the subcellular and tissue levels in plants: one published in *Annual Review of Plant Biology*, another in a focus issue of *Journal of Experimental Botany*, and a third provisionally accepted for publication in *Plant Physiology*.

Science objectives for 2024-2025:

In the coming year, we will continue efforts on all three aims. One primary focus will center on completing the work with DRP1A and LD biogenesis and advancing these results to publication stage. The structural characterization of Arabidopsis SEIPIN oligomeric complexes will be a priority as well and will be combined with additional approaches to discern the nature and function of SEIPIN protein complexes *in vivo*. Also, work is underway to identify additional protein candidates involved in lipid compartmentalization in jojoba seeds via a transcriptome-wide association study (TWAS) on approximately 25 jojoba accessions with varied wax content and composition. We anticipate that this will yield a list of potential lipogenic factors in addition to LDAP1 that could be important for wax production and accumulation. Finally, efforts to understand the compartmentalization of terpenes and unusual fatty acids will also be a natural extension of this work in the coming year.

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

The ideal collaborator for my project would have expertise in: Structural Biology, High-Resolution Ultrastructural Imaging, Cryo-Focused Ion Beam (FIB) Milling, Cryo-Electron Microscopy.

Publications supported by this project 2023-2024:

1. Horn PJ, Chapman KD (2024) Imaging plant metabolism *in situ*, Journal Experimental Botany, 75(6): erad423, https://doi.org/10.1093/jxb/erad423.
2. Guzha A, Whitehead P, Ischebeck T, Chapman KD (2023) Lipid Droplets: Packing Hydrophobic Molecules Within the Aqueous Cytoplasm. Annual Review of Plant Biology, **74**: 195-223. https://doi.org/10.1146/annurev-arplant-070122-021752.
3. Cai Y, Horn PJ. Packaging "Vegetable Oils": Insights into Plant Lipid Droplet Proteins. Plant Physiology. (*Provisionally Accepted*)

Molecular Mechanisms of Plant Cell Wall Loosening

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Overall research goals:

Plants have the astonishing ability to expand a photosynthetic canopy to collect sunlight and atmospheric CO_2 and package the harvested energy and carbon into cell walls, a large-scale renewable source of bioenergy and biomaterials. These processes depend on the ability of the growing cell wall to yield (expand) irreversibly to turgor-generated tensile stresses in the wall. Key mediators cell wall yielding are α-expansin proteins, but their novel wall-loosening effects are not yet understood at the molecular level; tests indicate expansins lack enzyme activity and do not mechanically 'soften' the wall. Wall loosening without wall softening appears to be a hallmark of α-expansin action. A key tenet in current models of growing cell walls is that cellulose microfibrils form a supramolecular network that increases in area by lateral sliding of cellulose microfibrils [doi 10.1126/science.abf2824]. We hypothesize that α-expansins promote cellulose-cellulose sliding. Because expression of plant expansins has proved intractable in many heterologous protein expression systems, we are using other approaches to explore expansin structure/function relations and expansin actions.

1. Using atomistic molecular dynamics simulations we are investigating potential interactions of α-expansin with wall structural polymers, specifically cellulose. Initial work is based on computational modeling of an α-expansin from *Arabidopsis thaliana*, AtEXPA4. The AtEXPA4 model enables us to identify residues potentially involved in pH and redox control of expansin protein dynamics and interactions with cellulose.
2. Predictions of the model are experimentally tested by genetic complementation of an *Arabidopsis thaliana* line that is defective in two *EXPA* genes required for root hair elongation (*EXPA7, EXPA18*). We are also testing the ability of *EXPA* genes from each of the 12 ancient *EXPA* clades to complement the short root-hair phenotype of the *expa7/18* mutant, to assess whether EXPA proteins are functionally equivalent.
3. An additional subproject is a screen of an expression library of 200 highly diverse microbial expansin genes for novel activities (binding targets, biophysical and biochemical actions). This exploratory work is based on the hunch (hypothesis) that the large diversity in microbial sequences reflects a large diversity of functionalities (substrates, physical actions, environmental optima) that evolved in the microbial world. The work is enabled by synthesis of the 200 constructs by JGI.

In short, the proposed work will enable us to test molecular-level concepts about the mechanism and regulation of α-expansin activity and to explore microbial expansins for novel, perhaps useful and insightful, activities by these enigmatic proteins. If successful, the outcome will yield deeper insights into the molecular basis of wall loosening, which is the key control of cell wall enlargement and limits accumulation of stored carbon and energy within plant cells.

Significant achievements: [2022-2024]:

1. An atomistic model of *At*EXPA4 was used to test for binding to cellulose-cellulose junctions. The protein can bind a single cellulose chain, not a crystalline surface. We modeled the interaction of EXPA4 with two cellulose chains under tensile stress. The residues in the chain that directly interact with EXPA4 underwent a twisting action, reducing chain-chain interactions and facilitating chain sliding *in silico*. The conserved active-site motif 'HFD' is involved in this action.
2. A chimeric *EXPA7-mCherry* construct complemented the short-root hair defect of the *expa7/18* double mutant. It produced a fluorescent protein that was trafficked to root hair tips.
3. Similarly, chimeric mCherry constructs with other EXPA genes complemented the short-root hair defect of the *expa7/18* double mutant, indicating they have similar wall-loosening action. These were driven with the root-hair specific promoter of AtEXPA7.

4. An exception to this general trend was obtained for AtEXPA13, which did not complement the mutant. AtEXPA13 is unusual in that the highly conserved active-site motif 'HFD' is modified to 'HFV'. AtEXPA13 is the sole member of EXPA clade IX in *A. thaliana*. Bioinformatic analysis of the EXPA gene family in other species revealed that other clade-IX orthologs likewise display the same HFD->HFV modification. This discovery suggests the possibility of a conserved divergence of EXPA action in Clade IX, reaching back to early angiosperm evolution. A similar story seems to be emerging for the closely-related Clade VIII EXPA20. Thus, some EXPA proteins may differ in the wall-loosening action mediated by the D in the HFD motif.
5. Following a similar protocol for the other expansin families (EXPB, EXLA, EXLB), none of them were competent to complement the *expa7/18* double mutant, suggesting a different activity.

Science objectives for 2024-2025:

1. Characterize the wall-loosening action of site-directed mutants of EXPA4 and EXPA7, using the root-hair complementation assay.
2. Monitor cellular trafficking patterns of additional EXPA-mCherry constructs to assess differences in patterns.
3. Characterize the binding and loosening action of bacterial expansins that are expressed adequately in *E. coli.*

My scientific area(s) of expertise is/are: Plant cell growth and hydraulics at cellular and whole-organ levels; plant cell wall structure and mechanics including macro-scale extensometry, micro-scale atomic force mechanics and imaging, and coarse-grained modeling of cell walls; evolution of gene families; structure of wall-binding proteins.

The ideal collaborator for my project would have expertise in: polymer mechanics; atomistic simulations, molecular physics of wall polysaccharides.

Publications supported by this project 2022-2024:

1. Cheung, A.Y., Cosgrove, D.J., Hara-Nishimura, I., Jurgens, G., Lloyd, C., Robinson, D.G., Staehelin, L.A., and Weijers, D. (2022). A rich and bountiful harvest: Key discoveries in plant cell biology. Plant Cell *34*, 53-71. 10.1093/plcell/koab234.
2. Cosgrove, D.J. (2022). Plant Cell Growth and Cell Wall Enlargement. eLS *2*, 1-14. https://doi.org/10.1002/9780470015902.a0029421.
3. Cosgrove, D.J. (2022). Building an extensible cell wall. Plant Physiology *189*, 1246-1277. 10.1093/plphys/kiac184.
4. Guo, K., Huang, C., Miao, Y., Cosgrove, D.J., and Hsia, K.J. (2022). Leaf morphogenesis: the multifaceted roles of mechanics. Mol Plant *15*, 1098-1119. 10.1016/j.molp.2022.05.015.
5. Coen, E., and Cosgrove, D.J. (2023). The mechanics of plant morphogenesis. Science *379*, eade8055. 10.1126/science.ade8055.
6. Cosgrove, D.J., Hepler, N.K., Wagner, E.R., and Durachko, D.M. (2023). Biomechanical Weakening of Paper and Plant Cell Walls by Bacterial Expansins. Methods in molecular biology (Clifton, N.J.) *2657*, 79-88. 10.1007/978-1-0716-3151-5_5.
7. Monschein, M., Ioannou, E., Koitto, T., Al Amin, L., Varis, J.J., Wagner, E.R., Mikkonen, K.S., Cosgrove, D.J., and Master, E.R. (2023). Loosenin-Like Proteins from Phanerochaete carnosa Impact Both Cellulose and Chitin Fiber Networks. Appl Environ Microbiol *89*, e0186322. 10.1128/aem.01863-22.
8. Boerjan, W., Burlat, V., Cosgrove, D.J., Dunand, C., Dupree, P., Haas, K.T., Ingram, G., Jamet, E., Mohnen, D., Moussu, S., et al. (2024). Top five unanswered questions in plant cell surface research. Cell surface (Amsterdam, Netherlands) *11*, 100121. 10.1016/j.tcsw.2024.100121.
9. Cosgrove, D.J. (2024). Structure and growth of plant cell walls. Nat Rev Mol Cell Bio *25*, 340-358. 10.1038/s41580-023-00691-y.
10. Cosgrove, D.J. (2024). Plant Cell Wall Loosening by Expansins. Annu Rev Cell Dev Biol *40 doi:* 10.1146/annurev-cellbio-111822-115334.

Versatility of electron donors to heterodisulfide reductase in hydrogenotrophic methanogens

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Overall research goals:

The overall goal of the proposed research is to understand the diversity of electron donors that participate in the energy generating metabolism of hydrogeotrophic methanogenic archaea. Specifically, the role of formate as an electron donor for methanogenesis. Our main focus is a protein complex that catalyzes the first and last steps of methanogenesis. This protein complex is composed of a hydrogenase, a formate dehydrogenase (Fdh), heterodisulfide reductase (Hdr), and formylmethanofuran dehydrogenase. Together, these enzymes couple endergonic and exergonic reactions in methanogenesis through flavin-based electron bifurcation. Interestingly, Fdh must not only donate electrons for initial CO_2 reduction during methanogenesis but must also serve as an electron donor for coenzyme F_{420} reduction to serve as reducing agent during intermediate steps in methanogenesis. Recently, we also started to explore the importance of trace metal bioavailability and metal co-factor synthesis for improved growth during nitrogen fixation in methanogenic archaea when hydrogen serves as the electron donor.

The goal of Aim 1 of the project is to understand how formate integrates into the core methanogenic pathway, how Fdh binds and transfers electrons to the site of flavin-based electron bifurcation in heterodisulfide reductase, and how Fdh can additionally reduce F_{420}. To accomplish this goal, we focus our studies on *Methanococcus maripaludis* due to its robust genetics system and ease of biochemical assays using tagged proteins. These organisms are metabolically versatile in that they can utilize H_2 or formate, as electron donors for metabolism. We first tested whether each isoform of Fdh in *M. maripaludis* is a direct electron donor for Hdr. We will additionally whether there is coordination between the competing reactions of F_{420} reduction and electron bifurcation.

In Aim 2, we expand to understand cryptic pathways of electron flow in methanogenesis. In mutant strain lacking all known paths of H_2-dependent F_{420} reduction or all known paths of H_2-dependent electron bifurcation, we still observe robust growth. We will monitor the H_2-dependent reactions in these mutant strains to purify the protein complexes catalyzing the activities in question. Through mass spectrometry, biochemical characterization, and mutagenesis, we will identify these cryptic electron flow paths.

Finally, Aim 3 of the proposal expands to understand formate metabolism at the organismal level. Methanogens often grow with partner organisms that produce H2 or formate as metabolic waste products. We will leverage competition experiments with genetically tagged and metabolically isolated strains to determine the importance of H_2 or formate in supporting these interactions

Significant achievements: [2022-2025]:

Aim1: We succeeded in purifying both Fdh1 and Fdh2 isoforms and verified that they coordinate with Hdr. Additionally, we showed that both have an ability to donate electrons to F_{420} and Hdr with a preference for F_{420} (i.e. this reaction occurs first when supplied substrates for both competing reactions). Finally, we found evidence that the Fdh2 isoform functionality is dependent upon the deletion of a molybdopterin biosynthesis gene (*moeA*). We are current working on understanding how the availability of this metal cofactor affects the overall formate metabolism or any cellular regulation that activates Fdh2 isoform functionality instead of Fdh1 isoform. Additionally, the cells that lack MoeA encoding gene shows a robust growth phenotype in nitrogen fixation compared to the wild-type strain. Preliminary evidence suggests that bioavailability of molybdenum in the media might contribute to the limited growth in the wild-type strain which was supplanted in the Δ*moeA* strain. We are doing additional biochemical analysis such as acetylene reduction assay and tagging the nitrogenase (*nifD*) to determine if there are any differences in nitrogenase activity in the wild-type and Δ*moeA* strains. We also would like to tease apart the interactions between the nitrogenase under trace metals bioavailability stress during nitrogen fixation with different electron donor source for methanogenesis such as hydrogen or formate.

Aim2: We tagged and expressed the Hdr proteins in the mutant background that is defective for H_2-dependent F_{420} reduction. We performed the initial purifications to verify that we can recover significant quantities of protein. Mass spectrometry analysis will be carried out to confirm the components of the purified Hdr complex. Additional experiments for this Aim are pending for the remainder of the project period.

Aim3: Ms. Day received a graduate fellowship to work at a DOE national lab and leveraged those funds and that opportunity to significantly expand the mutagenesis work originally proposed. She was able to analyze fitness in over 500 independent experiments and over 100 unique growth conditions. This led to identifying new genes important for methanogenesis and carbon, nitrogen, and sulfur assimilation, among many other novel findings.

Science objectives for 2024-2025:

- To understand metal availability affect formate-dependent electron flow and nitrogen fixation.
- Moving Aim 2 forward as purified Hdr complex protein is in hand

My scientific area(s) of expertise is/are: Microbial Physiology, Genetics, Biochemistry, Protein purification, Molecular Biology.

Publications supported by this project 2022-2025:

Day LA, Carlson HK, Fonseca DR, Arkin AP, Price MN, Deutschbauer AM, Costa KC. High-throughput genetics enables identification of nutrient utilization and accessory energy metabolism genes in a model methanogen. *mBio*. 2024 Aug 9:e0078124. doi: 10.1128/mbio.00781-24.

Abdul Halim MF, Fonseca DR, Niehaus TD, Costa KC. Functionally redundant formate dehydrogenases enable formate-dependent growth in *Methanococcus maripaludis*. *J Biol Chem*. 2024 Jan;300(1):105550. doi: 10.1016/j.jbc.2023.105550.

Abdul Halim MF, Hanson EH, Costa KC. *Methanococcus maripaludis*. *Trends Microbiol*. 2024 32(8):823-824. doi: 10.1016/j.tim.2024.04.007.

Zmuda AJ, Kang X, Wissbroecker KB, Freund Saxhaug K, Costa KC, Hegeman AD, Niehaus TD. A universal metabolite repair enzyme removes a strong inhibitor of the TCA cycle. *Nat Commun*. 2024 15(1):846. doi: 10.1038/s41467-024-45134-0.

Day LA, Kelsey EL, Fonseca DR, Costa KC. Interspecies Formate Exchange Drives Syntrophic Growth of *Syntrophotalea carbinolica* and *Methanococcus maripaludis*. *Appl Environ Microbiol*. 2022 88(23):e0115922. doi: 10.1128/aem.01159-22.

Structure and Function of the Methyl-coenzyme M Reductase Activation complex

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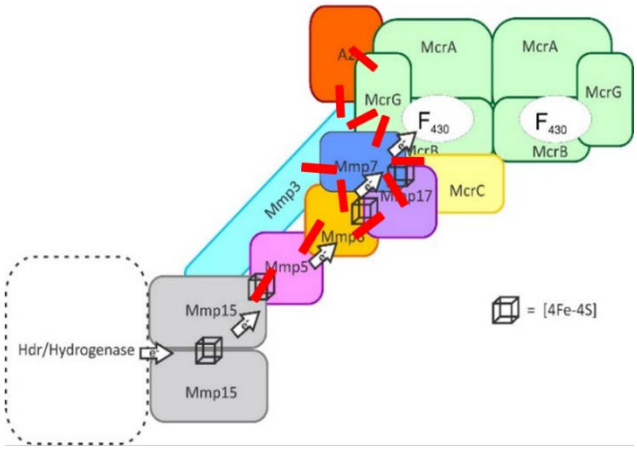
Overall research goals:

The major goal of this project is establishing a system to overexpress active Mcr. At present, we have expressed recombinant holoenzyme in the methanogen *Methanococcus maripaludis*. While the Mcr contains the correct subunit composition, PTMs and F_{430}, the in vitro activity is still low. We propose that the low activity is due to rapid inactivation upon preparation of cell extracts and purification. The proposed research will address a number of possible solutions. 1) Tagging the Mmp activation complex will allow rapid purification and further characterization of the complex. 2) Further exploration of the conditions for preparing cell extracts as well as rapid purification of Mcr may allow stabilization of the active form. 3) Further studies of Mcr assembly may identify other processes essential for Mcr activity in cells.

Significant achievements: 2023-2026

Methyl-coenzyme M reductase (Mcr) requires a large set of accessory genes to be able to function. These include the enzymes that make the unique nickel-containing corphin F_{430}, enzymes that are involved in the post-translational modification of up to five amino acids in Mcr, as well as an enzyme complex involved in the reductive activation of F_{430}. This last process, however, is not well understood, and here we present new insights into this process and propose that two enzyme complexes play a role in the activation of Mcr. The complexes might have variable compositions in different methanogens. The main components of the first complex are heterodisulfide reductase, F_{420}-nonreducing hydrogenase and tungsten- or molybdenum-containing formylmethanofuran dehydrogenase. Formate dehydrogenase and acetyl-CoA decarbonylase/synthase could also be associated.



Interaction observed in pull downs

The second complex contains McrC, methanogenesis maker proteins (Mmp) 3, 5, 6, 7, 15, and 17. Component A2 is also essential for activation but appears to interact directly with Mcr. An Alpha fold structural model of this complex was created by our colleague Steve Mansoorabadi of Auburn University, a schematic of which is shown above. To test the model, a series of pull-down experiments were conducted in *M. maripaludis*, where individual marker proteins were strep-tagged and proteins that co-purified identified by mass spectroscopy. The interactions observed between the proteins support many of the features of the model.

Science objectives for 2024-2025:

Characterization of the putative Mmp activation complex from *M. maripaludis*. In preliminary results, evidence was obtained for a large complex that included subunits of Mcr as well as Mmp proteins known to be required for Mcr activation. The complex will be purified and characterized to determine its protein and coenzyme composition. Conditions will be developed to preserve or stabilize any Fe-S clusters or other labile components.

Structure of heterodisulfide reductase containing complex. Samples will be sent to the group of Bonnie Murphy in Germany to obtained structural information using CryoEM.

***In vitro* and *in vivo* activation of the recombinant Mcr.** Further experiments will be performed to optimize the activation of the recombinant Mcr. Based upon recent progress isolating activated Mcr, further work will be performed to shorten and standardize these methods.

Investigating *Methanothermobacter thermoautotrophicus* as a high-yield expression system. Together with Bastian Molitor in Germany, methanogen marker protein 10 was expressed on a shuttle plasmid. These efforts will be expanded to Mcr. It will also be tested if tags can be added to the mcrC and hdrB genes.

My scientific area(s) of expertise is/are: Anaerobic biochemistry (Duin), spectroscopy (Duin) and microbial physiology (Whitman).

The ideal collaborator for my project 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 2023-2026:

1. Shao, N., Y. Fan, C.-W. Chou, S. Yavari, R.V. Williams, I.J. Amster, S.M. Brown, I.J. Drake, E.C. Duin, W.B. Whitman, Y. Liu (2022) Expression of divergent methyl/alkyl coenzyme M reductases from uncultured archaea. Communications Biol. 5: 1113. https://doi.org/10.1038/s42003-022-04057-6.
2. Costa, K.C., W.B. Whitman (2023) Model organisms to study methanogenesis, a uniquely archaeal metabolism. J. Bacteriol. 205**:** e00115-23**.** https://doi.org/10.1128/jb.00115-23.
3. Li, J., TS Akinyemi, N Shao, C Chen, X Dong, Y Liu, WB Whitman (2023) Genetic and metabolic engineering of Methanococcus spp. Curr. Res. Biotechnol. 5: 100115. https://doi.org/10.1016/j.crbiot.2022.11.002.
4. Sutherland-Smith, A.J., Carbone, V., Schofield, L.R., Cronin, B., Duin, E.C., R.S. Ronimus (2024) The crystal structure of methanogen McrD, a methylcoenzyme M reductase-associated protein. FEBS Open Bio, 14, 1222. https://doi.org/10.1002/2211-5463.13848.

Uncovering determinants of electron transfer insulation

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Overall research goals:

Efficiently controlling and manipulating electron transfer processes is pivotal for advancing energy storage, electronic devices, and catalysis. Microorganisms provide robust systems for studying electron transfer because biological systems must efficiently coordinate biological electron transfer in physiological functions from energy production to cellular signaling. Ferredoxins, small iron-sulfur proteins, regulate electron transfer between electron donors and acceptors, preventing undesired electron partitioning and maintaining optimal cellular function. To gain better control over electron transfer processes, we need to understand the role of ferredoxins and their underlying properties in controlling electron flow.

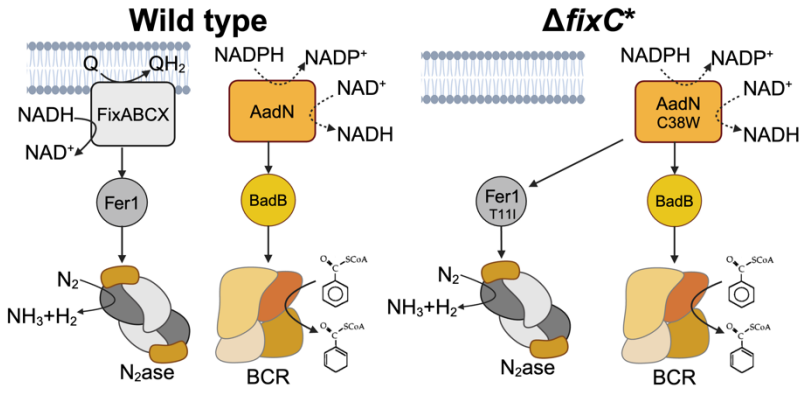

Figure 1. In *R. palustris,* the electron transfer pathway for nitrogen fixation and benzoate degradation are unable to interact with each other (left), but in *R. palustris* Δ*fixC**, mutations in *aadN* and *fer1* allow them to interact and support nitrogenase activity in the absence of FixABCX (right). N_2ase, nitrogenase and BCR, benzoyl-Co reductase. Not shown is that both N_2ase and BCR require ATP hydrolysis.

This proposal aims to investigate the properties of ferredoxin that allow it to regulate electron flow for complex, multielectron enzyme reactions like nitrogen fixation. We found we could disrupt electron transfer to nitrogenase, the key enzyme in nitrogen fixation. Using laboratory evolution, we found we connect the electron transfer pathway from one metabolic module in the anoxygenic phototroph, *Rhodopseudomonas palustris,* to the ferredoxin that delivers electrons to nitrogenase, restoring electron flow for nitrogen fixation. In so doing, we uncovered evidence for electron transfer insulation between metabolic pathways that could be overcome with only two mutations, one in a gene, *aadN*, which encodes a novel enzyme predicted to carry out electron bifurcation, and the other in a gene, *fer1*, which encodes a ferredoxin (Fd) that interacts with nitrogenase (Fig. 1). Building on previous data, we hypothesize that when two pathways utilize ferredoxins with similar midpoint potentials, factors like differences in electronic structure, abundance, and/or surface charge become pivotal in controlling electron flow. Three specific aims will address this central hypothesis.

Specific Aim 1. Determine how differences in the electronic structure of a ferredoxin controls intermolecular electron transfer.

Specific Aim 2. Determine how regulation of ferredoxins contributes to electron transfer insulation.

Specific Aim 3. Test the role of surface charge in controlling ferredoxin interaction with nitrogenase.

Significant achievements: [2024-2027]:

In the previous funding period, we identified that spin-spin coupling and the stabilization of various high spin states may contribute to the increased electron transfer observed between the Fer1 variant, $Fer1^{T11I}$, and the AadN variant, $AadN^{C38W}$ (Fig. 1). In Specific Aim 1, we plan to test additional variants of Fer1—both those compatible and incompatible with $AadN^{C38W}$—to investigate whether changes in the electronic structure of $Fer1^{T11I}$ correlate with its ability to interact with $AadN^{C38W}$. We are nearing completion of the purification and reconstitution of these ferredoxins and are preparing to conduct cyclic voltammetry and EPR to measure midpoint potentials and assess electronic structure differences.

Progress has also been made on Specific Aim 2. Testing an in-frame deletion mutant of Fer1 revealed that BadB, the cognate ferredoxin of AadN, does not compensate for the absence of Fer1. However, this may be because BadB is expressed only in the presence of aromatic compounds. RNA-seq analysis showed that both Fer1 and BadB are expressed in *R. palustris* under nitrogen-fixing conditions when grown with an aromatic compound. Despite both ferredoxins being expressed, BadB does not functionally replace Fer1. This was further confirmed when we replaced *fer1* with *badB* at the *fer1* locus. The resulting strain was unable to grow under nitrogen-fixing conditions. To understand why BadB cannot participate in electron transfer to nitrogenase, we subjected this strain to adaptive laboratory evolution and isolated suppressor mutants, which we plan to sequence for further insight.

Science objectives for 2024-2025:

- Complete purification, reconstitution, and characterization of Fer1 variants using EPR for Aim 1
- Sequence suppressors to gain insight into why BadB and Fer1 are not functional redundant and determine which mutations are required for suppressor phenotypes.

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

The ideal collaborator for my project would have expertise in: EPR, cyclic voltammetry.

Publications supported by this project 2024-2027:

1. Lewis, N.M., Kisgeropoulos, E. C., Lubner, C. E.$^{+}$, and **Fixen, K. R.**$^{+}$ (2024). Characterization of ferredoxins involved in electron transfer pathways for nitrogen fixation implicates differences in electronic structure in tuning 2[4Fe4S] Fd activity. *J Inorg Biochem* 254:112521. doi: 10.1016/j.jinorgbio.2024.112521
    - *Lubner and Fixen co-corresponding author*

Understanding and improving the oxygen-tolerance of Thi4 metalloenzymes

Andrew Hanson, Principal Investigator
Mark A. Wilson, Co-PI
David Obe (PhD Student); Rodrigo Campos da Silva, Anuran Gayen (Postdoctorals)
Horticultural Sciences Dept, University of Florida
Gainesville, FL 32611-0690
Email: adha@ufl.edu; Website: https://www.adhansonlab.org/

Overall research goals:

1. Evolve bacterial/plant Thi4s for more O_2-tolerance (i.e. less inactivation) and activity in low sulfide
2. Use cryo-EM and X-ray crystallography to determine 3D structures throughout evolution campaigns
3. Combine molecular dynamics simulations with structure data to uncover improvement mechanisms
4. Apply these mechanisms to design new mutational improvements, then test and further evolve them
5. Determine whether bacterial Thi4s prefer or require a persulfide or thiocarboxylate as sulfur donor

Significant achievements: Years of Current Award: 2023-2026

Goal 1:

Identification of bacterial catalytic Thi4 candidates to evolve for O_2-tolerance. With Dr. J. Zhou's group (University of Florida) we built computational pipelines that use only DNA sequence to explore (i) the average oxidation state of carbon (Z_C) in Thi4s and (ii) the presence of O_2-metabolism genes in the corresponding (meta)genomes as criteria for O_2-tolerant, mesophilic starting points for directed evolution campaigns. Z_C has been proposed to be highest (least negative) in proteins of organisms from O_2-rich, mesophilic environments (PMID:37289197). We found that Z_C values of >2,300 Thi4s ranged from -0.107 (relatively oxidized) to -0.302 (strongly reduced) and, consistent with expectation, that genes for cytochrome *c* or *o* oxidases (which need high O_2 levels) and cytochrome *bd* oxidase were more frequent in genomes encoding Thi4s with high Z_C values. Eight Thi4s were chosen using the above criteria and have been introduced into the yeast OrthoRep system for continuous directed evolution.

Plant catalytic Thi4s. We demonstrated that the catalytic Thi4s from barley (HvThi4) and oat (AsThi4) have substantial activity when expressed in the yeast OrthoRep system. Specifically, we tested whether the catalytic activity of these cereal Thi4s can complement a yeast *thi4*Δ strain. Codon-optimized AsThi4 or HvThi4 sequences under the control of the 10B2 promoter and the TP-DNAP-611 error-prone polymerase were introduced into strain BY4742-*thi4*Δ. The resulting strains grew at rates comparable to control strains expressing native yeast Thi4 via the OrthoRep machinery. This result makes it possible to deploy OrthoRep to improve the O_2-tolerance and enzymatic activity of the cereal catalytic Thi4s and hence to investigate the mechanistic basis of the improvements.

Goal 2: We reached a key milestone en route to understanding and improving O_2-tolerance of catalytic bacterial Thi4 metalloenzymes. We successfully determined the structure of wild-type *Saccharicrinis fermentans* Thi4 (SfThi4), whose O_2-tolerance we improved by directed evolution in the previous award. We determined the structure at exceptional resolutions using both X-ray crystallography (1.3 Å) and cryo-EM (2.9 Å). These high-resolution structures reveal two distinct ligands at the active site that appear to capture two different stages of the reaction. This is an important step in elucidating the enzyme mechanism and its O_2-sensitivity and opens the way to structural analysis of the O_2-tolerant evolved SfThi4.

Science objectives for 2024-2025:

1. Launch directed evolution campaigns with the selected bacterial Thi4s and cereal catalytic Thi4s.
2. Work to obtain crystal and/or cryo-EM structures of the evolved, O_2-tolerant D168G mutant of SfThi4.
3. Work to obtain crystal and/or cryo-EM structures of the wildtype *Mucinivorans hirudinis* catalytic Thi4 (MhThi4) and the O_2-tolerant MhThi4 mutants evolved in the previous and present awards.
4. Begin combining molecular dynamics simulations with emerging structure data to probe mechanisms.
5. Continue comparative genomic and genetic analysis of the role of persulfide-thiocarboxylate relay proteins in the function of suicide bacterial Thi4s.

My scientific area(s) of expertise is/are: Metabolic biochemistry and metabolic engineering, directed enzyme evolution.

The ideal collaborator for my project would have expertise in: The reaction mechanisms of mononuclear metal enzymes or sulfide transfer biochemistry.

Publications supported by this project 2023-2024:

1. U. Bathe, B.J. Leong, K. Van Gelder, G.G. Barbier, C.S. Henry, J.S. Amthor, A.D. Hanson, "Respiratory energy demands and scope for demand expansion and destruction." Plant Physiol. **191,** 2093 (2023). DOI: 10.1093/plphys/kiac493.
2. K.V. Gelder, E.R. Oliveira-Filho, J.D. García-García, Y. Hu, S.D. Bruner, A.D. Hanson, "Directed evolution of aerotolerance in sulfide-dependent thiazole synthases." ACS Synth. Biol. **12,** 963 (2023). DOI: 10.1021/acssynbio.2c00512.
3. A.D. Hanson, V. Lorenzo, "Synthetic biology─High time to deliver?" ACS Synth. Biol. **12,** 1579 (2023). DOI: 10.1021/acssynbio.3c00238.
4. K. Van Gelder, E.R. Oliveira-Filho, C.D. Messina, R.E. Venado, J. Wilker, S. Rajasekar, J.M. Ané, J.S. Amthor, A.D. Hanson, "Running the numbers on plant synthetic biology solutions to global problems." Plant Sci. **335,** 111815 (2023). DOI: 10.1016/j.plantsci.2023.111815.
5. E.R. Oliveira-Filho, C. Voiniciuc, A.D. Hanson, "Adapting enzymes to improve their functionality in plants: Why and how." Biochem. Soc. Trans. **51,** 1957 (2023). DOI: 10.1042/BST20230532.
6. J. Joshi, A.D. Hanson, "A pilot oral history of plant synthetic biology." Plant Physiol. **195,** 36 (2024). DOI: 10.1093/plphys/kiad585.
7. E.R. Oliveira-Filho, R. Campos-Silva, A.D. Hanson, "Running Fermi calculations as a superpower to gauge reality." Plant Physiol. Jun 14:kiae347 (2024). DOI: 10.1093/plphys/kiae347.
8. E.R. Oliveira-Filho, D.A. Rodionov, A.D. Hanson, "Comparative genomic and genetic evidence on a role for the OarX protein in thiamin salvage." ACS Omega **9,** 28888 (2024) DOI: 10.1021/acsomega.4c03514.

Understanding and improving the oxygen-tolerance of Thi4 metalloenzymes

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Significant achievements: Years of Current Award: 2023-2026

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4. Begin combining molecular dynamics simulations with emerging structure data to probe mechanisms.
5. Continue comparative genomic and genetic analysis of the role of persulfide-thiocarboxylate relay proteins in the function of suicide bacterial Thi4s.

My scientific area(s) of expertise is/are: Metabolic biochemistry and metabolic engineering, directed enzyme evolution.

The ideal collaborator for my project would have expertise in: The reaction mechanisms of mononuclear metal enzymes or sulfide transfer biochemistry.

Publications supported by this project 2023-2024:

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2. K.V. Gelder, E.R. Oliveira-Filho, J.D. García-García, Y. Hu, S.D. Bruner, A.D. Hanson, "Directed evolution of aerotolerance in sulfide-dependent thiazole synthases." ACS Synth. Biol. **12,** 963 (2023). DOI: 10.1021/acssynbio.2c00512.
3. A.D. Hanson, V. Lorenzo, "Synthetic biology─High time to deliver?" ACS Synth. Biol. **12,** 1579 (2023). DOI: 10.1021/acssynbio.3c00238.
4. K. Van Gelder, E.R. Oliveira-Filho, C.D. Messina, R.E. Venado, J. Wilker, S. Rajasekar, J.M. Ané, J.S. Amthor, A.D. Hanson, "Running the numbers on plant synthetic biology solutions to global problems." Plant Sci. **335,** 111815 (2023). DOI: 10.1016/j.plantsci.2023.111815.
5. E.R. Oliveira-Filho, C. Voiniciuc, A.D. Hanson, "Adapting enzymes to improve their functionality in plants: Why and how." Biochem. Soc. Trans. **51,** 1957 (2023). DOI: 10.1042/BST20230532.
6. J. Joshi, A.D. Hanson, "A pilot oral history of plant synthetic biology." Plant Physiol. **195,** 36 (2024). DOI: 10.1093/plphys/kiad585.
7. E.R. Oliveira-Filho, R. Campos-Silva, A.D. Hanson, "Running Fermi calculations as a superpower to gauge reality." Plant Physiol. Jun 14:kiae347 (2024). DOI: 10.1093/plphys/kiae347.
8. E.R. Oliveira-Filho, D.A. Rodionov, A.D. Hanson, "Comparative genomic and genetic evidence on a role for the OarX protein in thiamin salvage." ACS Omega **9,** 28888 (2024) DOI: 10.1021/acsomega.4c03514.

Dissimilatory Nitrite Reduction to Ammonium: Catalyzing Multi-Electron Reductions Using a Pentaheme Scaffold

Eric L. Hegg, Principal Investigator
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Overall research goals:

Pentaheme cytochrome *c* nitrite reductase (NrfA) catalyzes the remarkable six-electron reduction of NO_2^- to NH_4^+. Currently, there are several unanswered questions concerning (a) the precise molecular mechanism of NrfA, (b) the exact electron flow through this intriguing system, and (c) the importance of heme coordination as a way to tune reactivity. The long-term goals of this project are to (*i*) ascertain the precise enzymatic mechanism of NrfA, (*ii*) elucidate the strategy for the flow of electrons through NrfA and its redox partner NrfH, and (*iii*) determine the significance of the active site architecture. The specific objectives of this proposal are to characterize NrfA's physiological donor NrfH, examine the interaction between NrfA and NrfH as it relates to the activity and electron flow of the complex, and continue mechanistic studies on NrfA to identify some of the intermediates in the reaction cycle. To accomplish these objectives, we are employing a synergistic combination of biochemical, kinetic, spectroscopic, and electrochemical methods to trap and interrogate reaction intermediates. We are focusing our studies on the NrfA and NrfH enzymes from *Geobacter lovleyi*, a DNRA bacterium identified for its environmental relevance. Successful completion of this project will provide insight into how NrfA stores and regulates the flow of electrons, and it will also lay the foundation for subsequent detailed mechanistic studies to ascertain how this unique pentaheme enzyme orchestrates the challenging multi-electron and multi-proton reduction of NO_2^- to NH_4^+.

Significant achievements (2023-2024):

- After interrogating many expression vectors, growth conditions, expression tags, and bacterial hosts, we developed a heterologous expression system for *G. lovleyi* NrfH in *Escherichia coli*, and a purification protocol that involves the use of Strep-tag II affinity and size exclusion chromatography (in collaboration with Prof. Ekaterina Pletneva).
- We also developed a heterologous expression system and a purification system for several NrfA variants based in *E. coli* with upregulated cytochrome *c* maturation (ccm) proteins.
- We improved our non-turnover voltammograms technique via ITO electrodes to ascertain the reduction potentials for each heme in WT *Gl*NrfA and in a functional heme 5 knockout variant of *Gl*NrfA (in collaboration with Prof. Sean Elliott, Boston University).
- We developed a protocol to detect intermediates using stopped flow techniques and UV-Vis on a Ti(III) citrate partially reduced NrfA protein and successfully demonstrated spectroscopic changes observed from the intermediates.
- We determined the stability of the intermediates.

Science objectives for 2023-2024:
The specific goals of this 3-year research proposal are to:

Goal 1: Analyze the role of key NrfA protein-protein interactions.
- Determine the stoichiometry and structure of the NrfA-NrfH complex in solution.

Goal 2: Determine the flow of electrons to the active site.
- Determine the spectroscopic and electrochemical properties of *G. lovleyi* NrfH.
- Establish the role of NrfA-NrfH complex formation on electron transfer, storage, and distribution.

Goal 3: Elucidate the detailed mechanism of NO_2^- reduction
- Trap and characterize intermediates using a partially reduced, monomeric enzyme to obtain a frame-by-frame "snapshot" of the entire reaction cycle.

My project addresses BES cross-cutting priority areas by:
The proposed work aligns well with the Reaction Pathways in the Diverse Environments fundamental research theme outlined by the Chemical Sciences, Geosciences, and Biosciences Division, and it synergizes well with both the Charge Transport and Reactivity and the Chemistry in Aqueous Environments themes. In addition, our work is directly relevant to the BES – Physical Biosciences core mission to "further our understanding of the ways plants and non-medical microbes capture, convert, and store energy." Consistent with the Physical Biosciences vision, our work will "provide a better understanding of the structure/function, mechanistic, and electrochemical properties of enzymes that catalyze complex multielectron redox reactions."

My scientific area(s) of expertise is/are: Hegg: Role of metals in biological systems; mechanistic enzymology. Lehnert: spectroscopy and simulation, stopped-flow measurements, quantum-chemical calculations.

The ideal collaborator for my project would have expertise in: We established a collaboration with Professor Sean Elliott at Boston University for his expertise in protein film voltammetry to assess the redox potentials of the various hemes in both WT *G. lovleyi* NrfA and variants. We established a collaboration with Professor Ekaterina Pletneva at Dartmouth University for her expertise in structural and functional analysis of *c*-type cytochromes.

Publications supported by this project [2020-2022]:
K. Hird, J. Campeciño, N. Lehnert, E.L. Hegg, " Recent mechanistic developments for cytochrome *c* nitrite reductase, the key enzyme in the dissimilatory nitrate reduction to ammonium pathway" *J. Inorg. Biochem.* 112542 (2024). [DOI: 10.1016/j.jinorgbio.2024.112542]

Mechanistic Studies of Energy-Relevant Molybdenum Enzymes

Russ Hille, Principal Investigator
Michael W.W. Adams, Carolyn E. Lubner , Co-PI(s)
Steve Ortiz, Graduate Student
Gerti Schut, Postdoctoral Research Associate
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Overall research goals:

To define the kinetic reaction mechanisms of molybdenum enzymes and electron-bifurcating flavoproteins

Significant achievements: 2021-2024:

- Defined conditions for air-stabilization of *Cupriavidus necator* formate dehydrogenase FdsDABG
- Examined the reversible electrochemical interconversion of formate and CO_2 by FdsDABG
- Determined the reduction potentials for FdsDABG and assigned Fe/S EPR signals
- Characterized the kinetic behavior of the bifurcating NfnI from *Pyrococcus furiosus*

Science objectives for 2024-2025:

- Complete kinetic characterization of NfnII from *P. furiosus*
- Complete XAS and HERFD-XAS of the FdsDABG formate dehydrogenase from *C. necator*
- Undertake GC-MS investigation of the reaction mechanism of FdsDABG

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

mechanistic enzymology; rapid-reaction kinetics, EPR

The ideal collaborator for my project would have expertise in:

Someone who has interesting enzymes to investigate mechanistically.

Publications supported by this project 2021-2024:

Hakopian, S., Niks, D., and Hille, R. (2022) Air inactivation of formate dehydrogenase FdsDABG from *Cupriavidus necator*. *J. Bioinorg. Chem.* 231, DOI 10.1016/j.jinorgbio.2022.111788.

Graham, J.E., Niks, D., Zanee, G.M., Hille, R., Wall, J.D., & Raman, C.S. (2022) How a formate dehydrogenase responds to Oxygen: Unexpected O_2 insensitivity of an enzyme harboring tungstopterin, selenocysteine and [4Fe-4S] clusters. *ACS Catalysis* 12, 10449-10471.

Kirk, M.L., and Hille, R. (2022) Spectroscopic studies of mononuclear molybdenum centers. *Molecules* doi.org/10.3390/molecules27154802

Kalimuthu, P, Hakopian, S., Niks, D. Hille, R. & Bernhardt, P.V. (2023) The reversible electrochemical conversion of formate and CO_2 by formate dehydrogenase from *Cupriavidus necator*. *J. Phys. Chem. B.*, 127, 8382-8392. doi/10.1021/acs.jpcb.3c04652

Ortiz, S, Niks, D., Wiley, S. Lubner, C.E. & Hille, R. (2023) Rapid-reaction kinetics of the bifurcating NAD^+-dependent NADPH:ferredoxin oxidoreductase NfnI from *Pyrococcus furiosus*. *J. Biol. Chem.* **299**, 105403. doi.org/10.1016/j.jbc.2023.105403

Harmer, J., Hakopian, S., Niks, D., Hille, R., and Bernhardt, P. (2023) Redox characterization of complex molybdenum enzyme formate dehydrogenase from *Cupriavidus necator*". *J. Am. Chem. Soc.* **145**, 25850-25863. DOI: 10.1021/jacs.3c10199

Towards the Mechanism of N_2 Fixation by Nitrogenase

Brian Hoffman, Principal Investigator (DE- SP0047367)
Department of Chemistry and Department of Molecular Biosciences, Northwestern University, Evanston IL 60201
Email: bmh@northwestern.edu; Website: https://sites.northwestern.edu/hoffmanchem/

Overall research goals:

Biological nitrogen fixation — the reduction of N_2 to two NH_3 molecules —provides the nitrogen nutrient that supports more than half the human population. This process, which involves one of the most challenging chemical transformation in biology, the reduction of the N≡N triple bond, is catalyzed by nitrogenases, primarily the Mo-dependent enzyme (denoted MoFe). Our team has revealed key features of the catalytic mechanism, setting the stage for studies that will deepen and enrich our understanding of biological nitrogen fixation, a goal of significance to society. The industrial Haber-Bosch process for generating NH_3 from N_2 provides the remainder of human nitrogen nutrient. However, it, requires up to 3% of fossil fuel usage in the World, *and* emits CO_2 in the process. As a result, there is growing interest in developing a new generation of catalysts that can fix N_2 utilizing sustainably-derived electrons and protons instead of H_2, and operate under benign conditions. As nitrogenase is the paradigm for such a process, discovery of its molecular mechanism is imperative as a way to inform and inspire the synthesis of new catalysts.

Our team, which includes Dean, Raugei, and Seefeldt, has revealed the core of the mechanism by which the nitrogenase MoFe protein cleaves the N≡N triple bond, with the central advance being the discovery that that this endoergic cleavage becomes accessible for the intermediate (denoted E_4E(4H)) that has accumulated $4e^-/4H^+$ stored as two Fe-bridging hydrides, through the exoergic reductive-elimination of the hydrides to liberate H_2. Building on this foundation, we propose to broaden our mechanistic understanding through focused studies involving all three nitrogenase isozymes, using constructs prepared by Dean and Seefeldt, augmented by those of Daniel Suess and bioinorganic analogue complexes synthesized by Pat Holland. These efforts are broadly grouped into three main Aims.

(I) We will continue to exploit the remarkable biosynthetic achievement of Dean – targeted labeling of the central carbon of the nitrogenase catalytic cofactors. We shall extend our studies of the role of their CFe_6 core to all three nitrogenase isozymes, testing for hemilability of Fe-C bonds, as complemented by studies of model complexes prepared by Holland. In addition, we will explore the possibility that catalysis involves hemilabilitiy of S2B, which bridges Fe2 and Fe6 in the resting state, with Fe-S bond-breaking and even the possibility of loss/recovery of S2B

(II) Our mechanistic studies with Dean and Seefeldt have shown that the alternative nitrogenases function by the same mechanism as Mo-nitrogenase, while our spectroscopic studies revised the understanding of their electronic properties. This mechanistic equivalence means that the nature of the bound substrates/intermediates/products in an E_n intermediate in one isozyme corresponds to that of the others, and likewise for any tendency that either Fe-C bonding in the CFe_6 core or Fe-S2B bonds might show hemilability. The E_n *n = even* intermediates of Fe-Mo-co are amenable to EPR/ENDOR studies, they are EPR-silent in the alternative isozymes, while the reverse is true for the other two cofactors. Beyond this,

(III) Dean and Seefeldt have developed protocols for 'mixing and matching' the apoenzyme 'chassis' of one isozyme with the catalytic cofactor 'engine' of another, enabling us to determine: which isozyme characteristics are controlled by the 'engine' and which by the active-site environment provided by the 'chassis'.

Significant achievements: 2022-2024:

Our significant progress during this period can be illuminated by commenting on the peer-reviewed publications, in the order listed below; work in progress will be noted as objectives. Significant progress has been made in both of the two thrusts of the project: insights into the mechanism of substrate reduction at the nitrogenase active site and assembly of the active site. **(1)** We established the stoichiometry of

ATP utilization per electron transferred for the Fe protein (1+ or 0), resolving confusion from earlier studies. **(2)** Our initial effort toward determining the extent of S2B hemilability during catalysis. **(3)** Perhaps the major achievement, used ^{13}C and ^{95}Mo ENDOR to show that the FeMo-cofactor acts as a non-hemilabile 'heart of steel' in a suite of catalytic MoFe intermediates, rather than hemi-labile C-Fe bonds enabling substrate binding on Fe. **(4)** A fruit of the determination that all three isozymes follow the same catalytic mechanism. As the alternative nitrogenase isozymes (V- and Fe-based) are EPR active in the odd E states (E_1, E_3, etc.), while Mo-nitrogenase is EPR active in the even E states (E_0, E_2, etc.), thus enabling us to examine all of the E states of the catalytic cycle during N_2 reduction. **(5)** Discussed in objectives.

Science objectives for 2024-2025:

If any catalytic state were to exhibit hemilabile C-Fe bonds it would be the state formed upon H_2 reductive elimination with coupled N_2 binding, denoted E_4(2N2H). A major objective is to finalize preliminary results indicating that here too, the CFe cofactor core is a heart of steel. To help characterize the currently unknown mode of N_2 binding in this state shall pursue current studies of Fe-N_2 synthetic analogue complexes **(5)**. Finally, we expect to obtain mixed 'engine/chassis' enzymatic constructs to begin dissecting their respective roles in controlling catalysis.

Our scientific area(s) of expertise is/are: Paramagnetic resonance experimental and analysis techniques.

The ideal collaborator for my project would have expertise in: microbiology, biochemistry, and synthetic inorganic chemistry, and in high-level theory (QM/MM) applied to catalytic mechanism of metalloenzymes.

.

Publications supported by this project 2022-2024:

1. Yang, Z.-Y., Badalyan, A., Hoffman, B. M., Dean, D. R., and Seefeldt, L. C. (2023) The Fe protein cycle associated with nitrogenase catalysis requires the hydrolysis of two ATP for each single electron transfer event. *J. Am. Chem. Soc. 145*, 5637–5644.

2. Lukoyanov, D. A., Yang, Z.-Y., Shisler, K., Peters, J. W., Raugei, S., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2023) A conformational equilibrium in the nitrogenase MoFe protein with an α-V70I amino acid substitution illuminates the mechanism of H2 formation. *Faraday Discuss. 243*, 231–252.

3. Lukoyanov, D. A., Yang, Z.-Y., Pérez-González, A., Raugei, S., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2022) 13C ENDOR characterization of the central carbon within the nitrogenase catalytic cofactor indicates that the CFe6 core is a stabilizing "heart of steel." *J. Am. Chem. Soc. 144*, 18315–18328.

4. Lukoyanov, D. A., Harris, D. F., Yang, Z.-Y., Pérez-González, A., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2022) The one-electron reduced active-site FeFe-cofactor of Fe-nitrogenase contains a hydride bound to a formally oxidized metal-ion core. *Inorg. Chem. 61*, 5459–5464.

5.Yang, H., Sharma, A., Drena, A., Peters, J.S., Hoffman, B.M. (2024) N_2-reducing Nitrogenase Models, $P_3^EM(N_2)$ (M = Fe, Co; E = Si, B, C): EPR, ENDOR Characterization of Pseudo-Jahn-Teller Activity, E-M Hemilability, and Activation of Bound N_2. *In preparation.*

Engineering a Functional Equivalent of Nitrogenase for Mechanistic Investigations of Ammonia Synthesis

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Overall research goals:

The overarching goal of this project is to use NifEN of *Azotobacter vinelandii* as a mutational platform to construct partially defective or fully functional MoFe protein mimics for mechanistic investigations of ammonia synthesis by nitrogenase. Genetic methods (mutagenesis and homologous recombination) will be used to strategically reconstruct defective or functional mimics of MoFe protein, and biochemical (metal and enzymatic assays) and spectroscopic (EPR and XAS/EXAFS analyses) methods will be employed to monitor and analyze the (re)construction process. Success in generating partially defective nitrogenase variants on a NifEN template will facilitate capture of the reaction intermediates of N_2 reduction for mechanistic investigations of nitrogenase; whereas success in generating an active nitrogenase equivalent on a NifEN template will enable identification of all functional determinants for the catalytic activity of nitrogenase and provide a proof-of-concept for minimizing the essential *nif* gene set for future transgenic expression of nitrogenase via synthetic biology.

Significant achievements (2020-2024):

• **Catalytic abilities of NifEN.** We discovered the ability of NifEN to reduce N_2 at its surface-exposed L-cluster ([Fe_8S_9C]), a structural/functional homolog of the M-cluster (or cofactor; [(*R*-homocitrate)$MoFe_7S_9C$]) of NifDK. Further, we demonstrated the ability of the L-cluster-bound NifDK to mimic its NifEN counterpart and enable N_2 reduction. These observations, coupled with phylogenetic, ecological, and mechanistic considerations, led to the proposal of a NifEN-like, L-cluster-carrying protein as an ancient nitrogenase, the exploration of which could shed crucial light on the evolutionary origin of nitrogenase and related enzymes while providing insights into the reaction mechanism of nitrogenase (see ***manuscript #2*** below).

• **Heterologous expression of nitrogenase components in *Escherichia coli*.** One major hurdle for expressing an active Mo-nitrogenase in *E. coli* is to generate the complex metalloclusters (P- and M-clusters) within this enzyme, which involves some highly unique bioinorganic chemistry/metalloenzyme biochemistry that is not generally dealt with in the heterologous expression of proteins via synthetic biology; in particular, the heterologous synthesis of the homometallic P-cluster ([Fe_8S_7]) and M-cluster core (or L-cluster) on their respective protein scaffolds, which represents two crucial checkpoints along the biosynthetic pathway of a complete nitrogenase, has never been demonstrated by biochemical and spectroscopic analyses of purified metalloproteins.

As an initial successful outcome of this line of efforts, we have accomplished the heterologous formation of a P-cluster-containing NifDK protein upon co-expression of *Azotobacter vinelandii nifD*, *nifK*, *nifH*, *nifM* and *nifZ* genes, and that of an L-cluster-containing NifB protein upon co-expression of *Methanosarcina acetivorans nifB*, *nifS* and *nifU* genes alongside the *A. vinelandii fdxN* gene, in *E. coli* (see ***manuscript #3*** below). Our metal content, activity, EPR and XAS/EXAFS data provide the first conclusive evidence for the successful synthesis of P- and L-clusters in a non-diazotrophic host, thereby

highlighting the effectiveness of our metallo-centric, divide-and-conquer approach that individually tackles the key events of nitrogenase biosynthesis prior to piecing them together into a complete pathway for the heterologous expression of nitrogenase.

Building on the success of expressing the homometallic P- and M-cluster cores in *E. coli*, we have successfully pieced together a biosynthetic pathway for the heterologous synthesis of an active Mo-nitrogenase of *A. vinelandii* in *E. coli* (see ***manuscript #4*** below). Our metal, activity and EPR data demonstrate the integrity of the metallocenters in the purified nitrogenase enzyme; whereas our growth, nanoSIMS and NMR data illustrate the diazotrophic growth and ^{15}N enrichment by the *E. coli* expression strain, as well as an accumulation of extracellular ammonia upon deletion of the ammonia transporter that permits the subsequent incorporation of the accumulated ammonia into the cellular mass of a non-diazotrophic *E. coli* strain. It is important to note that, in this study, we have also successfully expressed and purified a biosynthetically competent, L-cluster containing form of NifEN from *E. coli*, which provides a useful tool for our continued efforts to engineer NifEN into a partially or fully functional MoFe protein equivalent.

Science objectives for 2025-2026:

Restoring the catalytically active M-cluster site of NifEN to enable the N_2-reducing activity for mechanistic studies.

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

The ideal collaborator for my project would have expertise in: Structural biologists, computational biologists and high-end spectroscopists

Publications supported by this project since 2020:

1. Solomon JB, Lee CC, Liu YA, Duffin C, Ribbe MW, Hu Y (2024) Ammonia synthesis via an engineered nitrogenase assembly pathway in Escherichia coli. ***Nat Catal*** 2024 Sep 19, doi:10.1038/s41929-024-01229-x
2. Lee CC, Górecki K, Stang M, Ribbe MW, Hu Y (2024) Cofactor maturase NifEN: A prototype ancient nitrogenase? ***Sci Adv*** 10(24):eado6169. doi: 10.1126/sciadv.ado6169
3. Solomon JB, Liu YA, Górecki K, Quechol R, Lee CC, Jasniewski AJ, Hu Y, Ribbe MW (2023) Heterologous expression of a fully active *Azotobacter vinelandii* nitrogenase Fe protein in *Escherichia coli*. ***mBio*** 14(6):e0257223. doi: 10.1128/mbio.02572-23
4. Quechol R, Solomon JB, Liu YA, Lee CC, Jasniewski AJ, Górecki K, Oyala P, Hedman B, Hodgson KO, Ribbe MW, Hu Y (2023) Heterologous synthesis of the complex homometallic cores of nitrogenase P- and M-clusters in *Escherichia coli.* ***Proc Natl Acad Sci USA*** 120(44):e2314788120. doi: 10.1073/pnas.2314788120
5. Hu Y, Lee CC, Grosch M, Solomon JB, Weigand W, Ribbe MW (2023) Enzymatic Fischer-Tropsch-type reactions. ***Chem Rev*** 123(9):5755-5797. doi: 10.1021/acs.chemrev.2c00612
6. Stripp ST, Duffus BR, Fourmond V, Léger C, Leimkühler S, Hirota S, Hu Y, Jasniewski A, Ogata H, Ribbe MW (2022) Second and outer coordination sphere effects in nitrogenase, hydrogenase, formate dehydrogenase, and CO dehydrogenase. ***Chem Rev*** 122(14):11900-11973. doi: 10.1021/ acs.chemrev.1c00914

Mechanism of Photochemical N_2 Reduction

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Overall research goals:

We have demonstrated that nanocrystal materials self-assemble with the Mo-nitrogenase MoFe protein into biohybrid complexes, which under illumination, support the catalytic reduction of N_2 and protons to ammonia and dihydrogen (*Figure 1*). The biohybrid system we have developed is a unique architecture and reporter for understanding the fundamental properties that control the N_2 reduction reaction kinetics of nitrogenase.

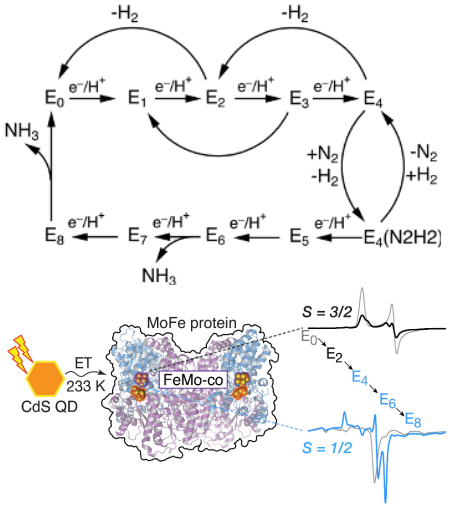

Figure 1. *Top*, N_2 reduction reaction cycle of nitrogenase. "E" refers to the oxidation states that follow successive electron transfer and reduction steps. *Bottom*, schematic of CdS quantum dot (QD) - MoFe protein complex. Photo-driven reduction is detected as changes in the EPR spectra of the catalytic FeMo-co cofactor (DOI:10.1063/5.0170405).

To understand how the components of the biohybrid system leads to functional photocatalysis, we have developed approaches for studying the binding interactions and thermodynamics (*Figure 2*), the interfacial electron transfer kinetics, and identifying MoFe protein reaction intermediates and kinetics. These approaches are informing on physical properties of nanocrystals, including the dimensions and surface chemistries, that control the binding interaction and photoexcited electron transfer efficiencies. Light-controlled electron delivery in the frozen state is being used to populate N_2 reduction reaction intermediates in pre-steady state for analysis by electron paramagnetic resonance (EPR) spectroscopy. A goal of our studies is to evolve an understanding of molecular assembly of MoFe protein with CdS nanocrystals and to elucidate the reaction mechanism and kinetics under photochemical electron delivery.

Significant achievements: [2023-2024]:

- Elucidated how the chemistry of sacrificial electron donors affects N_2 reduction reaction rates. *J. Inorg. Biochem.* 2024. DOI:10.1016/j.jinorgbio.2024.112484.
- Measured the nanocrystal-MoFe protein binding thermodynamics to understand molecular assembly. *Nano Letters*. 2023. DOI:10.1021/acs.nanolett.3c03205.
- Elucidated the kinetic stability of the N_2 reduction reaction intermediate E_4(2N2H) in pre-steady state reactions by cryo-annealing. *J. Am. Chem. Soc.* 2023. DOI:10.1021/jacs.3c06832.

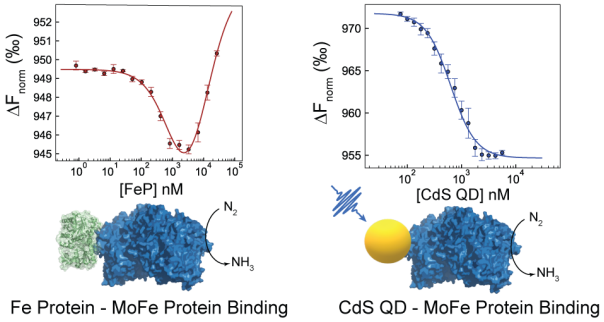

Figure 2. Binding curves obtained from MST for Fe protein and MoFe protein (left), and for CdS QD and MoFe protein (right). DOI:10.1021/acs.nanolett.3c03205.

- Demonstrated that photoexcited electron delivery to MoFe protein leads to the formation of catalytic intermediates by pre-steady state EPR studies. *J. Chem. Phys.* 2023. DOI:10.1063/5.0170405.

Science objectives for 2025-2026:

- We will further examine the population distributions of E_n intermediates by EPR under pre-steady state conditions to elucidate how photochemical electron flux controls MoFe protein reaction kinetics. The objectives are to (i) identify and define and the kinetic relationship of hydride bound intermediates by cryo-photolysis, (ii) develop kinetic model(s) of MoFe protein under different flux and substrate regimes, (iii) determine the activation energies of hydride and N_2 bound E_n intermediates.
- Use mass spectrometry and engineering of MoFe protein surface variants to map the localization of nanocrystal binding sites and understand the influence on interfacial electron transfer.
- Use capping ligand chemistry of nanocrystals to tune excited state lifetimes and elucidate the relationship to quantum efficiency of electron transfer, photochemical product selectivity and reaction kinetics.
- Elucidate the product formation rates of alternative VFe- and FeFe-proteins under photochemical activation and the pre-steady state reaction intermediates by EPR to understand how E-state kinetics are involved in control of substrate selectivity.

My scientific area(s) of expertise is/are: Biophysical and mechanistic studies of iron-sulfur metalloenzymes.

The ideal collaborator for my project would have expertise in: Expertise in Mössbauer or X-ray spectroscopy techniques of iron-sulfur cluster metalloenzymes.

Publications supported by this project [2022-2024]:

1. B.P. Jagilinki, M.A. Willis, F. Mus, R. Sharma, L.M. Pellows, D.W. Mulder, Z.-Y. Yang, L.C. Seefeldt, P.W. King, G. Dukovic, J.W. Peters. **2024**. "Microscale Thermophoresis (MST) as a tool to study binding interactions of oxygen-sensitive biohybrids." *Bio-protocol Journal.* **14**(15). DOI:10.21769/BioProtoc.5041.
2. A. Clinger, Z.-Y. Yang, L.M. Pellows, P.W. King, F. Mus, J.W. Peters, G. Dukovic, L.C. Seefeldt. **2024**. "Hole-scavenging in photo-driven N_2reduction catalyzed by a CdS-nitrogenase MoFe protein biohybrid system." *J. .Inorgan. Biochem.* **253**:112484. DOI:10.1016/j.jinorgbio.2024.112484.
3. L.M. Pellows, M.A. Willis, J.L. Ruzicka, B.P. Jagilinki, D.W. Mulder, Z.-Y. Yang, L.C. Seefeldt, P.W. King, G. Dukovic, J.W. Peters. **2023**. "High affinity electrostatic interactions support the formation of CdS quantum dot:nitrogenase MoFe protein complexes." *Nano Lett*ers. **23**:10466. DOI:10.1021/acs.nanolett.3c03205.
4. L.M. Pellows, G.E. Vansuch, B. Chica, Z-Y. Yang, J.L. Ruzicka, M.A. Willis, A. Clinger, K.A. Brown, L.C. Seefeldt, J.W. Peters, G. Dukovic and D.W. Mulder, P.W. King. **2023**. "Low-temperature trapping of N_2 reduction reaction inter-mediates in nitrogenase MoFe protein–CdS quantum dot complexes". *J. Chem. Phys.* **159**:235102. DOI:10.1063/5.0170405.
5. G.E. Vansuch, D.W. Mulder, B. Chica, J.L. Ruzicka, Z-Y. Yang, L.M. Pellows, M.A. Willis, K.A. Brown, L.C. Seefeldt, J.W. Peters, G. Dukovic, P.W. King. **2023**. "Cryo-annealing of photoreduced CdS quantum dot–nitrogenase MoFe protein complexes reveals the kinetic stability of the E_4(2N2H) intermediate." *J. Am. Chem. Soc*. **145**:21165. DOI:10.1021/jacs.3c06832.
6. A. Badalyan, Z.Y., Yang, M. Hu, M., T.L. Liu, L.C. Seefeldt. **2022**. "Tailoring electron transfer pathway for photocatalytic N_2-to-NH_3 reduction in a CdS quantum dots-nitrogenase system." *Sustain. Energy Fuels*. **6**:2256-2263. DOI:10.1039/D2SE00148A.
7. J.L. Ruzicka, L.M. Pellows, H. Kallas, K.E. Shulenberger, O.A. Zadvornyy, B. Chica, K.A. Brown, J.W. Peters, P.W. King, L.C. Seefeldt, G. Dukovic. **2022**. "The kinetics of electron transfer from CdS nanorods to the MoFe protein of nitrogenase." *J. Phys. Chem. C.* **126**: 8425-8435. DOI:10.1021/acs.jpcc.2c02528.
8. B. Chica, J. Ruzicka, L.M. Pellows, H. Kallas, E. Kisgeropoulos, G.E. Vansuch, D.W. Mulder, K.A. Brown, D. Svedruzic, J.W. Peters, G. Dukovic. **2022**. "Dissecting nitrogenase MoFe protein P-cluster P^{2+} to P^{+} conversion in CdS nanocrystal–MoFe protein complexes". *J. Am. Chem. Soc.* **144**:5708-5712. DOI:10.1021/jacs.1c13311.

Production of Ethylene and 3-Hydroxypropanoate from the Common Metabolite, 2-Oxoglutarate, by the Ethylene-Forming Enzyme (EFE)

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Overall research goals:

This project aims to provide a deeper understanding of the structure and mechanism of the unusual iron(II)- and 2-oxoglutarate-dependent (Fe/2OG) ethylene-forming enzyme (EFE), so that the enzyme can be used for the biocatalytic production of valuable commodity chemicals. We initially set out to understand how the reaction bifurcates between ethylene formation (EF), which accounts for ~ 70% of the reaction flux, and L-arginine (L-Arg) oxidation (RO) to guanidine and pyrroline 5-carboxylate, which accounts for the other ~ 30%. Along the way, we discovered a minor second branch off the EF pathway that yields a trace (1%) of the partially fragmented by-product, 3-hydroxypropanoate (3-HP). Both ethylene and 3-HP are valuable industrial chemicals. The main route to ethylene, steam cracking of higher hydrocarbons, is extremely energy intensive and will become even more costly as the world weans from fossil fuels. 3-HP is a "platform chemical" used, for example, in production of acrylates and directly as a monomer for biodegradable polyester; its renewable bioproduction has been hotly pursued. We seek to enable more efficient production of ethylene, higher alkenes, and 3-HP through a deeper understanding of the complex reaction pathway and how it is directed by the enzyme.

Our work from prior funding cycles established that the first branch point occurs as O_2 adds to the EFE•Fe(II)•2OG•L-Arg complex. It involves a partition between (1) net *replacement* by O_2 of the C1 carboxylate of 2OG to form a peroxysuccinatoiron(II) complex heading the RO pathway and (2) insertion by O_2 between C1 and C2 to form a succinylperoxocarbonatoiron(II) complex heading the EF pathway.[1,2] The former complex undergoes peroxide-bond heterolysis to form the ferryl intermediate that abstracts hydrogen from C5 of L-Arg to initiate its oxidation. The latter intermediate undergoes peroxide-bond *homolysis* to form a carbonatoiron(III) cofactor form and succinate-1-yl radical, which undergoes a C2-C3-cleaving Kolbe-like decarboxylation yielding the C3-C5-derived propanoate-3-yl radical. We proposed that this radical undergoes a novel radical γ coupling to an oxygen of the carbonate Fe(III) ligand, yielding a 2-carboxyethylcarbonatoiron(II) complex that undergoes a finishing Grob-like fragmentation to ethylene. Competition with the Grob-like step from elimination of CO_2 from the unstable carbonate monoester was invoked to explain the trace 3-HP produced. In the current funding period, we set out to obtain further experimental evidence for the polar-concerted nature of the EF step, an understanding of how to derail the step by mutagenesis to support production of 3-HP as the primary product, and insight into whether mutagenesis might enable the final step in cases of branched 2OG analogs that could, in principle, yield larger alkenes (e.g., propylene and isobutylene, which are also valuable compounds) but are fragmented by wild-type EFE only to analogs of 3-HP.

1. Copeland, R. A.; Davis, K.; Shoda, T.; Blaesi, E.; Boal, A.; Krebs, C; **Bollinger, J. M., Jr.** "An Iron(IV)-oxo Intermediate Initiating L-Arginine Oxidation but not Ethylene Production by the 2-Oxoglutarate-dependent Oxygenase, Ethylene-Forming Enzyme," *J. Am. Chem. Soc.* **2021**, *143*, 2293-2303.
2. Copeland, R. A.; Zhou, S.; Schaperdoth, I.; Tokufu, K. S.; **Bollinger, J. M., Jr.**; Krebs, C. "Hybrid Radical-Polar Pathway for Excision of Ethylene from 2-Oxoglutarate by an Iron Oxygenase," *Science* **2021**, *373*, 1489-1493.

Significant achievements: [2023-2026]

- Proof that 3-HP can be made as the primary product in variants of EFE harboring sterically impactful but electrostatically innocent substitutions near C3 and C4 of 2OG
- Demonstrations that (1) propylene can be made from (*4R*)-methyl-2OG, a substrate converted by wild-type EFE only to 2-methyl-3-HP, in high yield by a variant EFE harboring a bulk-diminishing substitution near C4 and (2) the variant consumes the 2OG analog considerably more efficiently than does wild-type EFE
- Verification by stopped-flow FTIR experiments that the C1 carboxylate of 2OG is converted not to CO_2 but rather to (bi)carbonate by *oxygenative rather than decarboxylative C1-C2 cleavage*
- Discovery by a combination of stopped-flow FTIR experiments and computational analysis that *ethylene formation preempts rather than follows C3• ↔ carbonate coupling*, i.e., that the novel coupling commits the reaction to 3-HP formation, whereas ethylene forms by a competing step still in the radical manifold

Science objectives for 2025-2026:

Multiple computational studies have implicated internal electric fields (IEFs) as important for directing the outcomes of enzymes in general and Fe/2OG oxygenases specifically. We are incorporating Stark-effect vibrational spectroscopic probes (e.g., cyanophenylalanine) to determine the IEF in EFE and evaluate the hypothesis, advanced by Christov and co-workers, that it can control the flux at the first branchpoint. In addition, we are attempting to make production of 3-HP and its larger derivatives more efficient by combining helpful substitutions (the starting point of a full-blown directed evolution effort). Crystallographic characterization of wild-type and variant EFEs with stable analogs of the succinylperoxycarbonate and 2-carboxyethylcarbonate ligands in the key intermediate states will allow us to deduce the structural basis for control of outcome by the substitutions we have identified.

My scientific area(s) of expertise is/are: The combined expertise of the PI team encompasses transient kinetics, enzyme engineering, structural biology, spectroscopy, and computational evaluation of enzyme dynamics and mechanisms.

The ideal collaborator for my project would have expertise in: We would benefit from collaboration with someone who has access to a state-of-the-art stopped-flow FTIR instrument that would allow us to define the kinetics of CO_2 production into the tens-to-hundreds of milliseconds time regime.

Publications supported by this project 2023-2026:

3. Burke, E. J.; Copeland, R. A.; Dixit, Y.; Krebs, C.; and **Bollinger, J. M., Jr.** "Steric Perturbation of the Grob-like Final Step of Ethylene-Forming Enzyme Enables 3-Hydroxypropionate and Propylene Production," *J. Am. Chem. Soc.* **2024**, *146*, 1977–1983.

Transmethylation reactions during methylotrophic methanogenesis in methanogenic Archaea

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Overall research goals:

Overall objectives: To understand the molecular and biochemical basis of biological methane and acetate formation from methylated compounds. This entails understanding of the enzymes that carry out methyl group transfer, the function of the 22nd amino acid pyrrolysine in this process, and how oxidized corrinoid proteins are reductively reactivated to the low potential Co(I) state using molecular hydrogen.

Methanogenesis from methylated amines in *Methanosarcina* spp. requires the prerequisite formation of methyl-CoM, the direct methane precursor. During our DOE sponsored project, we have discovered distinct enzymes have evolved for the methylation of CoM with trimethylamine (TMA), dimethylamine (DMA), and monomethylamine (MMA). Each methyltransferase preferentially binds TMA, DMA, or MMA as substrate to methylate a distinct cognate corrinoid binding protein. These distinct yet homologous corrinoid proteins are then demethylated by a single methyltransferase to methylate CoM. The three types of methylamine methyltransferases genes are not homologous, yet each contains an in-frame amber codon. We have shown this amber codon stands for pyrrolysine, an atypical genetically encoded amino acid, as well as how this residue is biosynthesized and genetically encoded. MttB, the TMA methyltransferase, is the founding member of a large superfamily composed of pyrrolysyl-proteins, and non-pyrrolysyl proteins. In collaboration with the Chan group we have obtained the structures of both MttB and MtbB, the DMA methyltransferase. We have carried out site directed mutagenesis of MttB and shown pyrrolysine is essential for efficient catalysis. We have shown that homologs of MttB lacking pyrrolysine are specific for larger methylamines, such as glycine betaine, choline, or *L*-carnitine. For example, we have recently characterized the novel choline dependent pathway of THF methylation in the human gut acetogen *Eubacterium limosum*. We have also carried out analysis of the active site of MtgB, a glycine betaine methyltransferase based on a substrate-bound structure obtained in collaboration with the Hao group.

In order for the cognate corrinoid proteins to accept methyl cations from these methyltransferases, the corrinoid cofactor must be in the highly reducing Co(I) state and return to this state after donating the methyl group to the CoM methylase. Adventitious oxidation of MttC to the Co(II) redox state inactivates the protein for methyl transfer. However, rescue is provide by the ATP dependent reductive activation of the Co(II)-corrinoid protein by the FeS protein, RamA. To accomplish this, RamA itself must first be reduced using molecular hydrogen by an unidentified protein we have termed the H_2-factor. We have undertaken the identification of the H_2-factor, which is a primary goal of this current period. We recently developed a novel assay which has proven reliable for anaerobic purification for

H_2-factor from cell extracts by column chromatography. At present rate of progress we hope to be able to identify this sole remaining uncharacterized protein of methylamine metabolism by end of this project period, setting us up for characterization of the protein in the coming period. Additionally, we have continued our collaboration with the Chan lab for structural characterization of the methylamine methyltransferases and are currently preparing a paper describing the structure of the dimethylamine methyltransferase. In the coming period we will also undertake experiments to further characterize RamA to gain insight into how this protein employs ATP hydrolysis to reduce the low potential (<-730 mV) Co(II)/Co(I) couple with the much higher redox potential of hydrogen (circa -400 mV).

Significant achievements: [2022-2025]

•In collaboration with the Chan group, we published the structure of MttB, the TMA methyltransferase.
•We carried out site directed mutagenesis to analyze the active site.
•In collaboration with the Hao group, we obtained a substrate bound structure of the non-pyrrolysyl MttB superfamily member MtgB, the glycine betaine methyltransferase.
•We carried out site directed mutagenesis to analyse the active site of MtgB.
•We completed the analysis of a novel choline dependent demethylation pathway from a human gut acetogen.
•In collaboration with the Chan group we obtained the structure of MtbB, the DMA methyltransferase.
•We developed an assay for the H_2-factor allowing RamA to reduce corrinoid proteins with H_2.
•We are now employing this assay to purify the H_2-factor from cell extract.

Science objectives for 2024-2025:

•Complete purification and characterization of the H_2-factor.

•Obtain protein sequence from the H_2-factor and thereby identify the encoding gene(s).

• Express H_2-factor gene(s) and reconstitute the reduction of corrinoid proteins with recombinant proteins, ATP, and hydrogen.

Test RamA ability to carry out ATP:Pi exchange.

My scientific area(s) of expertise is/are: microbiology and molecular biology of anaerobic bacteria and archaea; particularly the biochemistry of their enzymes related to one carbon metabolism.

The ideal collaborator for my project would have expertise in: structural and spectroscopic characterization of iron-sulfur and/or corrinoid proteins..

Publications supported by this project [2022-2025]:

1. Li, J., Kang P.T., Jiang, R., Lee, J.Y., Soares, J.A., Krzycki, J.A., Chan, M.K. Insights into pyrrolysine function from structures of a trimethylamine methyltransferase and its corrinoid protein complex. Commun Biol. 2023. 6(1):54. doi: 10.1038/s42003-022-04397-3. PMID: 36646841; PMCID: PMC9842639.
2. Jiang, R., Kountz, D.J., Zhang, L., Krzycki, J.A. A cobalamin-dependent pathway of tetrahydrofolate methylation with choline from the human gut acetogen *Eubacterium limosum*. 2024. Submitted.
3. Jiang, R., Picking, J., Chan, M.K., Hao, B., Krzycki, J.A. Mutagenic analysis of a pyrrolysyl and a non-pyrrolysyl MttB superfamily members. 2025. In preparation.
4. Li, J., Jiang, R, Krzycki, J.A., Chan, M. K. Structure of the pyrrolysyl-protein dimethylamine methyltransferase. 2025. In preparation.

Bioinorganic Chemistry of Nitrification: Structure and Function of Ammonia Monooxygenase

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Overall research goals:

Nitrification is a primary metabolism whereby microorganisms derive total energy for life from the oxidation of nitrogen- rather than carbon-based fuels. Despite the ubiquity of nitrifying organisms across practically all ecosystems, major gaps persist in understanding the fundamental biochemical steps comprising nitrification. Case in point, molecular level understanding of the very first step of nitrification remains elusive. This step, in both ammonia oxidizing bacteria (AOB) and archaea (AOA), is the conversion of ammonia (NH_3) to hydroxylamine (NH_2OH) by the multimeric integral membrane copper enzyme ammonia monooxygenase (AMO). No AMO has ever been purified in an active form. Some inferences may be drawn from homology, as AMO is a member of the copper membrane monooxygenase family, whose constituent enzyme particulate methane monooxygenase (pMMO) has been studied extensively, and for which numerous structures and copious reactivity data are available.

The goal of this project is to fill this major and persistent gap in understanding of the nitrogen cycle by solving the structure and studying the reactivity of the AMO family. The immediate goal is to obtain a purified and, ideally, active AMO. Two approaches are being pursued. One is to use *Mycobacterium smegmatis* as a recombinant expression host for AMO. A second approach is to reconstitute AMO from individually produced subunits.

Significant achievements: 2020-2024:

The key achievements of the funding period concern obtaining the individual subunits of AMO in appreciable yields and purity. This has not been straightforward. The amoA and amoC subunits principally comprise transmembrane domains and thus are insoluble in detergent-free buffer. Even in detergent, solubility is temporary and the subunits eventually precipitate. Consequently, there have been no reported preparations of amoA or amoC subunits nor of the related subunits from pMMO (pmoA and pmoC). By contrast, amoB comprises a large "soluble" component, and consequently examples of amoB and pmoB subunits have been purified and studied.

We found that we could use *E. coli* to express reasonable (1-5 mg protein/L media) quantities of amoA, amoB, and amoC as fusion proteins with maltose binding protein (MBP). These fusion proteins bore protease target sequences enabling liberation of the AMO subunits. We found could purify these proteins to homogeneity, and we also demonstrated successful cleavage of MBP. Until cleavage, the subunit chimeras appeared to be soluble However, we could not reconstitute the AMO quaternary structure, obtaining only evidence of aggregation from negative stain electron microscopy.

We later learned via small angle X-ray scattering (SAXS) that, despite their solubility, the MBP-amo subunit chimeras were aggregated—that is, they form soluble aggregates. Consequently, we redesigned the fusion proteins to bear the SUMO domain. These SUMO-amo subunit chimeras could also be purified to homogeneity, but importantly we found via SAXS that these constructs are not aggregated and appear properly folded in solution. Thus, we now have soluble, non-aggregated amo subunit chimeras that can be subject to biophysical characterization and with which we can proceed toward assembling the AMO complex.

Science objectives for 2024-2025:

This project is currently in a no-cost extension. Moreover, the graduate student, Alex Laughlin, who has been principally responsible for advancing the project will graduate near the time of the PI meeting. Given our

progress to date and the effort required to arrive at preparative methods for AMO subunits, we are eager to continue the project. However, given the difficulty and the fact that students in the Lancaster laboratory are all currently engaged in projects, we will be applying for renewal funding to enable hiring of a postdoctoral researcher with expertise in membrane protein manipulation. With useful quantities of properly solubilized and de-aggregated AMO subunits accessible, this researcher will be in a strong position to carry out assembly trials towards recapitulating functional AMO.

My scientific area(s) of expertise is/are: Bioinorganic chemistry, inorganic synthesis, inorganic spectroscopy, and structural biology.

The ideal collaborator for my project would have expertise in: Membrane protein purification/manipulation, cryoEM.

Publications supported by this project 2020-2024:

NA

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

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Narayanan Srividya, Co-Principal Investigator
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Overall Research Goals:

Plant-based terpenoid oils and resins are characterized by a high energy density and high degree of reduction and are thus viable "biocrude" feedstocks for fuels in the diesel and kerosene range. Furthermore, many specialty chemicals are also based on terpenoid backbones, including polymers, solvents, and diverse small molecules. Terpenoids thus have the potential to serve as chemical feedstocks in a non-food bioeconomy based on carbon and energy capture, allocation, conversion, and storage by plants, which is directly in line with the research mission of the Physical Biosciences area within the DOE-BES program. This proposal aims to unravel the mechanistic basis for selectivity in terpene synthases, which are enzymes that catalyze the formation of acyclic and cyclic hydrocarbons as the first committed step in the biosynthesis of terpenoids. Such knowledge will allow us to infer the mechanistic underpinnings of how plants produce highly complex, reduced chemical scaffolds.

Significant Achievements (2022-2024):

Specific Aim 1: Investigate the Mechanism of Monoterpene Synthases That Form Acyclic Products by Testing the Hypothesis That Early Carbocation Intermediates Are Not Sufficiently Stabilized, Thus Resulting in Early Reaction Termination. Sequence comparisons of putative active site residues across monoterpene synthases that generate acyclic hydrocarbon products (β-myrcene or (*E*)-β-ocimene) and those that release monocyclic hydrocarbon products ((-)-limonene, γ-terpinene or terpinolene) were employed to identify amino acids that might play roles in conferring specificity. We then generated a series of mutants designed to convert an enzyme that catalyzes a reaction with an acyclic product to variants that release a monocyclic product and vice versa. We are currently investigating if ligand docking and MD simulations might help explain these modulations of specificity. We are expecting to complete a manuscript reporting on these exciting findings before the end of the year.

Specific Aim 2: Evaluate the Mechanism of Monoterpene Synthases That Form Alcohols and Cyclic Ethers by Testing the Hypothesis That the Stabilization of Carbocation Intermediates in a Specific Pose Favors Water Capture Over Deprotonation. Sequence and structural comparisons of putative active site residues across monoterpene synthases that generate monocyclic hydrocarbon products ((-)-limonene, γ-terpinene or terpinolene) and those that form monoterpenoid alcohols or cyclic ethers (α-terpineol or 1,8-cineole) were employed to identify amino acids that might play roles in conferring specificity. We identified an active site expansion that accommodates a water molecule in the latter. We then generated a series of mutants designed to convert an enzyme that catalyzes a reaction with a monocyclic hydrocarbon product to variants that release a monoterpenoid alcohol or cyclic ether and vice versa. We are currently investigating if ligand docking and MD simulations might help explain these modulations of specificity. We are expecting to complete a manuscript reporting on these exciting findings before the end of the year.

Specific Aim 3: Examine the Mechanism of Monoterpene Synthases That Form Different Bicyclic Products by Testing the Hypothesis That Specificity is Determined by the Stabilization of Specific Carbocation Intermediates. Our recently published work centered on an evaluation of the determinants for the formation of monocyclic and bicyclic terpenes. Free energy simulations indicated that a common reaction intermediate, the α-terpinyl cation (ATC), preferentially adopts one of two different conformations in LMNS and BPPS, thus leading to the formation of monocyclic monoterpenes in the former and bicyclic products in the latter. We are currently investigating enzymes that generate a bicyclic product (β-pinene, sabinene, α-thujene or (-)-camphene) with high specificity to evaluate if differences in the stabilization of different carbocation intermediates can explain the formation of different end products in different monoterpene synthases.

Science Objectives for 2024-2026 (current funding period):

- Establish a mechanistic understanding of the determinants for the formation of different classes of products by monoterpene synthases. The emphasis is on investigating enzymes and their variants that generate an acyclic hydrocarbon, a monocyclic hydrocarbon, a monoterpenoid alcohol, a monoterpenoid cyclic ether or a bicyclic product.
- Investigate if a specific amino acid residue or the enzyme-bound diphosphate (hydrolyzed from the geranyl diphosphate substrate) is involved in the reaction termination as a catalytic base and if this differs in enzymes that generate different products.
- Begin developing a model, building on sequence characteristics and 3D modeled structures, to predict the functions of monoterpene synthases.

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

We are currently working with Simone Raugei and Hoshin Kim (PNNL), which has enabled us to make progress in an area (QM/MM and MD simulations) that requires unique computational expertise and resources. We are currently attempting to crystallize monoterpene synthases and selected variants. Should refractable crystals be obtained, we will be looking for a collaborator to perform X-ray crystallography.

Publications supported by this project (2022-2024):

1. Polito J. Lange I., Barton K., Srividya N., Lange B.M. (2024) Characterization of a unique pair of ferredoxin and ferredoxin NADP+ reductase isoforms that operates in non-photosynthetic glandular trichomes. ***Plants*** 13, 409 (completing work from previous funding period). https://www.mdpi.com/2223-7747/13/3/409
2. Srividya N., Kim H., Raugei S., Lange B.M. (2024) Chemical diversity in angiosperms - monoterpene synthases control complex reactions that provide the precursors for ecologically and commercially important monoterpenoids. ***Plant J.*** 119, 28-55. https://onlinelibrary.wiley.com/doi/full/10.1111/tpj.16743
3. Lange B.M., Srividya N., Lange I., Parrish A.N., Benzenberg L.R., Pandelova I., Vining K.J., Wüst M. (2023) Biochemical basis for the formation of organ-specific volatile blends in mint. ***Front. Plant Sci.*** 14, 1153. https://www.frontiersin.org/journals/plant-science/articles/10.3389/fpls.2023.1125065/full
4. Lange B.M., Srividya N. (2023) Cannabis monoterpene synthases – evaluating structure-function relationships. ***Phytochem. Rev.*** 22, 449-465. https://link.springer.com/article/10.1007/s11101-023-09861-4
5. Polito J.T., Lange B.M. (2023) Standard operating procedures for the comprehensive and reliable analysis of cannabis terpenes. ***Methods Enzymol.*** 680, 381-419. https://www.sciencedirect.com/science/article/pii/S0076687922002932
6. Kim H., Srividya N., Lange I., Huchala E.W., Ginovska B., Lange B.M., Raugei S. (2022) Determinants of selectivity for the formation of monocyclic and bicyclic products in monoterpene synthases. ***ACS Catal.*** 12, 7453-7469. https://pubs.acs.org/doi/full/10.1021/acscatal.2c01836
7. Vining K.J., Pandelova I., Lange I., Parrish A., Lefors A., Kronmiller B., Liachko I., Kronenberg Z., Srividya N., Lange B.M. (2022) Chromosome-level genome assembly of Mentha longifolia L. reveals gene organization underlying disease resistance and essential oil traits. ***G3 Genes|Genomes|Genetics*** 12, jkac112. https://academic.oup.com/g3journal/article/12/8/jkac112/6584825
8. Srividya N., Lange I., Richter J.K., Wüst M., Lange B.M. (2022) Selectivity of enzymes involved in the formation of opposite enantiomeric series of p-menthane monoterpenoids in peppermint and Japanese catnip. ***Plant Sci.*** 314, 111119. https://www.sciencedirect.com/science/article/pii/S0168945221003150

Understanding Nitrogenase Maturation and Activity in Methanogens

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Overall research goals:

The long-term objectives of this research are to: 1) elucidate the regulation and catalytic capabilities of methanogen nitrogenases, 2) identify the factors involved in the assembly and maturation of cofactors in methanogen nitrogenases, and 3) understand how electron transfer to nitrogenase is integrated with methanogenesis. Nitrogenase, a metalloenzyme system exclusive to bacteria and archaea, facilitates biological nitrogen fixation (diazotrophy) by converting dinitrogen (N_2) to ammonia (NH_3). All diazotrophs possess molybdenum (Mo)-nitrogenase (Nif), which includes iron-sulfur (Fe-S) clusters and the Mo-containing active site cofactor (FeMo-co). Some diazotrophs also have alternative nitrogenases where vanadium (V) or iron (Fe) replace Mo in FeMo-co. Bacterial V-nitrogenase (Vnf) and Fe-nitrogenase (Anf) exhibit different catalytic properties compared to Mo-nitrogenase. However, the catalytic properties of methanogen nitrogenases remain unknown. Nitrogenase is crucial for bioenergy research as it directly catalyzes biofuel production (e.g., H_2), serves as a model for understanding complex metallocofactor biogenesis, and its functional expression in plants could reduce the need for fossil fuel-derived fertilizers. We are using the genetically tractable *Methanosarcina acetivorans*, which contains Mo-, V-, and Fe-nitrogenases, as a model organism to achieve these long-term objectives. Initial results suggest that the assembly, activity, and regulation of nitrogenase in methanogens differ from those in bacteria, potentially offering new avenues to optimize or develop metalloenzyme-based energy production strategies.

Significant achievements: [2022-2024]:

- Related to objective 1: We used genetic and biochemical approaches to determine the functional importance and properties of nitrogenases in *M. acetivorans*.
  - We showed that Mo-nitrogenase is required for V-and Fe-nitrogenase usage, which to our knowledge is a regulatory aspect unique to methanogens (published).
  - We generated a *nif* deletion strain to allow for complementation studies to determine why Mo-nitrogenase is required for nitrogen fixation in the absence of Mo.
  - We optimized strains and protocols to purify *M. acetivorans* Mo-nitrogenase both from cells grown with and without Mo.
  - We have developed *M. acetivorans* strains that bypass fixed nitrogen regulation and express Mo-nitrogenase in cells growing with NH_3, which removes the dependence on Mo-nitrogenase for growth (unpublished).
  - We determined the regulatory factor that controls production of Fe-nitrogenase and generated a strain that produces Mo- and Fe-nitrogenases in cells grown with Mo (paper in preparation).
  - We used a hydrogenase-deficient strain to quantify *in vivo* H_2 produced during N_2 reduction by Mo-, V-, and Fe-nitrogenases in *M. acetivorans* (paper in preparation).
- Related to objective 2: We used genetic and biochemical approaches to ascertain the role of components of the ISC and SUF Fe-S cluster biogenesis systems and NifB to the maturation of nitrogenases in *M. acetivorans*.
  - We demonstrated that the minimal SUF system that is universally conserved in methanogens is not required for Fe-S cluster biogenesis and nitrogen fixation by *M. acetivorans* (published).
  - We showed that the gene encoding NifB, the radical SAM enzyme required for maturation of all three nitrogenases in bacteria, was shown to be essential to the viability of *M. acetivorans* (preprint/in revision)

- Related to objective 3: We used genetic, physiological, and biochemical approaches to examine the integration of nitrogenases in *M. acetivorans*.
    - We demonstrated that hydrogenase is required for hydrogen cycling resulting in a branched electron transport system during nitrogen fixation by non-hydrogenotrophic *M. acetivorans* (preprint/in revision).
    - We developed an affinity-based purification approach to identify interacting partner proteins to nitrogenase components and assembly proteins (unpublished).
    - We generated *in vivo* evidence that electron flow to nitrogenase significantly alters carbon flow and methanogenesis (paper in preparation).

Science objectives for 2024-2025:

- Continue to use genetics and biochemical approaches to determine why Mo-nitrogenase is required for nitrogen fixation in the absence of Mo.
- Continue to determine the biophysical/catalytic properties of purified *M. acetivorans* Mo-nitrogenase from cells grown with and without Mo.
- Continue to investigate the importance of ISC and SUF components to *M. acetivorans*.
- Continue to use affinity purification approaches and proteomics to identify:
    - Nitrogenase Fe-S cluster biogenesis and maturation proteins.
    - Nitrogenase redox partner proteins.

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

The ideal collaborator for my project would have expertise in: Structural Biology, Bioenergetics, Proteomics.

Publications supported by this project 2022-2024:

1. J. Saini, T.M. Deere, M. Chanderban, G.J. McIntosh, DJ Lessner. "*Methanosarcina acetivorans*" Trends Microbiol. **3**, 320-21 2023. DOI: 10.1016/j.tim.2022.10.001
2. M.C. Chanderban, C.A. Hill, A.E. Dhamad, and D.J. Lessner. Expression of V-nitrogenase and Fe-nitrogenase in *Methanosarcina acetivorans* is controlled by molybdenum, fixed nitrogen, and the expression of Mo-nitrogenase Appl. Environ. Microbiol. **89**, e0103323. DOI: 10/1128/aem.01033-23
3. J. Saini, T.M. Deere, D.J. Lessner. The minimal SUF system is not required for Fe-S cluster biogenesis in the methanogenic archaeon *Methanosarcina acetivorans.* Sci. Rep. **13**, 15120. DOI: 10.1038/s41598-023-42400-x
4. J.M. Hoerr, A.E. Dhamad, T.M. Deere, M. Chanderban, D.J. Lessner. Vht hydrogenase is required for hydrogen cycling during nitrogen fixation by the non-hydrogenotrophic methanogen *Methanosarcina acetivorans*. In revision (Pre-print available at https://www.biorxiv.org/content/10.1101/2021.10.12.464174v1)
5. J. Saini, A.E. Dhamad, A. Muniyasamy, A.J. Alverson, D.J. Lessner. The nitrogenase cofactor biogenesis enzyme NifB is essential for the viability of methanogens. In revision (Pre-print available at https://www.biorxiv.org/content/10.1101/2023.10.20.563283v1)

Characterizing Plant-Specific Features of Mitochondrial Respiratory Complexes

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Overall research goals:

Despite the centrality of respiration to plants' biomass accumulation, carbon flux and acclimation, the fundamental mechanisms by which plants' mitochondrial complexes produce electrochemical proton gradients for energy transduction remain unknown. A detailed functional and structural understanding of plant respiratory mitochondrial electron transport chain (mETC) complexes is essential to determine the fundamental mechanisms of biological energy transduction. By applying our expertise in mitochondrial-membrane-protein biochemistry and structure biology, we plan to characterize the plant-specific features of mitochondrial respiratory complexes and supercomplexes. Specifically, we plan to:

Aim 1: Examine plant-specific functions of complex I and its assembly intermediate CI*.
Aim 2: Examine conserved and plant-specific features of respiratory supercomplexes.
Aim 3: Determine the structure and molecular mechanisms of salicylic acid regulation of plant complex II.

This work will test several long-standing hypotheses, such as the catalytic function of complex I's carbonic anhydrase domain as well as test novel hypotheses triggered by the structures, such as the potential functional role of CI*. This project will shed light on the fundamental tenets of electron transfer coupled proton-pumping in plant mitochondria. It will generate approaches, materials and hypotheses for the continued mechanistic examination of further energy-converting enzymes in plants, significantly advancing the field of plant respiration and bioenergetics in general. Our research will also impact broader areas of plant physiology such as photosynthesis, biomass accumulation and stress response, as well as facilitate the development of novel inhibitors to be used as potential agricultural herbicides or pesticides.

Significant achievements: 2021-2024:

We performed a detailed kinetic characterization of complex I activity in plant mitochondrial membranes to determine whether plant complex I has an active-to-deactive transition like what is observed in other species. As the deactive state prevents reverse electron transport by complex I, our working hypothesis, based on thermodynamic considerations, was that plant complex I should have a deactive state. However, our results indicated that plant complex I does not have a mammalian-like deactive state but displays distinct behavior that has not been described in other systems. Mammalian complex I displays a state dependent sensitivity to the cysteine modifying reagent N-methyl maleimide (NEM). I.e., in mammals, complex I can adopt an NEM sensitive state (associated with the deactive state) and an NEM insensitive state (associated with enzyme turnover and hence the active state). However, we showed that the plant complex lacks an NEM insensitive state. I.e., it is always found in a state that is sensitive to NEM, even under turnover conditions. These activity measurements were further supported by our structure of intact plant complex I in SC I+III_2, which showed conformations of active site loops intermediate to what is seen in the active and deactive states of the mammalian complex. Together these data indicate that regulation of complex I activity in plants is very different to that in mammals and more work is needed to fully understand what is occurring in plants. We still

expect that plant complex I should be incapable of reverse electron transport but has evolved a distinct mechanism to prevent reverse electron transport compared to the mammalian complex.

We optimized an isolation protocol for SC I+III_2 from *V. radiata* mitochondria using digitonin extraction and SC stabilization in amphipathic polymers (amphipols) followed by sucrose gradient ultracentrifugation. Using this protocol, we optimized cryogenic electron microscopy (cryoEM) grid preparation and collected data that has resulted in a structure of the plant respiratory SC I+III_2 to ~3.0 Å resolution. The structure revealed a plant specific subunit on the intermembrane space side of the inner mitochondrial membrane that is not present in other known supercomplex structures, we coined this subunit P9. Unexpected interactions between the complex III_2 mitochondrial processing peptidase domain and the membrane arm of complex I were also observed. Although distinct in plants, these interactions are analogous to those seen in other organisms indicating a conserved interaction site. Importantly, this structure represents the first intact structure of plant complex I. We also obtained several ~4-6 Å reconstructions of the plant SC I+III_2 in alternative conformations lacking specific subunits of the "ferredoxin bridge domain" on CI. These structures are likely caused by disintegration of the complex after extraction from the membrane and emphasize the importance of complex III_2 in stabilizing the structure of complex I. Within the complex I structure specific active site loops were observed to adopt conformations not seen in structures of complex I from other species. These loops are known to be important in the active-to-deactive transition seen in mammals and hence the alternative conformations provide further evidence that something distinct is occurring in plants.

We have identified promising conditions for the solubilization of intact plant complex II using styrene maleic acid (SMA) polymers. We have been able to extract what appears to be intact complex II according to blue native PAGE and in-gel activity assays. Preliminary spectroscopic activity assays also indicate that the extracted complex II is functional. We are further optimizing our extraction conditions and activity assays to confirm these preliminary results. We aim to use the activity assays to develop a purification protocol for the complex. Given these promising initial results we are focusing our efforts on developing a purification protocol and generating detailed enzymatic characterizations of the plant complex. Once a biochemically active pure sample is produced, we will use the sample for cryoEM grid preparation and structure determination.

Science objectives for 2024-2025:

- Develop a reconstituted system to measure plant SC I+III_2 forward and reverse electron transport
- Develop a robust purification protocol for functional plant succinate dehydrogenase and solve its atomic structure.
- Develop a purification and reconstitution protocol for plant CI* and determine if it pumps protons

My scientific area(s) of expertise is/are: Membrane protein biochemistry and reconstitution, structural biology, single particle cryogenic electron microscopy, enzyme kinetic characterization.

The ideal collaborator for my project would have expertise in: Plant genetics, plant cell culture, EPR spectroscopy.

Publications supported by this project 2021-2024:

1. Maldonado, M., Fan, Z., Abe, K. M. & Letts, J. A. Plant-specific features of respiratory supercomplex I + III_2 from Vigna radiata. *Nat. Plants* **9**, 157–168 (2023). DOI: 10.1038/s41477-022-01306-8
2. Meyer, E. H., Letts, J. A. & Maldonado, M. Structural insights into the assembly and the function of the plant oxidative phosphorylation system. *New Phytol* (2022) doi:10.1111/nph.18259.

Transformative Biohybrid Diiron Catalysts for C-H Bond Functionalization

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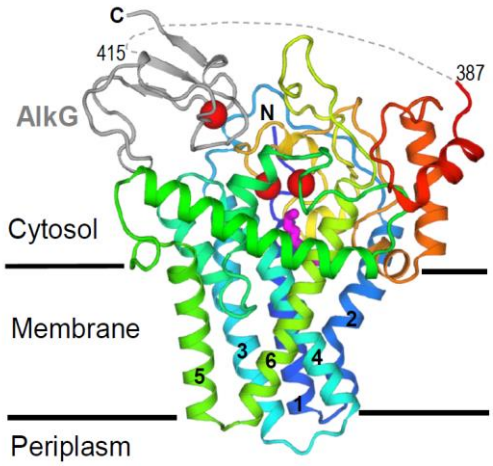
Overall research goals:

The functionalization of C-H bonds in hydrocarbons, such as naturally abundant alkanes in crude oils, in a selective and environmentally benign manner is a grand challenge of chemical manufacturing. C-H bonds in alkanes are inert, and their chemical activation and functionalization in industrial manufacturing require heat, pressure, and the use of precious transition-metal catalysts. Due to similar dissociation energies of different C-H bonds in an alkane, it is also challenging to achieve selective functionalization of bonds at specific positions. Our primary objective is to advance science to enable catalytic biohybrid systems to replace high-temperature/pressure catalysis for C-H functionalization with precious metals by low-temperature electrochemically driven biocatalysts using earth-abundant iron for selective C-H functionalization tunable to a variety of products.

To investigate and develop transformative biohybrid catalysts, we are employing a multidisciplinary approach of structural biology, biochemistry, theoretical chemistry, computational modeling, electrochemistry, artificial intelligence and machine learning (AI/ML), and in operando X-ray absorption spectroscopy. We are 1) investigating the structure and function of the nonheme diiron monooxygenase biocatalysts to understand structural determinants for alkane substrate specificity, activity, selectivity, and electron transfer reactions; 2) employing quantum chemical calculations, computational modeling, X-ray absorption spectrum, and AI/ML to reveal the underlying mechanistic details of the biocatalytic C-H bond functionalization to guide the design and development of tunable transformative biohybrid catalysts; 3) elucidating the electron transfer and redox processes for transformative biohybrid catalysis through electrochemical driven synthesis.

Significant achievements: 2022-2024

- **Structural basis for enzymatic terminal C-H bond functionalization:** We determined the structure of a natural fusion between AlkB and AlkG from *Fontimonas thermophila* (*Ft*AlkBG). The structure reveals the mechanistic basis for its selectivity towards and functionalization of alkane terminal C-H groups. AlkB structure contains a dodecane substrate and a diiron active site. AlkG docks on AlkB through electrostatic interactions and transfers electrons to the diiron center for catalysis. To understand the substrate entry and selectivity, we performed molecular dynamics simulations and identified key hydrophobic residues as gating residues in selecting and mediating substrate alkane entry. This research provides a landmark structural view of the diiron-center structure, alkane binding, and electron transfer of AlkBG. The structure serves as the basis for the creation of novel biocatalysts for the selective production of chemical feedstocks and value-added products from abundant alkanes.



Structure of a *Ft*AlkBG complex. *Ft*AlkB is shown as a rainbow cartoon with colors ranging from blue at the N-terminus to red at the C-terminus. *Ft*AlkG is shown as a gray cartoon. Three irons, two in *Ft*AlkB and one in *Ft*AlkG are shown as red spheres. A substrate dodecane is shown as magenta sticks. The six transmembrane helices are labeled 1-6.

- **Density functional theory for understanding nonheme diiron complexes:** We developed a computational protocol that provides an efficient approach to predicting Mössbauer parameters,

namely the isomer shift (δ) and quadrupole splitting ($|\Delta E_Q|$), and probing the local electronic environment of nonheme diiron complexes and enzyme active sites. Building on the modeling of nonheme diiron complexes and enzyme active sites**,** we employed QM cluster calculations to probe the activation of the AlkB diiron active site by electron transfer reactions and following oxygen and C-H activation chemistry. One significant finding was the impact of the medium's polarity on the energetics of electron transfer and chemical steps in the oxygen and C-H activation mechanism.

Science objectives for 2024-2025:

- **Structure and function:** In FY24, we successfully reconstituted *Po*AlkB and *Po*AlkB-AlkG fusion into MSP (membrane scaffold protein) nanodiscs. We also produced a triple mutant (VLI, V129M, L132V, and I233V) that can convert short-chain gas alkanes (C3 and C4) to liquid alcohols. In FY25, we will perform cryoEM screening of these samples for structure determination. We also plan to determine the cryoEM structures of the enzyme in a reduced state, i.e. Fe^{2+} using sodium dithionite, and intermediate states to reveal the mechanisms of catalysis. In addition, we will improve the selectivity and activity of the VLI triple mutant to transform C3 and C4 alkanes to their corresponding alcohols through directed evolution.
- **Computation and modeling:** We will probe the activation of the AlkB nonheme diiron active site by electron transfer reactions and following oxygen and C-H activation chemistry using hybrid QM/MM calculations. We will also use molecular dynamics to simulate the processes of oxygen binding and product release, from which we will identify key residues mediating these critical processes.
- **X-ray absorption and machine learning:** To prepare for the XAS-protein prediction model implementation, we manually extracted iron-contained protein structures and their corresponding XAS data from the literature. We will experiment with various transformer and diffusion models to enable spectrum-to-structure prediction and vice versa. On X-ray absorption, we will study *Po*AlkB catalysis under reaction conditions to capture intermediates for understanding the catalysis mechanism involving oxygen binding, iron redox and electron transfer.
- **Electrocatalysis development:** We developed a polymer composite and optimized polymer content, enzyme content, and crosslinker conditions to ensure direct electron transfer, but also maintain enzyme catalytic activity. In FY25, we will use our redesigned electrochemical cell to study direct bioelectrocatalysis of the *Po*AlkB, the *Po*AlkB-AlkG fusion protein, and mutants.

My scientific area(s) of expertise is/are: Structural biology, X-ray crystallography, cryo-electron microscopy.

The ideal collaborator for my project would have expertise in: Chemical synthesis.

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

1. Chai, J., Guo, G. R., McSweeney, S. M., Shanklin, J. & Liu, Q. "Structural basis for enzymatic terminal C-H bond functionalization of alkanes." *Nat. Struct. Mol. Biol*. 30, 521–526, (2023). https://doi.org/10.1038/s41594-023-00958-0
2. Atiya, B., Liu, Q., Shanklin, J., & Ertem, M. "Predicting Mössbauer parameters of nonheme diiron complexes with density functional theory." *Inorg. Chem.* (2023). https://doi.org/10.1021/acs.inorgchem.3c00969

Elucidating the Mechanistic Determinants of Flavin-Based Electron Bifurcation

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Overall research goals:

Electron bifurcation is a biological mechanism to drive a thermodynamically unfavorable redox reaction through direct coupling with an exergonic reaction. The overarching goal of this project is to develop a fundamental understanding of how electrons are controlled, temporally, spatially, and energetically, in the novel flavin-based electron bifurcating (FBEB) class of enzymes. Specifically, our work focuses on investigation of the FBEB enzymes NADH-dependent ferredoxin:$NADP^+$ oxidoreductase (Nfn, Fig. 1) and the bifurcating [FeFe]-hydrogenase (Hyd-Bfu). The objective of this project is to delineate the physical and electronic determinants of flavin electron bifurcating sites to generate a detailed, mechanistic framework that leads to a robust understanding of how biocatalysts transform electrochemical potential into chemical bonds. This is being addressed in three aims to: (I) Elucidate the physical features responsible for tuning of bifurcating flavin cofactors. (II) Investigate the impact of spatial configuration and coupling between bifurcating flavin cofactors and the initial acceptors of bifurcated electrons. (III) Understand how bifurcating enzymes impart independent control of the two bifurcated electrons.

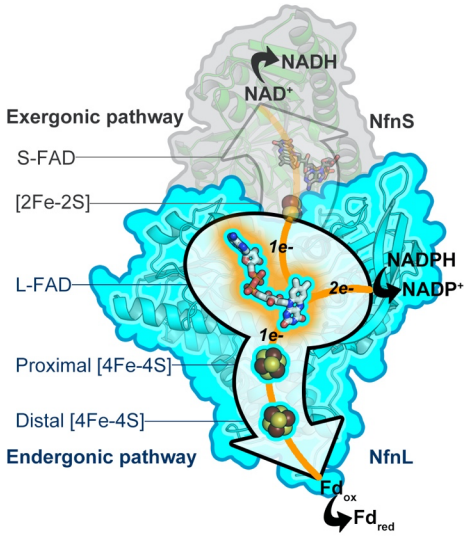

Fig. 1. Nfn from *Pyrococcus furiosus* (*Pf*) (PDB 5JFC, NfnL: large subunit in blue, NfnS: small subunit in grey). Following oxidation of NADPH, the bifurcating flavin, L-FAD (center), performs two one-electron transfers, first to the site-differentiated [2Fe-2S] of NfnS, then to the site-differentiated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

Significant achievements (2019-2024):

Over the preceding years, our work has focused on salient aspects of each of the three project aims as well as expansion to apply this foundational knowledge to the study of the new Bfu family of FBEB:

- *Defining the overall thermodynamic and kinetic landscape of bifurcation in Nfn:* We were able to measure and assign all of the reduction potentials of the bifurcating and accessory flavins and the FeS clusters, as well as measure the initial kinetics of the bifurcation reaction in Nfn. This represents the only bifurcating system with a complete, empirically measured energy landscape. Our work revealed an unusually negative redox regime for Nfn, while reframing the current understanding of bifurcating energy landscapes and points to molecular features that regulate electron flux across the entire enzyme.
- *New methodology to probe reactive flavin intermediates.* 405 nm illumination of NfnL at 230 K in the presence of NADPH enabled the accumulation of an anionic semiquinone radical. This radical is stable for weeks when stored in liquid nitrogen. This represents a new approach to probe and study reaction intermediates at the L-FAD bifurcating site which have previously proven difficult to study because of its 10-picosecond lifetime under ambient conditions. Furthermore, we observed accumulation of the proximal cluster, the distal cluster, and spin-spin coupling between these two cofactors in an illumination-time dependent manner, indicating that photo-driven formation of the anionic semiquinone drives electron transfer along the low-potential pathway. *This allows for 1)*

trapping and rigorous assessment of unstable reaction intermediates in flavin-based electron bifurcating enzymes and 2) probing discrete redox cofactor reduction steps and accompanying redox cofactor interactions that are integral to the fidelity of energy transfer.

- *Mapping the electronic properties of FeS redox cofactors that regulate electron flow in the Bfu family of electron bifurcating enzymes.* Low-field EPR spectra of *T. maritima* Hyd-BfuB afforded the identification of half-field transitions, indicating an unusually large coupling strength between two redox cofactors. By considering our collective EPR data within the context of published structures, we have ascribed the coupled cofactors as two FeS clusters placed along the low potential pathway that is found in all structurally characterized Bfu enzymes. Higher spin-states of FeS cluster(s) were also found in *T. sibiricus* Nfn-BfuABC, including a S = 9/2 or 7/2 FeS cluster with an uncommon *g* ~ 12 signature. *These findings advance our knowledge of how FeS cluster properties can be utilized for controlling energy transfer in the Bfu family of bifurcating enzymes*.

Science objectives for 2024-2025:

- Explore how the molecular and atomic determinants of electron bifurcation constitute the extensive degree of synchronization required for electron bifurcation activity and plasticity across the Nfn and Bfu enzyme families.
- Elucidate how the interactions between two conserved Arg residues and the bifurcating flavin enable generation and control of the high-energy, short-lived intermediate central to the FBEB mechanism.
- Understand how the electronic structure of the primary bifurcated-electron acceptors controls the repression of short-circuit events, and thus leads to the high fidelity of the FBEB reaction.
- Explore novel mechanisms for accomplishing pathway coupling and fidelity of electron bifurcation within the newly described Bfu family, specifically [FeFe]-Hyd-Bfu and Nfn-Bfu enzymes.

My scientific area(s) of expertise is/are: **Lubner:** Ultrafast optical spectroscopy, bioelectrochemistry, biophysical and biochemical analyses of redox enzymes and photosynthetic systems. **Vansuch:** physical (bio)inorganic chemistry, electron paramagnetic resonance spectroscopy, infrared and visible transient absorption spectroscopy, photochemistry of metalloenzymes.

The ideal collaborator for my project would have expertise in: Mass spec, Cryo-EM, MCD.

Publications supported by this project (2021-2024):

1. Imran, S. M. S., Wiley, S. A., Lubner, C. E. "Electrochemistry of flavin-based electron bifurcation: 'Current' past and 'potential' futures." *Curr. Opin. Electrochem.*, 47, 101536 (2024). DOI: 10.1016/j.coelec.2024.101536
2. Lewis, N. M., Kisgeropoulos, E. C., Lubner, C. E., Fixen, K. R. "Characterization of ferredoxins involved in electron transfer pathways for nitrogen fixation implicates a role for spin-spin coupling in tuning 2[4Fe-4S] Fd activity." *J. Inorgan. Biochem.*, 254, 112521 (2024). DOI: 10.1016/j.jinorgbio.2024.112521
3. Ortiz, S., Wiley, S., Niks, D., Lubner, C. E., Hille, R. "Rapid-reaction kinetics of the bifurcating NAD^+-dependent NADPH:ferredoxin oxidoreductase NfnI from *Pyrococcus furiosus*." *J. Biol. Chem.* 299(12) 105403 (2023). DOI: 10.1016/j.jbc.2023.105403
4. C. E. Wise, A. E. Ledinina, C. E. Lubner, "Site-Differentiated Iron-Sulfur Cluster Ligation Affects Flavin-Based Electron Bifurcation Activity." *Metabolites*, 12(9), 823 (2022). DOI: 10.3390/metabo12090823
5. H. Wu, M. D. Pun, C. E. Wise, B. R. Streit, F. Mus, A. Berim, A. Islam, D. A. Gang, J. L. DuBois, C. E. Lubner, C. E. Berkman, B. M. Lange, J. W. Peters, "The pathway for coenzyme M biosynthesis in bacteria." *Proc. Natl. Acad. Sci. U.S.A.*, 119 (36) e2207190119 (2022). DOI: 10.1073/pnas.2207190119
6. C. E. Wise, A. E. Ledinina, D. W. Mulder, K. J. Chou, J. W. Peters, P. W. King, C. E. Lubner, "An Uncharacteristically Low-Potential Flavin Governs the Energy Landscape of Electron Bifurcation." *Proc. Natl. Acad. Sci. U.S.A.*, 119 (12) e2117882119 (2022). DOI: 10.1073/pnas.211788211
7. C. E. Wise, A. E. Ledinina, J. L. Yuly, J. H. Artz, C. E. Lubner, "The role of thermodynamic features on the functional activity of electron bifurcating enzymes." *Biochim. Biophys. Acta – Bioenergetics*, 1862, 148377 (2021). DOI: 10.1016/j.bbabio.2021.148377

Post-translational modifications in archaeal redox biology

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Overall research goals:

Post-translational modifications (PTMs) are of interest in biotechnology and metabolic engineering to expand the molecular toolbox available for control of enzymatic activity, protein stability, and intra- and intermolecular protein interactions. To maximize this potential, advanced understanding of PTMs in regulating cellular responses and altering protein structure and function is needed.

The long-term objective of this project is to discern the PTMs that regulate metabolic pathways and responses of archaea to environmental cues such as redox potential shifts. In this study, the focus will be on ubiquitin-like (Ubl)-ligation and lysine acetylation in the halophilic archaeon *Haloferax volcanii*. We find lysine acetylation and Ubl ligation share overlapping protein targets. Furthermore, these PTMs are more abundant when the archaeon is challenged with oxidant. These PTM responses are robust and provide a unique perspective to understand redox biology in archaeal cells.

Significant achievements: [07/15/2022-07/14/2025]:

- Observed changes in lysine acetylation abundance and patterns in response to different carbon sources, oxidant exposure, and the *Δsir2* mutation.
- Mapped lysine acetylome occupancy and quantified protein targets in both wild type and *Δsir2* mutant cells using SILAC-based LC-MS/MS analysis.
- Conducted purification and detailed biochemical, genetic, and physiological analysis of proteins modified by lysine acetylation and ubiquitin-like modifications (Ubl), with focus on: a 2Fe-2S ferredoxin and a flavin-based oxidoreductase involved in NAD(P)H electron flow, as well as glycerol kinase (GlpK), a central metabolic enzyme in glycerol versus glucose utilization.
- Purified and characterized lysine acetyltransferases Pat1 and Pat2, as well as the lysine deacetylase Sir2.
- Demonstrated that Pat2, rather than Pat1, catalyzes the acetylation of GlpK at lysine 153 in vitro.
- Found that GlpK is acetylated at lysine residues and exhibits higher Vmax when purified from cells grown on glycerol or fructose compared to glucose or peptide-rich media.
- Identified co-occurrence patterns of GlpK, Pat1, and Pat2, and modeled their 3D structures, supporting the finding that Pat2 is responsible for GlpK acetylation.
- Revealed that Sir2 co-purifies with the TrmB-like chromatin-binding protein OxsR, which is involved in oxidative stress responses.
- Found deletion of the GNAT acetyltransferase gene homologs *pat2* and *elp3* can be achieved (contrary to published work).

Science objectives for 2024-2025:

In this next year, we plan to continue our work towards achieving the following objectives:

Aim 1: Identify and quantify lysine acetylome shifts during redox stress and determine the impact of deacetylases, Ubl ligation and proteasomes. *Hypothesis*: Quantitative analysis of the lysine acetylome during HOCl stress will provide a global perspective in archaeal redox biology. The *Δsir2* and Ubl ligation mutants are predicted to have an increased occupancy of acetylated lysine residues, particularly during oxidant challenge. Mutation of the conserved active site residues and Zn-finger motif of Sir2 are hypothesized to have a similar effect. The *Δhda1* mutation will stabilize certain lysine acetylation sites; however, its influence on the oxidant-dependent shifts of the lysine acetylome will be modest.

Aim 2: Determine the protein substrates of Pat1 and Pat2 and how these single GNAT (Gcn5-related N-acetyltransferase) domain proteins are regulated. *Hypothesis:* Pat1 and Pat2 catalyze the addition of acetyl-/acyl-groups to specific lysine residues. Reactive oxygen/nitrogen species (ROS/RNS) modify the N-terminal Cys-rich motif of Pat1 and alter its lysine acetyltransferase activity, while Pat2 activity is sequestered by UspA binding.

Aim 3: Examine how lysine acetylation and Ubl ligation may influence electron transfer between a [2Fe-2S]-type ferredoxin (Fdx) and a Fdx reductase homolog. *Hypothesis*: Oxidant challenge impacts the PTM occupancy of lysine residues at the intersubunit interface of a Fdx and Fdx reductase homolog. This in-turn alters electron transfer between NAD(P)H and Fdx which influences downstream biological pathways related to redox homeostasis.

My scientific area(s) of expertise is/are: microbial physiology, metabolism, proteomics, transcriptomics, biochemistry, genetics, protein synthesis and purification, post-translational modifications, regulatory mechanisms, extremophiles, archaea, bioconversion, proteases, proteasomes, DNA/RNA modifying enzymes.

The ideal collaborator for my project would have expertise in: electrochemistry of biomolecules including those with Fe-S clusters and flavin-cofactors; structural biologist who analyzes 'unusual' proteins (*e.g.*, salt-loving proteins from haloarchaea have a highly acidic shell and function in environments of low water activity including organic solvents).

Publications supported by this project (07/15/2022-07/14/2025):

1. Hackley RK, Hwang S, Herb JT, Bhanap P, Lam K, Vreugdenhil A, Darnell CL, Pastor MM, Martin JH, Maupin-Furlow JA, Schmid AK. 2024. TbsP and TrmB jointly regulate *gapII* to influence cell development phenotypes in the archaeon *Haloferax volcanii*. *Mol Microbiol*. Jan 11. doi: 10.1111/mmi.15225.
2. Martinez Pastor M, Sakrikar S, Hwang S, Hackley RK, Soborowski AL, Maupin-Furlow JA, Schmid AK. 2024. TroR is the primary regulator of the iron homeostasis transcription network in the halophilic archaeon *Haloferax volcanii*. *Nucleic Acids Res*. Jan 11;52(1):125-140. doi: 10.1093/nar/gkad997.
3. Jia, H, S Dantuluri, S Margulies, V Smith, R Lever, T Allers, S Chen, and JA Maupin-Furlow. 2023. RecJ3/4-aRNase J form a Ubl-associated nuclease complex functioning in survival against DNA damage in *Haloferax volcanii*. *mBio*. 14(4):e0085223. doi: 10.1128/mbio.00852-23.
4. Johnsen U, Ortjohann M, Reinhardt A, Turner JM, Stratton C, Weber KR, Sanchez KM, Maupin-Furlow J, Davies C, Schönheit P. 2023. Discovery of a novel transcriptional regulator of sugar catabolism in archaea. *Mol Microbiol.* 120(2):224-240. doi: 10.1111/mmi.15114.
5. Couto-Rodríguez RL, Koh J, Chen S, Maupin-Furlow JA. 2023 Insights into the lysine acetylome of the haloarchaeon *Haloferax volcanii* during oxidative stress by quantitative SILAC-based proteomics. *Antioxidants* 12(6):1203. doi: 10.3390/antiox12061203.
6. Maupin-Furlow, JA. 2024. HvJAMM1 desampylase. In *Handbook of Proteolytic Enzymes*. 4th Edition. N Rawlings and DS Auld (Eds). Elsevier. ISBN: 9780443288494.
7. Hepowit NL, Maupin-Furlow JA. 2023. Application of archaea in deubiquitinase-like enzyme discovery and activity assay. *Methods Mol Biol*. 2591:151-169. doi: 10.1007/978-1-0716-2803-4_10.
8. Mondragon, P, S Hwang, L Kasirajan, R Oyetoro, A Nasthas, E Winters, RL Couto-Rodriguez, A Schmid, and JA Maupin-Furlow. 2022. TrmB family transcription factor as a thiol-based regulator of oxidative stress response. *mBio* 13(4):e0063322. doi: 10.1128/mbio.00633-22.
9. Zhang H, Gong X, Zhao Q, Mukai T, Vargas-Rodriguez O, Zhang H, Zhang Y, Wassel P, Amikura K, Maupin-Furlow J, Ren Y, Xu X, Wolf YI, Makarova KS, Koonin EV, Shen Y, Söll D, Fu X. 2022. The tRNA discriminator base defines the mutual orthogonality of two distinct pyrrolysyl-tRNA synthetase/tRNAPyl pairs in the same organism. Nucleic Acids Res. 50(8):4601–15. doi: 10.1093/nar/gkac271.
10. Couto-Rodriguez, R.L.1, Gal, D.1, McMillan, L.J.1, Koh, J., Chen, S., Maupin-Furlow, J.A. 2022. Quantitative mass spectrometry by SILAC in *Haloferax volcanii*. In: Ferreira-Cerca, S. (Ed) Archaea. Methods in Molecular Biology, vol 2522. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-2445-6_16
11. Egan, MS, K Hogan, J Maupin-Furlow and M Pohlschroder. 2022. The best of both worlds: Discovery-driven learning through a lab-seminar approach. J Microbiol Biol Educ. 23(3):e00031-22. doi: 10.1128/jmbe.00031-22.

Gating electron transfer in biological energy storage and conversion

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Overall research goals:

Elucidate the requirements for electron transfer bifurcation with the objective of making bifurcation portable and applicable in man-made materials and devices.

To this end we are -1- examining protein-flavin interactions responsible for the very different redox activities of the two flavins in bifurcating electron transfer flavoproteins (ETFs), -2- developing spectroscopic and computational tools for probing flavin electronic structure and interactions with the protein, and -3- developing NMR and neutron scattering in conjunction with molecular dynamics calculations, to learn how the domain-scale motions of ETFs are coupled to elements of catalytic turnover, and elucidate their role in gating internal electron transfer between the two flavins, *vs* external electron transfer to partner proteins.

ETFs mediate electron transfer (ET) between different metabolic pathways, for example conveying electrons from fatty acid oxidation to the respiratory electron transfer chain in mitochondria (canonical ETFs). A non-covalently bound FAD in a mobile 'head' domain, was shown to mediate ET and is therefore called the ET-flavin (Fig. 1). More recently recognized bifurcating ETFs employ 2-electron (2e) sources and mediate electron transfer bifurcation ('bifurcation', Bf), so they are called BfETFs, and the FAD that accepts the incoming 2e from NAD(P)H is called the Bf-FAD. Exergonic ET from the Bf- to the ET-flavin drives endergonic ET of the second electron from Bf-FAD to a low-E° carrier such as ferredoxin or flavodoxin semiquinone. The spent high-E° (low energy) electron on the ET-flavin is transferred to a higher-E° partner such as butyryl CoA dehydrogenase (BCD). Bifurcation's extraordinary production of a more strongly reducing product based on less reducing substrate provides cells with vital energy versatility including ability to drive nitrogen fixation. However, Bf requires the enzyme to strictly limit electron flow so that only 1e per pair accesses the exergonic path.

80 ° rotation of the head domain is observed in some crystal and cryo-EM structures (open conformation, Fig. 1). This repositions the ET-flavin to be >35 Å from the Bf-flavin, severing ET between the two flavins of ETF and instead enabling ET between the ET-flavin and BCD. Recent data indicate that the ET-flavin's E°s are also altered, and the open conformation has only been observed in complexes with partner, whereas the closed conformation is observed for ETF alone. Thus, coupling between flavin redox state, partner binding and domain reorientation may gate ET in BfETFs.

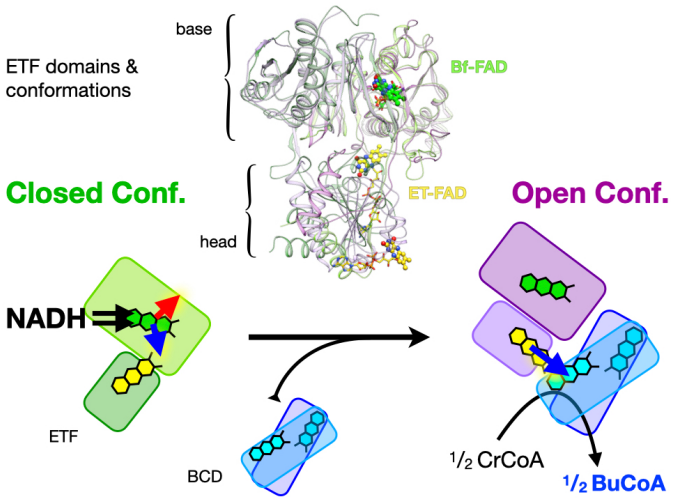

Figure 1. Overlay of crystal structures of open (burgundy) and closed (green) conformations of ETF (above). The head domain rotates carrying the ET-flavin from a position near the Bf-flavin in the closed conformation to an exposed position accessible to partners in the open conformation. Below: cartoon with exergonic ET (blue arrows) and endergonic ET (red) in each conformation. High-E° partner protein BCD is in blue.

Significant achievements: [2020-2025]:

We employed Small Angle Neutron Scattering (SANS) to characterize bifurcating ETF in solution and discovered that up to 50% of the population adopts an extended conformation in solution that cannot be

explained by any existing crystal or cryo-EM structure. To explain the data, it was necessary to consider a second conformation of ETF with a much larger radius of gyration (R_g). Such conformations are observed in molecular dynamics (MD) trajectories; they retain intact head and base domains as in solid-state structures, but the head is detached from the base and explores a wide range of orientations relative to the base. Inclusion of such extended conformations in the models produces robust fits to SANS with ensembles of as few as 2 conformations. Crucially, the same fitting protocol is applicable to the different SANS obtained from oxidized BfETF, BfETF reduced with dithionite and BfETF reduced with NADH. Thus, OX-BfETF in solution is best described as 44% compact (as in crystal structures, R_g = 24.9 Å) and 56% extended (R_g = 32.6 Å) whereas reduction by either agent results in RED-BfETF in solution best described as 80% compact (25.5 Å) and 20% extended (R_g=34.8 Å).

SANS does not perturb flavin oxidation states, so it can be used to compare oxidation states of ETF. X-rays, electron beams and light with $\lambda < 500$ nm (excitation for fluorescence techniques) all reduce flavins. Therefore they are not applicable.

Our first SANS paper has just been reviewed; we expect to have revisions submitted before the P.I. meeting.

Science objectives for 2024-2025:

We will compare the conformation of ETF in complex with partners *vs* free (1) in conjunction with (2) Quantum Mechanics / Molecular Mechanics (QM/MM) and molecular dynamics (MD) computation to elucidate coupling between conformational change and flavin redox states.

The open conformation of ETF has only been observed in complexes of ETF with partner. To compare ETF's conformations in solution ± partner, we will use deuterated BCD to form a complex with protiated ETF and adjust the degree to which solvent is deuterated to achieve zero contrast between BCD and solvent. Under this 'matched' condition, scattering from BCD will be indistinguishable from scattering from solvent, causing it to be eliminated by the subtraction of solvent scattering that is included in data processing. The scattering from ETF will be unaffected and will reflect the conformation of ETF in complex with BCD. Comparison with the scattering from ETF alone will reveal how ETF's conformation changes upon binding to BCD. We will first conduct extensive trials with different concentrations of proteins and solution conditions to optimize population and homogeneity of the complex. These exploit size-exclusion chromatography (SEC) and NMR to characterize protein complexes formed. We have observed NMR signatures of ETF conformational change and are exploiting these in the absence of BCD as well.

We are combining our SANS with accelerated MD with advanced sampling methods and a genetic algorithm our collaborators optimized for use with SANS data. The computations seek to identify interactions that can couple flavin oxidation state to relative stabilities of protein conformations.

My scientific area(s) of expertise is/are: Protein engineering, Spectroscopy of Flavins, Strategic NMR spectroscopy, Small Angle Neutron Scattering, QM/MM computation.

The ideal collaborator for my project would have expertise in: Multifield EPR capability, X-ray crystallography, organism-level engineering, cryo-EM.

Publications supported by this project 2020-2025:

1. Non-covalent interactions that tune the reactivities of the flavins in bifurcating electron transferring flavoprotein" by M. González-Viegas, R. K. Kar, A.-F. **Miller** and M.-A. Mroginski (2023) *Journal of Biological Chemistry, 299:104762.* https://doi.org/10.1016/j.jbc.2023.104762
2. Structure, dynamics and redox reactivity of an all-purpose flavodoxin" by S. Khan, A. Ansari, M. Brachi, D. Das, W. Housseini, S. Minteer, **Miller, A.-F.** (2024) *J. Biol. Chem. 300* (4) doi.org/10.1016/j.jbc.2024.107122

Structure and function of the nitrogenase-like methylthio-alkane reductase that converts volatile organic sulfur compounds into hydrocarbons

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Overall research goals:

Sulfur is an essential element of life. Cellular organic and inorganic sulfur compounds such as catalytic cysteine residues in proteins, coenzyme A, sulfide, and iron-sulfur clusters directly participate in primary energy metabolism pathways. Recently, we discovered nitrogen fixation-like genes and corresponding proteins that reductively cleave volatile organic sulfur compounds (VOSCs) of the form CH_3-S-R for microbial sulfur acquisition and energy metabolism when other sulfur sources like sulfate and sulfide become limiting. This Methylthio-Alkane Reductases (MAR) system was initially observed to catalyze the cleavage of dimethylsulfide (CH_3-S-CH_3) into methane and methanethiol and cleave methylthio-ethanol (CH_3-S-CH_2-CH_2-OH) into ethylene and methanethiol. These industrially useful methane and ethylene hydrocarbons are released from the cell after cleavage from their respective VOSC by MAR. Coordinately, the methanethiol is further utilized for methionine biosynthesis to fulfill the cell's organic sulfur needs for growth and energy metabolism. While sequence homology and phylogenetic analysis shows that MAR is a relative of true nitrogenases that fix nitrogen gas into ammonia, the reaction requirements, metallocofactor identities, substrates, inhibitors, structure, and mechanism of MAR catalysis remain largely unknown.

In this project employ an interdisciplinary approach, combining microbiology, biochemistry, and biophysics to characterize the structure and function of methylthio-alkane reductases. This is achieved through the following primary goals: **1.)** Fully elucidate the *Rhodospirillum rubrum* MAR holoenzyme and precursory complexes to understand the subunit composition and stoichiometry and probe the role of the identified MAR metallocofactor synthesis enzyme. **2.)** Quantify in vitro activity requirements, substrate specificity, carbon monoxide inhibition, and hydrogen evolution to provide insights as to whether MAR follows a similar mechanism to that of nitrogenase. **3.)** Enumerate amino acid residues involved in catalysis through amino acid substitutions and structural modelling through crystallographic and cryo-EM analyses to uncover the structure-function relationship using native and amino acid-substituted enzyme variants. **4.)** Determine the identity of the active-site metallocofactors(s) through EPR and complementary spectroscopy techniques, and probe the catalytic mechanism using isotopic labeling and inhibitor analysis.

This will ultimately result in a structural and mechanistic model of MAR catalysis for biological sulfur acquisition from ubiquitous VOSCS and concomitant hydrocarbon production. This in turn will provide essential understanding of the nitrogenase superfamily's mechanistic diversity for catalyzing distinct carbon-, nitrogen-, and sulfur-based reactions.

Significant achievements: [2023-2024]:

We have completed our initial analysis of the subunit composition, metallocofactor identity, activity requirements of Methylthio-alkane reductase (MAR) for cleavage of VOSCs. To achieve this, we constructed a native over-expression system for *mar* genes in *Rhodospirillum rubrum,* because natural abundances of MAR is too low to reasonably purify. This overexpression system also enabled recovery through affinity tag purification. The following key observation were made:

1) Thr catalytic enzyme complex of MAR is composed of MarH, MarD, and MarK analogous to Nitrogenase NifH, NifD, and NifK. MarH is the ATP-dependent dimeric reductase that delivers electrons to the MarDK tetramer for catalysis. Consistent with *in vivo* observations, purified MarHDK is catalytically active with dimethylsulfide and methylthioethanol, producing methane and ethylene, respectively. Catalysis requires ATP, an electron donor, and anoxic conditions, and is

inhibited by CO. Catalytic rates of purified MarHDK is consistent with the VOSC metabolism rates and MAR protein abundances *in vivo* that are required to support cellular sulfur metabolism.

2) MarDK possesses an accessory protein identified as *R. rubrum* protein A3441. A3441 contains a long glycine rich N-terminal region and a winged helix C-terminal region. It has no sequence homology to the alternative nitrogenase (Vnf/Anf) G-subunit, CowN protein for CO-protection, or Shethna II proein for O_2 protection. Both genetic and *in vitro* MarHDK activity analysis indicate that the A3441 protein is not required for catalysis. Based on initial data, A3441 may be involved in fidelity of metallocofactor insertion into MarDK or involved in complexing multiple MarDK catalytic complexes together. The latter is of keen interest as *R. rubrum* also possesses a second nitrogenase-like system distinct from MarDK that has specificity for C-S cleavage of ethylthio-alkane VOSCs. Experiments are underway to determine if A3441co-localizes these two nitrogenase-like systems as a one-stop shop for VOSC cleavage.
3) MarDK possesses multiple distinct metallocofactors. ICP-MS of the purified MarDK reveals 32 Fe per tetramer, and no Mo or V. This is analogous to the metal content of the Anf iron-only nitrogenase, and consistent with the MarDK primary amino acid sequence which possesses the same conserved cysteines used by NifDK to coordinate the P-cluster and conserved cysteine and histidine to coordinate the M-cluster. The presence of P- and M-like metallocofactors is corroborated by Cryo-EM structural analyses revealing an electron density consistent with a P-like cluster at the MarDK-interface and a pocket for binding an M-like cluster. Coordinately, EPR measurements of MarDK under turnover conditions show features highly-similar to the iron-only nitrogenase FeFe-cofactor under turnover conditions, suggesting the present of an iron-only M-like cluster. We designate these P- and M-like clusters as Mar1- and Mar2-clusters, respectively.

We have also completed initial structure-function analyses through site specific amino-acid substitusion and through quantifying substrate diversity.

1) The MAR system, as quantified *in vivo* has a broad substrate range. MarHDK catalyzes reductive C-S cleavage of methylthio-alkanes up to ~ 7 carbons in alkyl chain length. In addition, methylthio-alkanes with aromatic and alcohol side chains also serve as substrates. Ethylthio-alkanes, propylthio-alkanes and larger VOSCs are very poor substrates, indicating MarDK is specific with methylthio-alkane VOSCs.
2) Site-specific substitution of MarDK amino acid residues reveal key cysteines responsible for coordinating the metallocofactors, and residues potentially involved in substrate coordination.

Science objectives for 2024-2025:

In the next period, we will **1)** Probe the synthesis pathway of the M-like Mar2 metallocofactor through Mar gene expression and protein purification in the presence or absence of identified metallocofactor synthesis proteins. **2)** Continue to probe the MarDK structure-function relationship through site-specific substitutions and Cryo-EM structural analyses. **3)** Fully characterize the MarDK and MarH metallocofactor identities and probe the catalytic mechanism through CO inhibition studies using bioinorganic spectroscopy.

My scientific area(s) of expertise are: Microbial metabolism, genetics and metabolic engineering. Analytical chemistry including liquid/gas chromatography and mass spectrometry. Enzymology and protein structure-function.

The ideal collaborator for my project would have expertise in: Structural biology of oxygen sensitive, metallocofactor containing enzymes. Specifically, preparation of nitrogenase quaternary complexes for Cryo-EM under anaerobic conditions.

Publications supported by this project 2023-2024:

1. Structural basis for the cleavage of volatile organic sulfur compounds into hydrocarbons by the nitrogenase-like methylthio-alkane reductase (in submission)

Novel microbial-based enzymatic CO_2 fixation mechanisms: conformational control of enzymatic reactivity

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Overall research goals:

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

The project is divided into three aims to probe three fundamental mechanisms of conformational control of reactivity. The first aim examines the mechanism of how acetone carboxylase (AC) couples ATP binding and reaction of the substrate's acetone and bicarbonate with large-scale conformational changes, which protect ATP hydrolysis and the delivery of reactive intermediates to a remote metal site. In addition, the project aims to address how highly conserved Acs from different microbial sources bind different metals preferentially. The second aim investigates refining an energy landscape and conformational gating electron flow in FixABCX, an electron bifurcating member of the electron transfer flavoprotein family of enzymes. The final objective investigates critical fundamental questions on the mechanism of electron bifurcation in the Bfu family of flavin-based electron bifurcating enzymes.

Significant achievements: (2023-2024):

ATP-Dependent acetone carboxylation – overcoming Irving-William Series metal binding tendencies for metal complex formation. Acetone carboxylases (ACs) are metal-dependent enzymes that convert acetone and bicarbonate into acetoacetate, incorporating acetone into biomass. ACs are ~300kD heterohexameric enzymes that carboxylate acetone in an ATP-dependent manner. In previous DOE-supported work, we have structurally characterized AC from *Xanthobacter autotrophicus,* revealing that the sites for ATP binding and generation of phosphoenol acetone and carboxy phosphate intermediates are separated by nearly 40 angstroms. We combined MS, EPR, and enzyme activity measurements during the funding period to reveal long-range reciprocal communication between the two sites. We implicated that either ATP binding or the activation of substrates triggers a conformational change that opens a conduit for intermediates to travel to the metal sites where the binding of intermediates at the metal site results in a displacement of the metal-ligand that triggers a reciprocal conformational change that closes the conduit and opens a cleft for product release. A revised manuscript describing this work has been submitted to the Journal of Biological Chemistry. We have also verified that highly conserved ACs from *X. autotrophicus* and *Aromatoleum aromaticum* require Mn(II) and Fe(II), respectively. This is unexpected since the active site coordination and second sphere protein environment are highly conserved. The Mn(II) requirement for the *X. autotrophicus* AC contradicts Irving-Williams Series metal complex stability predicted behavior (Shisler et al., 2024). We are investigating possible determinants for the different metal binding preferences and reactivity requirements and believe that solvent access and metal binding site solvation may play a role.

Defining the FixABCX energy landscape

During nitrogen fixation, *Azotobacter vinelandii*, generates low potential electrons in the form of reduced ferredoxin (Fd) and flavodoxin (Fld) using two distinct mechanisms via the enzyme complexes Fix and Rnf1. Fix has a unique mechanism of action called flavin-based electron bifurcation, which couples the exergonic reduction of quinone with the endergonic reduction of Fd/Fld. At the same time, Rnf1 uses the proton motive force to provide the additional energy required for the reaction (Alleman and Peters, 2023). We have recently successfully optimized the purification of the FixABCX protein complex, which is expressed heterologously in *E. coli.* This task took us nearly two years, but now we can obtain sufficient quantities for spectroscopic and structural studies. We have been focused on defining the energy landscape and the pathway of electron flow. One of the critical knowledge gaps is the role of the FixX subunit in the electron transfer pathway. As a result of previous structural modeling, MS, and EPR studies, we proposed that the FixX subunit (2[4Fe-4S]) was involved in the endergonic half-reaction and the electron path toward the reduction of Fd or flavodoxin. We based this on the presence of low-potential FeS cluster or clusters in FixX. Mike Adams' group recently determined the cryo-EM structure of a homolog of the in which an alternative pathway of electron flow and role for FixABCX was implicated with one cluster of the 2[4Fe-4S] cluster containing FixX was participating in the exergonic pathway and the reduction of menaquinone. We now have small-angle X-ray scattering data under different conditions, as well as a moderate-resolution cryo-EM structure that is more in line with the results from Adams' lab. In addition, we have performed UV/Vis and EPR studies on the FixABCX and FixX expressed on its own, indicating that FixX exists with two [4Fe-4S] clusters having disparate reduction potentials.

Science objectives for 2025-2026:

1) Elucidate the structural determinants of AC metal preference
2) Complete the energy landscape studies on FixABCX
3) Elucidate the structure of FixABCX in different ligand-bound states and oxidation states
4) Optimize heterologous expression of Bfu electron bifurcating hydrogenase

My primary 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:

Publications supported by this project: 2023-2024:

1. A.B. Alleman, J.W. Peters "Mechanisms for generating low potential electrons across the metabolic diversity of nitrogen-fixing bacteria" Appl. Environ. Microbiol. DOI: 10.1128/aem.00378-23 (2023).
2. N.R. Boyer, M. Tokmina-Lukaszewska, F. Mus, M.B. Batista, R. Dixon, B. Bothner, J.W. Peters "Structural insights into redox signal transduction mechanisms in the control of nitrogen fixation by the NifLA system *Proc. Natl. Acad. Sci. USA* DOI: 10.1073/pnas.2302732120 (2023).
3. J.S. Martin del Campoa, J. Rigsbee, M.B. Batista, F. Mus, L.M. Rubio, O. Einsle, J.W. Peters, R. Dixon, D.R. Dean, P.C. Dos Santos "Overview of physiological, biochemical, and regulatory aspects of nitrogen fixation in *Azotobacter vinelandii*" *Crit. Rev. Biochem. Mol. Biol.* DOI: 10.1080/10409238.2023.2181309 (2023).
4. M. Tokmina-Lukaszewska, Q. Huang, L. Berry, H. Kallas, J.W. Peters, L.C. Seefeldt, S. Raugei, B. Bothner "Fe protein docking transduces conformational changes to the MoFe protein active site FeMo-co in a nucleotide-dependent manner" *Commun. Chem. DOI*: 10.1038/s42004-023-01046-6 (2023)
5. K.A. Shisler, W.M. Kincannon, J.R. Mattice, J. Larson, A. Valaydon-Pillay, F. Mus, S.A. Stoian, S. Raugei, B. Bothner, J.L. DuBois, J.W. Peters "Homologous acetone carboxylases strictly select Fe(II) or Mn(II) as the catalytic cofactor" mBio DOI: 10.1128/mbio.02987-23 (2024).
6. J.R. Mattice, K.A. Shisler, J.R. Malone, N.A. Murray, M. Tokmina-Lukaszewska, J.L. DuBois, J.W. Peters, B. Bothner "Long range allosteric communication is essential in acetone carboxylase" *Revised manuscript submitted J. Biol. Chem.* (2024).

Alterations in Electron- and Proton-Transfer Circuits of *cbb*$_3$ Oxidases at the Onset of Denitrification

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Overall research goals:

Bacterial enzymes cytochrome *cbb*$_3$ oxidases catalyze O_2 reduction when concentrations of this electron acceptor are limited. In these environments, denitrification pathways get activated and concentrations of intermediate products of this process (*e. g.* nitric oxide (NO)) rise. As many other bacterial proteins that function at low or no O_2, *cbb*$_3$ oxidases have an extended chain of hemes in their structures. Knowing how increasing NO levels affect redox properties of multiheme structures is important for understanding electron flow in proteins functioning in these environments and how it is regulated. *Cbb*$_3$ oxidases catalyze reduction of both O_2 and NO. However, while O_2 reduction by these enzymes is associated with proton pumping, a key process in cellular energy conversion, NO reduction is not.

In this project the following effects are examined: of (1) NO binding on redox properties of diheme components of *cbb*$_3$ oxidase; (2) conformational changes triggered by NO binding on abolishing proton pumping of the enzyme; and (3) the oxidation state of neighboring hemes and complex formation in directing electron flow through a triheme component of the enzyme, to support either O_2 or nitrite reduction. To probe these effects, spectroscopic and electrochemistry experiments as well as computational analyses of electron-transfer paths are being employed.

Significant achievements 2023-2026:

Variants of M-to-H cytochrome c_4 proteins were prepared and characterized by spectroelectrochemistry, CD spectra, and unfolding studies. Spectral changes upon NO binding were examined. Because evolutionary relationships identified stronger redox cooperativity in one of c_4 variants, we will use it for subsequent NO binding studies.

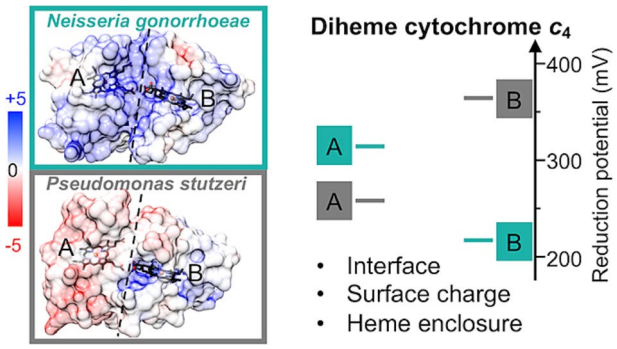

Fig. 1. Surface charge distribution and reduction potentials of *Neisseria gonorrhoeae* in comparison to those for the c_4 protein from *Pseudomonas stutzeri*.

Structural features and interactions of the proteins of *cbb*$_3$ oxidase from *Neisseria* bacteria with triheme CcoP were evaluated. Crystal structure of c_4 was solved (**Figure 1**), studies of reduction potentials of its M-to-H mutants ambiguously assigned reduction potentials of the two hemes in c_4. Interactions with modeled c_1, CcoP, and c_5 proteins were evaluated. Cross-linking studies have identified that CcoP interacts with c_4. Reduction potentials measurements suggest favorable ET from c_4 to CcoP but unfavorable from CcoP to c_2. The diheme ΔC CcoP fragment was crystallized but diffracted only at moderate resolution, optimization trials are in progress.

Science objectives for 2024-2026:

NO interactions with the developed c_4 and CcoP variants will be probed, equilibrium constants for NO binding to specific hemes in ferric and ferrous diheme proteins will be determined.

Kinetics of NO-induced dissociation of the Met ligand will be examined and associated structural changes will be identified.

Proton pumping ability of mutants of cbb_3 oxidase with replacements mimicking those in cNORs will be examined.

Complexes of *Neisseria* cytochromes will be characterized by mass-spectrometry (HDX and cross-linking) and subsequent modeling. Stopped-flow studies with fluorescently labeled proteins will identify ET rate constants for specific hemes. Structural studies of c_2 will be pursued.

My scientific area(s) of expertise is/are: biochemistry of heme proteins, spectroscopy, thermodynamics and kinetics of redox reactions.

The ideal collaborator for my project would have expertise in: cryo-EM, HDX mass-spectrometry, modeling of proton pumping.

Publications supported by this project 2023-2026:

1. F. Zhong, M. E. Reik, M. J. Ragusa, E. V. Pletneva, "The structure of the diheme cytochrome c_4 from *Neisseria gonorrhoeae* reveals multiple contributors to tuning reduction potentials" J. Inorg. Biochem. **253**, 112496 (2024). https://doi.org/10.1016/j.jinorgbio.2024.112496.

Enzymology of methanogenesis: Mechanism of Methyl-CoM Reductase
DE-FG02-08ER15931

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Overall research goals:

Our research program is poised to provide the long-awaited structure of the active Ni(I) state of MCR and to describe the intermediates and transition states for each step in the MCR mechanism. Our study will also illuminate the proposed MCR mechanism of C-S and S-S bond activation and cleavage involving Ni(I)-sulfonate interaction and long-distance through-bond electron transfer. The anticipated impact of our studies is to inform the design of more efficient catalysts for industrial alkane biosynthesis, biogas generation, and the rational design of methanogenesis inhibitors. Aim 1 is to determine the first structure of active Ni(I)-MCR using X-ray free electron laser serial crystallography (XFEL), X-ray diffraction (XRD), and neutron diffraction methods. Aim 2 is to generate a movie of the catalytic cycle, which will furnish detailed structures of the substrate-bound and intermediate states and will be staged with active MCR, in-house synthesized native and "slow" substrates, CoBSH substrate analogs designed to test the proposal for long-range electron transfer in the MCR mechanism and trap the proposed methyl- and sulfur-based radical intermediates during catalysis.

Significant achievements: January 1, 2024- Dec. 31 2024:

We have optimized conditions to determine the structure of the highly oxygen-sensitive active Ni(I)-MCR by XRD and XFEL methods. These studies were performed in collaboration with the groups of J. Kern, J. Yano, and V. Yachandra (KYY) at Berkeley; R. Sarangi at SSRL; and A. Cohen at SSRL. Methods have been developed to perform all steps in growth of the organism (*Methanothermobacter marburgensis*), activation, and purification of MCR, crystal growth, and structure determination under strictly anaerobic conditions. We have optimized methods to validate the "state" of the crystals by EPR, UV-visible, X-ray absorption (XAS), and activity measurements. For serial crystallography using XFEL, we prepare a "shower" of several hundred thousand tiny (~50 μm) green Ni(I) crystals. We validate the "state" of the crystals *during* XRD structure determination by XRD using simultaneous "in-line" UV-visible measurements. For XFEL, we validate the "state" of the crystal using *simultaneous* X-ray emission spectroscopic measurements as the pulsed 30 fsec X-ray laser beam (for structure determination) hits the sample. All measurements of the crystal are accompanied by parallel X-ray absorption experiments by M. Abernathy and R. Sarangi. (SSRL). In addition, we have grown large (~0.1-0.5 mm3) deuterated green crystals of Ni(I)-MCR and will optimize the data collection protocol for neutron diffraction.

This technology has allowed determination of the first structures of active Ni(I)-MCR using XFEL and XRD. Using both methods, we obtained coinciding and complementary 1.6 A structures. For XRD, we grow and mount our green crystals in anaerobic sealed capillaries. For XFEL structures are obtained at room-temperature without radiation damage. Our results reveal that inactive Ni(II)-MCR is in a "locked-in" state containing hexacoordinate F430, while active N(I)-MCR is highly dynamic. In the Ni(I) state, major changes at the active site including ligand coordination and in the F430 radiate throughout the entire protein structure. These results are being prepared for publication.

Science objectives for 2024-2025:

We plan to react Ni(I) crystals with substrates and analogs to will furnish detailed structures of the substrate-bound and intermediate states of MCR during its catalytic cycle. These states will be staged with active MCR reacted under various methods with in-house synthesized native and "slow" substrates, and CoBSH substrate analogs. These experiments should allow us to generate a movie of the catalytic cycle, test the proposal for long-range electron transfer in the MCR mechanism, and trap the proposed methyl- and sulfur-based radical intermediates during catalysis. Computational experiments are being performed in collaboration with the group of S. Raugei at PNNL. Perform spectroscopic, kinetic, and structural measurements of site-directed mutants of MCR.

My scientific area(s) of expertise is/are: Biochemistry, enzyme mechanisms, bioinorganic chemistry, structural biology.

The ideal collaborator for my project would have expertise in: mutagenesis of methanogens, structural biology (XFEL, XAS, XES, XRD).

Publications supported by this project (2024-2027):

1. Ohmer, C. J., Abernathy, M., Marchany-Rivera, D., Bogacz, I., Kaminsky, C., Doyle, M. D., Chen, P. Y., Keable, S. M., Makita, H., Simon, P. S., Massad, R., Fransson, T., Chatterjee, R., Bhowmick, A., Paley, D. W., Moriarty, N. W., Brewster, A. S., Gee, L. B., Alonso-Mori, R., Moss, F., Fuller, F. D., Batyuk, A., Sauter, N. K., Bergmann, U., Cohen, A., Sarangi, R., Yachandra, V. K., Yano, J., Kern, J. F., Ragsdale, S. W., The crystal structure of the active Ni(I) state of MCR, in preparation.
2. Bojana Ginovska, Simone Raugei, Christopher Ohmer, Macon Abernathy, Ritimukta Sarangi, Stephen Ragsdale, Methyl-Coenzyme M Reductase, Accounts of Chemical Research: Special Issue – Upgrading C1 Feedstocks to Value-Added Chemicals and Fuels (ed: Nilay Hazari, Jenny Y. Yang, Hannah Shafaat), in preparation.

Enzymatic Energy Conversion, FWP 66476

Simone Raugei, Principal Investigator
Marcel Baer, Bojana Ginovska, Lance Seefeldt, Co-PI(s)
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Overall research goals:

The Physical Biosciences program at PNNL aims to understand better the core principles employed by enzymes to control the flow of energy and matter to achieve remarkable specificities, efficiencies, and catalytic rates. The program integrates state-of-the-art theory and computation with experimental efforts across the U.S. Department of Energy's Basic Energy Sciences Physical Biosciences community to fill critical gaps in knowledge about how enzymes orchestrate spatial and temporal events to direct electrons, protons, and substrates for selective conversions and allosteric regulation.

Our research is divided into three general themes to fill outstanding gaps in knowledge and explore the transferability of concepts learned: (1) understand how dynamic confinement imposed by the enzyme scaffold controls specificity and selectivity; (2) understand how electrons and protons are delivered and accumulated at the active site; and (3) understand how mechanical energy and electro- or thermochemical energy are interconverted in biomolecules.

Significant achievements: (Years of Current FWP: 2022-2024)

Identifying Residues that Contribute to Controlling Product Selectivity in MTSs. Using two Using (4S)-(-)-limonene synthase (LMNS) and (+)-bornyl diphosphate synthase (BPPS) as models, we combined simulations with experimental testing to understand how these enzymes determine regio- and stereo-selectivity. Our simulations revealed that LMNS and BPPS use a common reaction intermediate, the α-terpinyl cation (ATC), but favor different conformations, linear in LMNS and half-chair in BPPS. This leads LMNS to produce monocyclic monoterpenes and BPPS to produce bicyclic products. We found that interactions between ATC and active site residues influence the enzyme's preferred conformation, aligning with experimental results. Additionally, the binding of the substrate geranyl diphosphate (GPP) affects the stereochemical outcome, with each enzyme showing different preferences for GPP's handedness, which correlates with the types of terpene products produced. Integrating simulations and experiments helped us pinpoint how active site residues control the ratio of product types in these enzymes.

Characterization of the Central Carbon in the Nitrogenase FeMo- Cofactor. Substrates and inhibitors of Mo-dependent nitrogenase bind and react at Fe ions of the active-site FeMo-cofactor contained within the MoFe protein α-subunit. The cofactor contains a CFe6 core, a carbon-centered within a trigonal prism of six Fe atoms, whose role in catalysis is unknown. Targeted 13C labeling of the carbon (Dean, VirginiaTech) enabled ENDOR spectroscopy (Hoffman, Northwestern University) to sensitively monitor the electronic properties of the Fe-C bonds for several reaction intermediates and inhibited states. It showed that all exhibit near-zero ^{13}C isotropic hyperfine coupling constants. Density functional theory analysis of the C-Fe bonds showed this occurs because of a (3 spin-up/3 spin-down) spin-exchange configuration of CFe6 Fe-ion spins that produce cancellation of large spin transfers to carbon in each Fe-C bond (Figure 2). The persistent structure and Fe-C bonding of the CFe6 core indicate it does not provide a functionally dynamic (hemilabile) "beating heart." Instead, it acts as "a heart of steel," stabilizing the structure of the FeMo-cofactor during catalysis.

Mapping the Mechanism of T6P-Mediated Inhibition of Plant Metabolic Sensor Kinase SnRK1. Trehalose 6-phosphate (T6P) regulates plants by influencing the SnRK1 kinase, which adjusts the cell's energy balance. When activated, SnRK1 phosphorylates proteins to manage energy use. T6P inhibits the activation of KIN10, a SnRK1 subunit, by blocking its interaction with GRIK1.

We conducted a comprehensive computational study using molecular dynamics (MD) simulations alongside in vitro assays. Our analysis identified the T6P binding site on KIN10. Docking studies and

simulations pinpointed a specific region on KIN10 with clusters of positively charged residues where T6P binds. The binding of T6P blocks the conformational rearrangement of KIN10's activation loop, which is essential for its phosphorylation by GRIK1. This inhibition mechanism was supported by mutagenesis experiments (Shanklin at Brookhaven National Laboratory).

The weakening of T6P affinity for GRIK1 and the prevention of phosphorylation and activation under high-sugar conditions maintain SnRK1's basal activity. This mechanism ensures the cell shifts toward anabolic processes like fatty acid synthesis when sugar levels are high, linking cellular energy status to metabolic regulation.

Science objectives for 2024-2024:

Gathering fundamental understanding to predict, manipulate, and design biochemical processes that underpin innovations for energy conversion.

My scientific area(s) of expertise is/are: Computational Chemistry and Biophysics.

The ideal collaborator for my project would have expertise in: experimental characterization of enzymatic mechanisms and protein redesign. In this regard, we are already fruitfully collaborating with John Shanklin (BNL), Steven Ragsdale (U. Michigan), M. Lange (Washington State University), John Peters (University of Oklahoma), Brian Hoffman (Northwestern University), Brian Bothner (Monta State University), Cara Lubner (NREL), and David Beratan (Duke University).

Publications supported by this project ,:

1. Tzeli, D., Golub, P. ; Brabec, J.; Pernal, K. ; Veis, L. ; de Jong, W. A. ; Raugei, S. ; Xantheas, S. S., The importance of correlation on the geometry and the electronic structure of [2Fe−2S] Systems: An accurate study on the [Fe2S2(SCH3)4]2-,3-,4-, [Fe2S2(SCys)4]2-, [Fe2S2(S-p-tol)4]2-, and [Fe2S2(S-o-xyl)4]2- complexes. *The Journal of Chemical Theory and Computation* **2024**, just accepted.
2. Blanford, J.; Zhai, Z.; Baer, M. D.; Guo, G.; Liu, H.; Liu, Q.; Raugei, S.; Shanklin, J., Molecular mechanism of trehalose 6-phosphate inhibition of the plant metabolic sensor kinase SnRK1. *Science Advances* **2024**, *10*, eadn0895. DOI: 10.1126/sciadv.adn0895
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4. Richardson, J. A.; Kim, H.; Kas, J. J.; You, X.; Andersen, A.; Ginovska, B.; Bhattacharjee, A.; Sarangi, R. X-ray absorption spectroscopy and theoretical investigations of the effect of extended ligands in potassium organic matter interaction. *Journal of Chemical Physics* **2024**, *160*, 044114. DOI: 10.1063/5.0183603.
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Light-driven activation of small molecules by nitrogenase hybrids

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Overall research goals:

Nitrogenase is a versatile metalloenzyme that is capable of ambient activation and reduction of a wide range of small molecules, such as N_2, CO, and CO_2. In this project, we propose to generate a light-driven electron transport system for small molecule activation based on nitrogenase proteins and their homologs from phototrophic bacteria, taking advantage of the similarities and distinctions among these homologous proteins that can be utilized to combine key elements with different functionalities and distinct locations to harvest light and drive reduction of substrates at the nitrogenase cofactor center. Success in generating light-driven nitrogenase hybrids for small molecule activation will facilitate time-resolved mechanistic investigation of nitrogenase-catalyzed reactions.

Significant achievements (2021-2024):

I. Introducing the "fishing pole" of *Av*NifEN into *Rc*BchNB for L-cluster attachment. In this project, we propose to introduce the L-cluster-binding site of *Av*NifEN into *Rc*BchNB and thereby create a light-driven electron transport pathway in the direction of Pchlide→[Fe_4S_4] cluster→L-cluster. The crystal structure of *Av*NifEN indicates the presence of a flexible loop containing two Cys ligands ($Cys^{\alpha 15}$, $Cys^{\alpha 25}$) at the surface of its α1-domain, which resembles a "fishing pole" for the attachment of the L-cluster. In comparison, the corresponding sequence in *Rc*BchNB is shorter than that in *Av*NifEN and carries only one Cys ligand. Substitution of the "fishing pole" sequence of *Av*NifEN for the corresponding N-terminal sequence of the α1-domain of RcBchNB should allow us to generate a hybrid with the ability to bind both the L-cluster and Pchlide. Recently, we expressed an *Rc*BchNB variant carrying the "fishing pole" of *Av*NifEN (designated *Rc*BchNB①) heterologously in *E. coli* and purified large batches of this protein. As expected, the as-purified *Rc*BchNB① protein displayed the [Fe_4S_4] cluster-specific S=1/2 EPR feature. Incubation of *Rc*BchNB① with the isolated Pchlide or L-cluster, followed by re-purification of the protein from the mixture, resulted in species that showed VIS absorbances and EPR features indicative of the protein-bound Pchlide and L-cluster, respectively. Excitingly, driven by visible light-active CdS@ZnS (CZS) core-shell quantum dots (QDs), the C_2H_2-reducing activity of the L-cluster-reconstituted *Rc*BchNB① exceeded that of the mature V-nitrogenase (*Av*VnfDGK). This observation provides a strong proof-of-concept for the feasibility to engineer a catalytically competent system on the basis of this template. Building on this initial result, we will now test whether the catalytic activity of this system can be accomplished by light activation of the protein-bound Pchlide.

II. Light-driven transformation of carbon monoxide into hydrocarbons using CdS@ZnS:*Av*VnfDGK nitrogenase biohybrids (*see* manuscript #1 below). Hydrogenation of carbon monoxide (CO) to longer-chain hydrocarbons (>C2) is a multi-electron involved, energy-intensive and kinetically challenging process. Contrary to the industrial Fisher-Tropsch (FT) process that requires high temperature and high pressure, the enzymatic hydrogenation of CO can be accomplished by the V-cluster of the vanadium (V)-dependent nitrogenase under ambient conditions, although this process also requires the expensive ATP and reducing agent(s). During the last reporting period, we have shown that the visible light-active CdS@ZnS (CZS) core-shell quantum dots (QDs) can photosensitize the catalytic component (VnfDGK) of V-nitrogenase, where photogenerated electrons can replace ATP and the reductase component of V-nitrogenase and drive the

enzymatic CO reduction into hydrocarbons. Our efforts to engineer surface ligands have enabled successful molecular and opto-electronic coupling between QDs and the VFe protein, with the optimal design of CZS:VFe biohybrids achieving a high-efficiency (quantum yield > 56 %), ATP-independent, photon-to-fuel production and an electron turnover number of >900, 72% compared to the ATP-dependent transformation of CO into hydrocarbons by the native V-nitrogenase enzyme.

Science objectives for 2025-2026:

We will characterize *Rc*BchNB① (*see* I above). Additionally, we will generate the P-cluster site in *Rc*BchNB① to enhance the electron flux and the catalytic ability of the engineered enzyme system.

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

The ideal collaborator for my project would have expertise in: Structural biologists, computational biologists, high-end spectroscopists (e.g., Mössbauer spectroscopy).

Publications supported by this project since 2021:

1. Ding Y, Lee CC, Hu Y, Ribbe MM, Nagpal P, Chatterjee A (2023) Light-driven transformation of carbon monoxide into hydrocarbons using CdS@ZnS : VFe protein biohybrids. ***ChemSusChem*** 16(20):e202300981. doi: 10.1002/cssc.202300981
2. Hu Y, Lee CC, Grosch M, Solomon JB, Weigand W, Ribbe MW (2023) Enzymatic Fischer-Tropsch-type reactions. ***Chem Rev*** 123(9):5755-5797. doi: 10.1021/acs.chemrev.2c00612
3. Stripp ST, Duffus BR, Fourmond V, Léger C, Leimkühler S, Hirota S, Hu Y, Jasniewski A, Ogata H, Ribbe MW (2022) Second and outer coordination sphere effects in nitrogenase, hydrogenase, formate dehydrogenase, and CO dehydrogenase. ***Chem Rev*** 122(14):11900-11973. doi: 10.1021/acs.chemrev.1c00914

Missing links in biological methane and ammonia oxidation

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Overall research goals:

The central hypothesis of this project is that unidentified protein components contribute to particulate methane monooxygenase (pMMO) activity in methanotroph cells. These "missing links" could facilitate loading, assembly, and stabilization of the copper active site, localization and arrangement of pMMO in intracytoplasmic membranes, and/or the delivery of electrons and protons to the active site. The strategy for identifying these missing links has focused on genes frequently present in the operon that encodes the core pMMO and ammonia monooxygenase (AMO) subunits. In particular, several lines of evidence indicate that the PmoD protein and its homologs encoded in AMO operons are important players in biological methane and ammonia oxidation by pMMO and AMO, respectively. First, PmoD/AmoD proteins occur exclusively in methane-oxidizing and ammonia-oxidizing bacteria. Second, the genes *pmoD*, *amoD*, and *amoE* are located in *pmo*/*amo* operons and coregulated with the genes encoding the pMMO/AMO subunits. Third, deletion of *pmoD* in *Methylosinus trichosporium* OB3b impairs cell growth under pMMO-utilizing conditions. Fourth, PmoD is a copper-binding protein, consistent with potential functions in copper delivery or redox chemistry. PmoD consists of an N-terminal periplasmic copper-binding domain that forms a Cu_A-like site with unique structural and electronic properties as well as a C-terminal transmembrane helix. Full-length PmoD has not been biochemically characterized. The goal of this project is to pair biochemical studies of full-length PmoD, both alone and in the presence of pMMO, with genetic deletion or mutation of PmoD from the same species.

Significant achievements: 2023-2024:

- We developed a system for genetic manipulation of *Methylocystis* sp. Rockwell.
- We initiated genetic disruption of *pmoD* in both *M.* sp. strain Rockwell and *Methylotuvimicrobium buryatense* 5GB1C.
- We expressed and purified the periplasmic domains of two PmoD proteins from *M. buryatense* 5GB1C.
- We determined that an additional helix present in the crystal and cryoEM structures of *M.* sp. Rockwell pMMO is not PmoD as hypothesized, and were able to assign its sequence.
- We detected a previously unknown subunit in *Nitrosomonas europaea* AMO.

Science objectives for 2024-2025:

In the remaining 6 months of funded research, we will follow up on our recent identification of the exogenous helix in *M.* sp. Rockwell pMMO.

My scientific area(s) of expertise is/are: bioinorganic chemistry, structural biology (crystallography and single particle cryoelectron microscopy), copper proteins, biological methane and ammonia oxidation.

The ideal collaborator for my project would have expertise in: cryoelectron tomography (cryoET), lipidomics, microbial genetics

Publications supported by this project 2023-2024:

1. Tucci, F.J.; Rosenzweig, A. C. Structures of methane and ammonia monooxygenases in native membranes, submitted.

Investigating Microbial Extracellular Electron Uptake from Redox Active Solid Substrates: Mechanisms for Gaining Electrons from Minerals, Electrodes, or Other Microbes

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Overall research goals:

The overarching goal of this work is to advance our understanding of the genetic and biochemical basis of microbial extracellular electron uptake (EEU) in microbes ranging from model systems to environmentally isolated strains.

In *Shewanella oneidensis* MR-1—a model system capable of bi-directional extracellular electron transfer, we recently identified two distinct genes critical for EEU that are not involved in traditional extracellular electron transfer to minerals. We hypothesize these proteins are involved linking electron flow between anaerobic (i.e., mineral reducing) and aerobic electron transport chains. To investigate function in these proteins, we propose to test purified proteins biochemically and electrochemically, specifically using thin film electrochemical techniques that will allow us to quantify redox potential and test activity with different quinones. The results of this work will help us understand the role of these proteins in EEU and bi-directional electron transfer—processes important for understanding *Shewanella* physiology, and/or its utility for biotechnology applications.

This work will also apply our previously used high throughput genetic techniques (i.e., Tn-Seq) to investigate a novel mechanism of electron uptake in an environmentally isolated marine sediment microbe, *Pseudomonas stutzeri*—a facultative autotroph capable of processive electron uptake when compared with other model systems. As this microbe lacks homologs to currently characterized extracellular electron transfer pathways, we hypothesize it uses an uncharacterized mode of EEU. Using differential growth conditions comparing heterotrophic, lithotrophic and electrotrophic growth, this work will uncover genes essential for EEU in *P. stutzeri*. Though exploratory, we expect this work to point to a novel EEU pathway, that has the potential to expand our understanding of the metabolic and biochemical nature of extracellular electron uptake.

Stemming from our previous proteomic work in the methanogenic Archaea *Methanosarcina barkeri*, this work will investigate phenotypes for extracellular electron uptake in proteins implicated in EEU. In addition to their unique cell structure and cell physiology, our previous electrochemical work in *Methanosarcina* demonstrated a low redox potential electron uptake process that is coupled directly to CO_2 fixation to methane, which is an exciting process for biofuel applications. Our proteomic studies in this system highlighted two proteins that are extracellular and/or redox active that we propose to investigate genetically using CRISPRi-dCas9 genetic techniques in *M. barkeri* (and/or *M. acetivorans*). Confirming a role for these proteins in EEU will help provide insights into more mechanistic investigations of their function, as well as the potential for engineering enhanced electron uptake rates in *Methanosarcina*.

The expected outcomes of this work will expand our understanding the mechanisms of EEU in diverse microbial systems. We will gain insight into novel redox processes, as well as the ecology and biochemistry of extremely important, applied and environmentally relevant groups of microbes.

Significant achievements: [Year 1]:

Aim 1: Graduate student Jin-Sang Yu has made progress performing localization work in *Shewanella* electron up-take genes, demonstrating they are membrane associated. He is currently working on purification techniques and will begin biochemical characterization of these proteins in the coming year.

Aim 2: Joshua Sackett is currently training new graduate student Allision Brown (Fall 2024) in electrochemical techniques for our marine sediment isolates including *Pseudomonas stutzeri.*

Aim 3: Saranya Sriram is working on electrochemical techniques in *Methanosarcina acetivorans* and will be using this strain to test previously constructed thioredoxin mutants in the coming year.

Science objectives for 2024-2025:

Aim1: Jin-Sang Yu is transferring to MSU in Jan 2024. He will take his qualifying exam shortly after. His current work is focusing on characterization of SO0400 and SO3662. His goal is to complete spectroscopic analysis of purified proteins (UV-vis absorbance, EPR, etc.) and start redox characterization assays using thin-film electrochemical techniques.

Aim 2: Allison Brown will also transfer to MSU in Jan 2024. She has just started her training, but is developing the tool kit to design and run TN-seq experiments in *P. stutzeri*. Ideally a transposon library will be generated in the coming year.

Aim 3: Saranya Sriram will return from maternity leave in October. She will begin screening the *M. acetivorans* mutants we have in the lab for phenotypes in electron uptake on cathodes and if time allows electrode/iron reduction.

My scientific area(s) of expertise is/are: Microbiology, physiology, bioelectrochemistry, genetics in bacteria.

The ideal collaborator for my project would have expertise in: People interested in or experienced in characterization of diverse redox proteins, and or experts in genetics in *Methanosarcina*.

Regulated Electron Flux Through Archaeal Energy Production Systems

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Overall research goals:

Energy production and energy conservation strategies supporting the growth of hyperthermophilic Archaea often push - and have expanded the known limits - of energy conversation mechanisms. Many advances in our understanding of archaeal energy systems have emerged from the genetically-tractable order *Thermococcales*, wherein the roles of individual enzymes, soluble and membrane-bound respiratory complexes, electron-carriers, regulatory factors, and competing and complementary catabolic pathways have been probed by a combination of ever-increasingly complex genetic, biochemical, and –omics approaches. *Thermococcales*-encoded, unique, extant energy conversation strategies likely mimic the energy transaction strategies present in early life on Earth and further represent idealized energy conversation mechanisms to support life in the extremes. ***Our long-term research goal is to determine the interplay, competition, and regulation of archaeal energy production with respect to regulated electron flux in the model species Thermococcus kodakarensis***.

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

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

Significant achievements: Award has been on-going since 2017:

- Liman GLS, Lennon CW, Mandley JL, Galyon AM, Zatopek, Gardner AF, Santangelo TJ. Intein-splicing can control archaeal DNA replication. 2024 *(in press at Science Advances)*.
- Liman GLS, Garcia AA, Fluke KA, Davidson SC, Welander PV, Santangelo TJ. Tetraether archaeal lipids promote long-term survival in extreme conditions. 2023. Mol Micro. Feb 19.
- Caffrey PJ, et.al. Thermococcus kodakarensis TK0353 is a novel AP lyase with a new fold. 2023. J Biol Chem. Nov25:300(1).
- Watts EA, et al. Histones direct site-specific CRISPR spacer acquisition in model archaeon. Nature Microbiol. 2023. Sept 8(9):1682-1694.
- Liman GLS, Stettler ME, Santangelo TJ. Transformation techniques for the anaerobic hyperthermophile Thermococcus kodakarensis. Methods in Mol Biology. 2022. 2522:87-104.
- Scott KA, Williams SA, Santangelo TJ. Thermococcus kodakarensis provides a versatile hyperthermophilic archaeal platform for protein expression. Meth in Enzy. 2021. 659:243-273.
- Zatopek KM, et. al. The hyperthermophilic restriction-modification systems of *Thermococcus kodakarensis* protect genome integrity. Frontiers in Microbiology. 2021. May 20; 12:657356.
- Burkhart BW, et al. Distinct physiological roles for the three ferredoxins encoded in the hyperthermophilic archaeon Thermococcus kodakarensis. mBio. 2019. 10(2):e02807-18.
- Liman GLS, et al. A linear pathway for mevalonate production supports growth of Thermococcus kodakarensis. Extremophiles. 2019. s00792-019-01076.
- Speed MC, et al. An Archaeal Fluoride-Responsive Riboswitch Provides an Inducible Expression System for Hyperthermophiles. Appl Environ Microbiol. 2018 Mar 19;84(7).
- Gehring AM, Sanders TJ, Santangelo TJ. Markerless Gene Editing in the Hyperthermophilic Archaeon Thermococcus kodakarensis. Bio Protoc. 2017 Nov 20;7(22).

Science objectives for 2021-2022:

(1) Determine the impacts on H_2 production and cellular fitness that result from forcing a change in the primary electron donor to the membrane-bound hydrogenase (MBH).

(2) Establish the physiological consequences, fitness impacts, and energy-production alterations due to dramatically limiting electron flux to lipid maturation pathways, inclusive of the alternative pathways and Fd-isoforms now employed to dispose of excess reductant.

(3) Establish the impacts of hybrid Fd-isoforms on in vivo electron flux, cellular fitness, and energy conservation mechanisms.

My scientific area(s) of expertise is/are: archaeal biology & genetics, ferredoxin activity, electron flux.

The ideal collaborator for my project would have expertise in: modeling electron flux on the organismal scale.

Publications supported by this project: see significant achievements above.

Elucidating the Catalytic Mechanism of Microbial CO_2 Fixation

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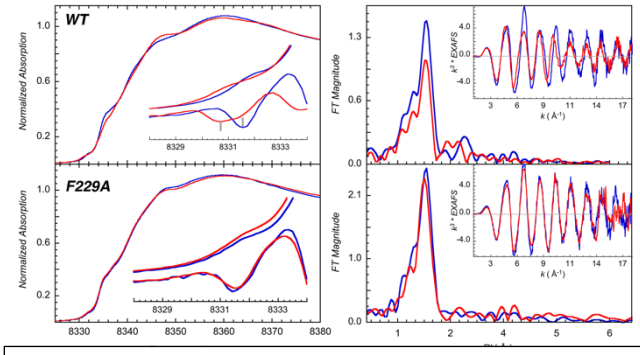
Overall research goals:

The Wood-Ljungdahl pathway (WLP) enables anaerobic microbes to conserve energy during autotrophic fixation of carbon dioxide into acetyl-CoA - a universal metabolic building block. The sole pathway known to both conserve energy and fix carbon, the WLP was present in the last universal common ancestor and may have fueled the emergence of life. The key enzymes in this pathway, CO dehydrogenase (CODH) and acetyl-CoA synthase (ACS), purify as a stable CODH/ACS complex. CODH catalyzes reduction of CO_2 to CO while ACS condenses CO with a methyl group and coenzyme A to generate acetyl-CoA. For over two decades, researchers have searched for the unique series of organometallic intermediates in ACS proposed to facilitate the C-C and C-S fusions. This has been made difficult by the presence of a large number of metals in the CODH/ACS complex (3 Ni and 14 Fe per heterodimer), its lower (~30%) than optimal Ni-occupancy and activity, and its extreme oxygen sensitivity. In collaboration with the Ragsdale group at University of Michigan, who are perfecting the biochemistry to trap high-purity and high-conversion intermediate species, this FWP employs a combination of x-ray absorption spectroscopy techniques and density functional theory methods to elucidate the mechanism of anaerobic biological CO_2 fixation by this most energy efficient CO_2 sequestration pathways in nature.

Significant achievements: [09/21-8/24]:

Using a recombinant "ACS-only" (lacking CODH) construct that affords near full metal occupancy and activity of the active site, the active site of A-cluster of ACS was investigated using a combination of spectroscopic and theoretical methods. The proposed Ni-methyl- and Ni-acetyl intermediates of acetyl CoA synthase were trapped and characterized completing the identification and characterization of all proposed organometallic intermediates in the WLP (previously, the PI had solved the structure of the CO bound intermediate to ACS). In a collaboration with several experts in the field, the structural and electronic properties of methyl- and acetyl-ACS were characterized by a range of spectroscopic methods e.g., UV-visible, X-ray absorption, Mössbauer, electron paramagnetic resonance and electron nuclear double resonance, combined with DFT calculations. The results demonstrate that Nip in the A-cluster undergoes major geometric and redox changes as it transits the resting Ni(II), active Ni(I), tetrahedral Nip(I)-CO and planar methyl- and acetyl-Nip intermediates.



Ni K-edge XAS (left) and Fourier-transforms and EXAFS (inset) (right) data for WT(top) and F229A(bottom) for unexposed (blue) and CO exposed (red) Acetyl CoA Synthase. The smaller Ala disrupts the alcove and CO binding is not observed (no change in data upon CO exposure).

We continued working on the enzymatic systems in the Wood-Ljungdahl pathway, extending our investigation from the immediate active site for substrate binding (the $[Fe_4S_4Ni_2]$ cluster of ACS) to the role of the secondary sphere residues

around the A-cluster in tuning CO binding to the proximal Ni site. The mechanism of A-cluster catalysis includes a CO tunnel and C-C and C-S bond formation via an organometallic reaction sequence involving Ni-CO, -methyl, and -acetyl intermediates followed by reaction of CoA with Ni-acetyl to generate acetyl-CoA. In this follow up study, we collaboratively, show that an alcove adjacent to the A-cluster is essential for autotrophic growth on CO_2 and H_2. Disrupting the ACS alcove by a Phe-to-Ala substitution prevents autotrophic growth and disrupts interactions with CO without affecting metal occupancy or methylation activity. Thus, the alcove is integral to the ACS active site and is essential for CO binding, CO_2 fixation, and growth by the WLP. These results provide evidence for the hypothesis that tunnels play a great role in the functions of metalloenzymes that metabolize small molecules that they should be considered to be extensions of the metallocofactor active site.

Science objectives for 2025-2026:

The focus in the upcoming year will be on the active site of CODH which reversibly converts CO_2 to CO at a unique $NiFe_3S_4$-FCII active site. This active site has been extensively interrogated by crystallographic techniques poised in various redox states and several intermediate bound forms. However, questions about the mode of substrate binding, atomic-resolution structural and electronic descriptions of the $NiFe_3S_4$-FCII core and the involvement of the ferrous iron II component have remained elusive. High resolution EXAFS studies are ongoing that will help establish the structural models for the resting oxidized (C_{Ox}), one-electron reduced (C_{Red1}) and three-electron reduced (C_{Red2}) species. The XAS data, combined with theoretical calculations will explain the integral role of the Fe-S cluster in stabilizing the charge upon subsequent reduction steps from C_{Ox} to C_{Red2}. In particular, a formally Ni(0) state is expected in C_{Red2}, in which the Fe-S cluster is likely to play an important role in stabilizing the excess charge. These studies will be followed by characterization of substrate analogues. The binding of CO will be modeled with CN^-, which will help ascertain the binding mode of CO (bent versus linear). Similarly, investigations on the OCN^- bound forms of C_{Red2} will shed light on the binding mode of CO_2 to the C_{Red2} state, helping resolve structural disputes regarding the crystallographic CO_2-bound C_{Red2} structure. Moving forward, the goal is to ultimately trap the CO and CO_2 bound species using single and double mutants and characterize them with X-ray methods.

My scientific area(s) of expertise is/are: Synchrotron-based X-ray spectroscopy methods, Theoretical calculations that correlate the spectroscopic data to electronic structure and to obtain structure-function correlation.

The ideal collaborator for my project would have expertise in: My ideal collaborator is a biochemist or chemist who is working on complex metalloenzyme or model systems, preferably those involved in complex redox transformations and is interested in structural biology and mechanistic understanding of metalloenzyme catalysis.

Publications supported by this project 09/21-8/24:

1. Can, M.; Abernathy, M. J.; Wiley, S.; Griffith, C.; James, C. D.; Xiong, J.; Guo, Y.; Hoffman, B. M.; Ragsdale, S. W.; Sarangi, R. Characterization of Methyl- and Acetyl-Ni Intermediates in Acetyl CoA Synthase Formed during Anaerobic CO_2 and CO Fixation. *J. Am. Chem. Soc.* **2023**, *145* (25), 13696-13708. DOI: 10.1021/jacs.3c01772.
2. Wiley, S.; Griffith, C.; Eckert, P.; Mueller, A. P.; Nogle, R.; Simpson, S. D.; Kopke, M.; Can, M.; Sarangi, R.; Kubarych, K.; et al. An alcove at the acetyl-CoA synthase nickel active site is required for productive substrate CO binding and anaerobic carbon fixation. *J. Biol. Chem.* **2024**, *300* (8), 107503. DOI: 10.1016/j.jbc.2024.107503
3. Grosjean, N., Yee, E.F., Kumaran, D. *et al.* A hemoprotein with a zinc-mirror heme site ties heme availability to carbon metabolism in cyanobacteria. *Nat. Commun*. **2024**, 15, 3167. DOI: 10.1038/s41467-024-47486-z

Structure and function of the bacterial carbon concentrating machinery

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Overall research goals:

Cells compartmentalize biochemical reactions as a means of improving pathway rate, yield, and toxicity. This strategy is epitomized in the bacterial microcompartment known as the carboxysome, which acts in the physiological strategy known as the CO_2 Concentrating Mechanism to insure fast and efficient carbon assimilation in microbes. Microcompartments are simplified, protein-based organelles. Understanding their assembly and function could provide a way to improve the catalysis of engineered biochemical pathways. The carboxysome plays a central role in the Calvin Cycle – the most important pathway in nature for the capture and conversion of bioenergy-related molecules – and it is likely that principles and/or components from this system could be used to improve photosynthesis in plants. The overarching goal of our work is to develop a mechanistic understanding of essential components of the bacterial CO_2 Concentrating Mechanism, including the carboxysome, in order to understand how its function emerges from the activities of individual components. Specifically, we propose to answer three open questions which have arisen through our past work on this system. Firstly, we will investigate how the protein CsoS2 acts as a scaffold to help assemble the entire carboxysome. This will be rigorously tested in follow-up experiments wherein we attempt to build carboxysomes *de novo* from individual components. Finally, we will investigate the structure and function of the DAB carbon transporter which provides the critical carbon substrates and powers the overall system.

Significant achievements: 2023-2024

- Discovered and structurally characterized the mechanism of loading for the alpha-carboxysome carbonic anhydrase.
- Identified and characterized the mechanism of size control of carboxysomes.
- Developed a novel in vivo selection system for rubisco and completed the first deep mutational scan of the enzyme.
- Developed collaboration with the W.E. Moerner Lab (Stanford) to investigate the function and structure of single carboxysome complexes using a Brownian motion trap.

Science objectives for 2024-2025:

- Continue moving towards biochemical reconstitution of entire carboxysome.
- Finish structure-function studies of the DAB complex.
- Pursue structural studies of a trapped shell intermediate in carboxysome assembly.
- Use rubisco selection system to further evolve rubisco variants with altered enzyme kinetic parameters.

My scientific area(s) of expertise is/are: biochemistry, protein engineering, genome editing.

The ideal collaborator for my project would have expertise in: Mechanistic enzymology and/or spectroscopy.

Publications supported by this project 2022-2024:

1. Prywes N, Philips NR, Oltrogge LM, Lindner S, Tsai YCC, de Pins B, Cowan AE, Taylor-Kearney LJ, Chang HA,

Hall LN, Bellieny-Rabelo D, Nisonoff HM, Weissman RF, Flamholz AI, Ding D, Bhatt AY, Shih PM, Mueller-Cajar O, Milo R, Savage DF. A map of the rubisco biochemical landscape. bioRxiv. 2024. p. 2023.09.27.559826. Available from: https://www.biorxiv.org/content/10.1101/2023.09.27.559826v2

2. Valentin-Alvarado LE, Fakra SC, Probst AJ, Giska JR, Jaffe AL, Oltrogge LM, West-Roberts J, Rowland J, Manga M, Savage DF, Greening C, Baker BJ, Banfield JF. Autotrophic biofilms sustained by deeply sourced groundwater host diverse bacteria implicated in sulfur and hydrogen metabolism. Microbiome. BioMed Central; 2024 Jan 26;12(1):15. PMCID: PMC10811913
3. Valentin-Alvarado LE, Appler KE, De Anda V, Schoelmerich MC, West-Roberts J, Kivenson V, Crits-Christoph A, Ly L, Sachdeva R, Greening C, Savage DF, Baker BJ, Banfield JF. Asgard archaea modulate potential methanogenesis substrates in wetland soil. Nat Commun. 2024 Jul 31;15(1):6384. PMCID: PMC11291895
4. Turnsek JB, Oltrogge LM, Savage DF. Conserved and repetitive motifs in an intrinsically disordered protein drive α-carboxysome assembly. J Biol Chem. Elsevier BV; 2024 Jul 4;(107532):107532. PMID: 38971311
5. Oltrogge LM, Chen AW, Chaijarasphong T, Turnšek JB, Savage DF. 2024. α-Carboxysome Size Is Controlled by the Disordered Scaffold Protein CsoS2. Biochemistry 63: 219–229. http://dx.doi.org/10.1021/acs.biochem.3c00403.
6. Blikstad C, Dugan EJ, Laughlin TG, Turnšek JB, Liu MD, Shoemaker SR, Vogiatzi N, Remis JP, Savage DF. 2023. Identification of a carbonic anhydrase-Rubisco complex within the alpha-carboxysome. Proc Natl Acad Sci U S A 120: e2308600120. http://dx.doi.org/10.1073/pnas.2308600120.
7. Prywes N, Phillips NR, Tuck OT, Valentin-Alvarado LE, Savage DF. 2023. Rubisco Function, Evolution, and Engineering. Annu Rev Biochem 92: 385–410. http://dx.doi.org/10.1146/annurev-biochem-040320-101244.
8. Wang RZ, Nichols RJ, Liu AK, Flamholz AI, Artier J, Banda DM, Savage DF, Eiler JM, Shih PM, Fischer WW. 2023. Carbon isotope fractionation by an ancestral rubisco suggests that biological proxies for CO2 through geologic time should be reevaluated. Proc Natl Acad Sci U S A 120: e2300466120. http://dx.doi.org/10.1073/pnas.2300466120.
9. Lavania AA, Carpenter WB, Oltrogge LM, Perez D, Turnšek JB, Savage DF, Moerner WE. 2022. Exploring Masses and Internal Mass Distributions of Single Carboxysomes in Free Solution Using Fluorescence and Interferometric Scattering in an Anti-Brownian Trap. J Phys Chem B 126: 8747–8759. http://dx.doi.org/10.1021/acs.jpcb.2c05939.
10. Carpenter WB, Lavania AA, Borden JS, Oltrogge LM, Perez D, Dahlberg PD, Savage DF, Moerner WE. 2022. Ratiometric Sensing of Redox Environments Inside Individual Carboxysomes Trapped in Solution. J Phys Chem Lett 13: 4455–4462.

Nitrogenase Reduction of N_2

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Overall research goals:

The research objectives of our joint project are to reveal molecular level insights into the mechanism of nitrogenase catalyzed reduction of N_2 to NH_3. Further, we seek to gain insights into the complex array of proteins and steps involved in the installation of the active site metal clusters of nitrogenase. The outcomes of these studies are expected to provide foundational information about the assembly and reactivity of the complex metal clusters of nitrogenase, giving guidance to the design of next generation N_2 catalysts.

Significant achievements: 2022-2024:

Progress over the last two years has resulted in 9 peer-reviewed publications (below). Significant progress has been made in both of the two thrusts of the project: insights into the mechanism of substrate reduction at the nitrogenase active site and assembly of the active site. An important advance has come from developing an approach to selectively label the C in the middle of the nitrogenase active site, FeMo-cofactor. By labelling with ^{13}C and trapping substrate reduction intermediates by freezing during turnover, we have been able to apply advanced spectroscopic techniques (^{13}C-ENDOR in collaboration with Brian Hoffman) to gain new insights into the spin states of the C-Fe_6 core of FeMo-co during turnover. Findings so far indicate that the six C-Fe bonds at the center of FeMo-cofactor remain intact in the intermediate states trapped and that the spin states of the 6 core Fe atoms remain balanced. These findings contrast with earlier proposals that the C-Fe bonds might be hemi-labile to create substrate binding sites on Fe atoms. We have also discovered that the alternative nitrogenase isozymes (V- and Fe-based) are EPR active in the odd E states (E_1, E_3, etc.). This finding complements the earlier finding that the Mo-nitrogenase active site is EPR active in the even E states (E_0, E_2, etc.). Thus, we are now in a position to examine all of the E states of the catalytic cycle during N_2 reduction, providing important mechanistic insights. We have recently been able to elucidate the stoichiometry of ATP utilization per electron transferred for different oxidation states of the Fe protein (1+ or 0), resolving confusion from earlier studies about the energy transduction mechanism. We have also put forward a new kinetic model for substrate reduction by Mo-nitrogenase, revealing new aspects of the kinetic mechanism. New insights have also been gained on proteins involved in controlling synthesis and installation of the active sites of all three nitrogenase isozymes (Mo, V, Fe). For example, we have found that a protein designated AnfO, encoded within the Fe-only nitrogenase system, controls the fidelity of FeFe-cofactor insertion by specifically preventing the incorporation of FeV- or FeMo-cofactor into FeFe protein during its maturation. Gene shuffling approaches have permitted the production, at scale, of hybrid species for which FeV-cofactor is inserted into the MoFe protein, FeMo-cofactor has been inserted into VFe protein, and either FeV-cofactor or FeMo-cofactor has been inserted into the FeFe protein. These constructs are expected to advance an understanding of the role of the apical heterometal, and their corresponding protein contributions, to nitrogenase catalysis. Gene shuffling approaches are also being used to define how molecular scaffolds involved in FeMo-cofactor and FeV-cofactor assembly provide specificity for heterometal incorporation into their cognate catalytic cofactors.

Science objectives for 2024-2025:

- Selectively label with ^{13}C the central C for all three nitrogenase isozymes (Mo, V, Fe) and trap substrate intermediates, including states during N_2 reduction. Analysis of these trapped states by ^{13}C-ENDOR will reveal the spin states of the C-Fe_6 core at different steps in the reaction pathway. These studies are expected to advance our understanding of the mechanism of nitrogenase reduction of substrates.
- Finalize a full kinetic model that advances on the model put forward by Thorneley and Lowe over 40 years ago. The full model will provide predictions of the populations of all of the E states under different reaction conditions, thus enhancing our efforts to trap intermediates to reveal mechanistic features.
- Advance the analysis of auxiliary proteins that are associated with, but not essential for, the maturation of the three nitrogenase isozymes.

Our scientific area(s) of expertise is/are: Metalloenzyme mechanism, catalysis, Fe-S clusters, electron transfer, N_2 reduction, bacterial genetics.

The ideal collaborator for my project would have expertise in: Spectroscopic methods for paramagnetic metal clusters such as ENDOR (Hoffman); application of high-level theory (QM/MM) on catalytic mechanism of metalloenzymes (Raugei).

Publications supported by this project 2022-2024:

1.Chen, T., Ash, P. A., Seefeldt, L. C., and Vincent, K. A. (2023) Electrochemical experiments define potentials associated with binding of substrates and inhibitors to nitrogenase MoFe protein. *Faraday Discuss. 243*, 270–286.

2. Yang, Z.-Y., Badalyan, A., Hoffman, B. M., Dean, D. R., and Seefeldt, L. C. (2023) The Fe protein cycle associated with nitrogenase catalysis requires the hydrolysis of two ATP for each single electron transfer event. *J. Am. Chem. Soc. 145*, 5637–5644.

3. Tokmina-Lukaszewska, M., Huang, Q., Berry, L., Kallas, H., Peters, J. W., Seefeldt, L. C., Raugei, S., and Bothner, B. (2023) Fe protein docking transduces conformational changes to MoFe nitrogenase active site in a nucleotide-dependent manner. *Commun. Chem. 6*, 1–8.

4. Lukoyanov, D. A., Yang, Z.-Y., Shisler, K., Peters, J. W., Raugei, S., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2023) A conformational equilibrium in the nitrogenase MoFe protein with an α-V70I amino acid substitution illuminates the mechanism of H2 formation. *Faraday Discuss. 243*, 231–252.

5. Stappen, C. V., Jiménez-Vicente, E., Pérez-González, A., Yang, Z.-Y., C. Seefeldt, L., DeBeer, S., R. Dean, D., and Decamps, L. (2022) A conformational role for NifW in the maturation of molybdenum nitrogenase P-cluster. *Chem. Sci. 13*, 3489–3500.

6. Pérez-González, A., Jimenez-Vicente, E., Salinero-Lanzarote, A., Harris, D. F., Seefeldt, L. C., and Dean, D. R. (2022) AnfO controls fidelity of nitrogenase FeFe protein maturation by preventing misincorporation of FeV-cofactor. *Molecular Micro. 117*, 1080–1088.

7. Lukoyanov, D. A., Yang, Z.-Y., Pérez-González, A., Raugei, S., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2022) 13C ENDOR characterization of the central carbon within the nitrogenase catalytic cofactor indicates that the CFe6 core is a stabilizing "heart of steel." *J. Am. Chem. Soc. 144*, 18315–18328.

8. Lukoyanov, D. A., Harris, D. F., Yang, Z.-Y., Pérez-González, A., Dean, D. R., Seefeldt, L. C., and Hoffman, B. M. (2022) The one-electron reduced active-site FeFe-cofactor of Fe-nitrogenase contains a hydride bound to a formally oxidized metal-ion core. *Inorg. Chem. 61*, 5459–5464.

9. Harris, D. F., Badalyan, A., and Seefeldt, L. C. (2022) Mechanistic insights into nitrogenase FeMo-cofactor catalysis through a steady-state kinetic model. *Biochemistry 61*, 2131–2137.

Developing a molecular level understanding of carbon monoxide dehydrogenase/acetyl coenzyme A synthase through model metalloenzymes

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Overall research goals:

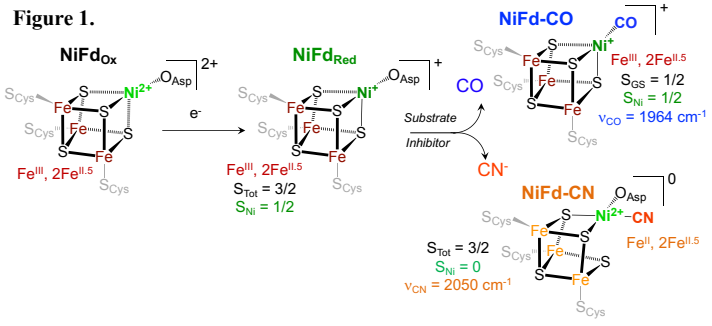
This project will provide mechanistic insight into the bifunctional metalloprotein complex, carbon monoxide dehydrogenase (CODH)/acetyl coenzyme A synthase (ACS), which catalyzes the selective reduction of carbon dioxide to carbon monoxide and generation of new energetic carbon-carbon and carbon-sulfur bonds from one-carbon precursors. These reactions represent valuable targets for carbon dioxide utilization and the selective synthesis of liquid fuels from CO_2. However, we lack a fundamental understanding of how these enzymes work. In this project, we will develop and characterize protein-based models of CODH and ACS as structural, functional, and mechanistic mimics to reveal the biochemical principles by which CODH and ACS operate.

In **Aim 1**, a robust ferredoxin scaffold will be used to construct a site-differentiated nickel-iron-sulfur cluster-containing protein as a model for CODH. Detailed electronic structure characterization will be pursued using complementary spectroscopic tools, and function will be assessed using solution-phase and electrochemical experiments. **Aim 2** will build from prior work within the group that demonstrated electronic and mechanistic parallels between a modified nickel-substituted azurin model and ACS. New variants will be pursued to develop functional models of ACS, allowing resolution of the key protein-derived factors influencing reactivity. Specific contributors to substrate channeling will be investigated in **Aim 3**. The development of functional, robust enzymes that catalyze CO_2 reduction and carbon-carbon bond formation will provide catalyst design guidelines for sustainable generation of liquid fuels from CO_2.

Significant achievements: [2022-2024]:

Using a suite of complementary techniques, including EPR, X-ray absorption, and resonance Raman spectroscopy, we have interrogated the electronic structure of the [3Fe-4S-Ni] cluster in a ferredoxin-based model of CODH (NiFd) across multiple redox and substrate-bound states. We find that the three Fe ions serve as a redox buffer for electronic structure changes occurring upon ligand binding at the metal center, implicating electronic isomerism as a mechanism to support the multielectron transformations catalyzed by native CODH (**Figure 1**).

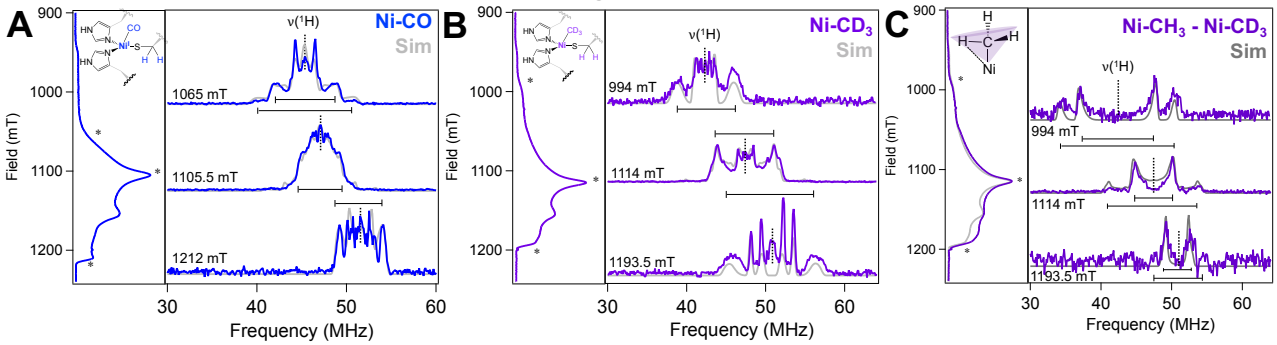
Figure 1.



We have used pulsed EPR and sulfur K-edge X-ray absorption spectroscopies in conjunction with CASSCF computational analyses to probe the effects of covalency on the electronic structures of isolable intermediates within our functional ACS model, Ni-substituted azurin (NiAz). Minimal hole character is delocalized onto the cysteine ligand, even in electron-deficient and formally high-valent states, rationalizing the electronic basis promoting two-electron reactivity at the nickel center (**Figure 2A-2B**). The S = ½ Ni-CH_3 Az species, previously suggested to have an "inverted" Ni^{I}-$^{+}CH_3$ electronic structure, is indicated from high-level calculations to also possess significant Ni^{II}-$^{\bullet}CH_3$ character. The multireference nature of this formal Ni^{III} species likely suppresses the canonical organometallic reactivity that otherwise would be destructive to the protein scaffold. Interestingly, the bound methyl

group is found to be static within the NiAz pocket, rather than freely rotating, and is canted towards the metal center, reflecting the asymmetry in the electronic structure (**Figure 2C**).

Figure 2.



Science objectives for 2024-2025:

Aim 1

- Construct primary and secondary sphere mutants in *Pf* Fd and metallate with nickel and iron to model the CODH active site with higher structural and electronic fidelity than in wild-type *Pf* Fd
- Use electrochemical and solution-phase assays to characterize reactive substrate binding in NiFd and variants using CODH substrates, inhibitors, and alternative substrates
- Apply multifrequency EPR and variable-field Mössbauer spectroscopy (in collaboration with Katlyn Meier at U. Miami) to characterize electronic structure and substrate-active site interactions in NiFd

Aim 2

- Complete EPR and XAS analysis connecting covalency to reactivity in NiAz ACS model
- Develop complementary approaches for introducing catalytic thioester synthesis activity into NiAz

Aim 3

- Generate mutations that perturb sterics and electronics of substrate binding channel in NiAz
- Quantify effects of channel mutations on substrate binding kinetics and thermodynamics
- Apply pulsed EPR techniques to investigate how outer sphere mutations change electronic structure of substrate-bound species
- Establish correlations between CO affinity, CO inhibition, and thioester synthesis across NiAz mutants

My scientific area(s) of expertise is/are: Metalloenzyme spectroscopy, especially EPR, pulsed EPR, resonance Raman spectroscopy.

The ideal collaborator for my project would have expertise in: Anaerobic X-ray crystallography, particularly in the presence of gaseous substrates.

Publications supported by this project 2022 – 2024 :

1. Lewis, L. C.; Sanabria-Gracia, J. A.; Lee, Y.; Jenkins, A. J.; Shafaat, H. S.* Electronic isomerism in a heterometallic nickel–iron–sulfur cluster models substrate binding and cyanide inhibition of carbon monoxide dehydrogenase. *Chem. Sci.* **2024**, 15, 5916–5928 DOI: 10.1039/D4SC00023D. ***Selected as Pick of the Week and selected for Front Cover***.
2. Shafaat, H. S.; Manesis, A. C.; Yerbulekova, A. How to Build a Metalloenzyme: Lessons from a Protein-Based Model of Acetyl Coenzyme A Synthase. *Acc. Chem. Res*. **2023**, 56, 984–993 DOI: 10.1021/acs.accounts.2c00824.

Mechanisms and Regulation of Carbon Allocation and Storage in Plants.

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Overall research goals:

We address the Department of Energy (DOE) Basic Energy Sciences mission: "To provide foundational knowledge on processes that convert renewable resources into fuels, chemicals and other energy-enriched products". The BNL group focuses on uncovering foundational mechanisms governing the allocation of photosynthetically fixed carbon within central metabolism of plants, and how that carbon flows into lipid and phenylpropanoid biosynthetic pathways to create the major highly reduced end products: triacylglycerols and lignin. There are parallel goals for both lipids and lignins, first to understand the enzymology and genetics of the two pathways, to control the composition of the final storage compounds and second, to understand the molecular basis for various aspects of metabolic feedback regulation within each of the pathways as a foundation for reengineering plant metabolism to deregulate it and thereby accumulate more of the desired storage compounds. Lipids and lignins are highly reduced carbon compounds that consume high levels of energy in the form of reducing equivalents. Since all plant storage compounds originate from sugars, their production are under tight homeostatic regulation by SnRK1, a conserved sugar signaling sensor-kinase that plays a major role in controlling the balance between anabolism and catabolism. We are working to understand the molecular mechanisms that govern SnRK1 activity in controlling key regulatory processes related to both lipid and lignin synthesis along with other more specific modes of biochemical regulation of key steps in their synthesis.

Significant achievements: Current award 2023-2024:

We described a much-expanded role for WRINKLED1 as a master transcriptional activator of triacylglycerol synthesis that now includes genes in glycolysis, fatty acid synthesis and TAG assembly. We also identified a role for CDK8 in activating WRINKLIED1. Several studies addressed how energy in the form of electrons is allocated to distinct intracellular processes. For example, tissue-preferential recruitment of electron transfer chains for cytochrome P450-catalyzed phenolic biosynthesis was reported. We also reported on the diversity of cytochrome b_5 in green lineages, and how some b5s have a strong preference for delivering electrons to specific metabolic processes for example, syringyl lignin synthesis. Our publication of the structure of an alkane omega hydroxylase protein with its bound redox donor and substrate in the active site cavity provided insights into the supply of electrons necessary for the activation of terminal methyl groups within alkanes that are highly resistant to oxidation. In collaboration with other CSGB colleagues, A method for predicting Mössbauer Parameters of nonheme diiron complexes was developed with CSGB colleagues in BNL's artificial photosynthesis group using density functional theory. In collaboration with PNNL Physical Biosciences colleagues we used a combination of computation and biophysical characterization to define the molecular mechanism of inhibition of SnRK1 by trehalose 6-phosphate, the signaling molecule proxy for cellular sugar availability. In a related SnRK1 study we showed that GRIK phosphorylates and activates KIN10 which intriguingly also promotes its degradation.

Science objectives for 2024-2027:

In lipid work: structure-function studies to determine reaction outcomes of soluble and integral membrane desaturase and related diiron enzymes and understanding posttranslational regulation of anabolism and fatty acid synthesis using an integrated structure/computation approach. Characterization of tgd mutants and TGD proteins. Analysis of phosphatidic acid phosphohydrolase (PAH) function and regulation, and the

Identification of molecular factors in lipid droplet assembly. Increasing seed oil content by improving precursor and energy cofactor metabolism. Characterizing new gene targets of WRINKLED1 and increasing seed oil content by overexpressing a mammalian pyruvate kinase. For phenylpropanoids: dissecting regulatory cascades that control phenylpropanoid synthesis in response to cellular carbon status. Elucidating the CB5D-F5H, and CPR-CB5-F5H protein complexes and determining biological functions of cytochrome b_5 proteins leading to the formation of other reduced carbon storage compounds. For electron flow: elucidating mechanisms governing the specificity of cytochrome b_5s in phenylpropanoid synthesis.

My project addresses BES cross-cutting priority areas by:

Building detailed knowledge of carbon capture, conversion and storage in plants that lays the foundations for new energy technologies to advance DOE missions in energy and the environment. Specifically, we focus on the discovery, design, and understanding of biochemical processes to harness nature to benefit people and society. Aspects of this work involves collaboration with CSGB computational colleagues.

Our scientific areas of expertise are: PIs in this program have expertise in: structure-functions studies on enzyme specificity, cellular homeostasis by the biochemistry of sensor kinase regulation of central metabolism and storage of reduced carbon, metabolic flux analysis, metabolic modeling, mechanisms of regulation of lipid and phenylpropanoid metabolism and intracellular lipid trafficking.

The ideal collaborator for my project would have expertise in:. Computational theory of electron transfer.

Publications supported by this project -Selected recent publications (from 45 in present funding cycle)

Chai, J., Guo, G., McSweeney, S.M., **Shanklin, J., Liu, Q**. (2023). Structural basis for enzymatic terminal C–H bond functionalization of alkanes. *Nature Structural & Molecular Biology* **30**, 521.

Kuczynski, C., McCorkle, S., Keereetaweep, J., **Shanklin, J**., **Schwender, J**. (2022). An Expanded Role for the Transcription Factor WRINKLED1 in the Biosynthesis of Triacylglycerols during Seed Development. *Front. Plant Sci.* 2022; 13: 955589

Yu, L., Shen, W., Fan, J., Sah, SK., Mavraganis, I., Wang, L., Gao, P., Gao, J., Zheng, Q., Meesapyodsuk, D., Yang, H., Li, Q., Zou, J., **Xu, C.** A chloroplast diacylglycerol lipase modulates glycerolipid pathway balance in Arabidopsis (2023). *Plant J. 115, 335–350*

Zhai, Z., Blanford, J., Cai, Y., Sun, J., Liu, H., Shi, H., **Schwender, J., Shanklin, J**. (2023) CDK8 positively regulates oil synthesis by activating WRINKLIED1 transcription. *New Phytologist* 238, 724

Zhao, X., Zhao, Y., Gou, M., and **Liu C-J*** (2023) Tissue-preferential recruitment of electron transfer chains for cytochrome P450-catalyzed phenolic biosynthesis***. Science Advances 9, eade4389***

Banerjee, A., Liu, Q. **Shanklin, J**., and Ertem, M.Z. (2024) Predicting Mössbauer Parameters of Nonheme Diiron Complexes with DFT. 2023. Inorganic Chemistry 62, 11402-11413

Blanford, J., Zhai, Z., Baer, MD., Guo, G., Liu, H., **Liu, Q**., Rauge, S., **Shanklin, J**., (2024) Molecular mechanism of trehalose 6-phosphate inhibition of the plant metabolic sensor kinase SnRK1. Science Advances. 10, eadn0895

Zhao, X., Zhao, Y; Zeng, Q-y; **Liu, CJ.** (2024) Cytochrome b5 diversity in green lineages preceded the evolution of syringyl lignin biosynthesis. *Plant Cell*. 36, 2709

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

Significant achievements: [2024-2027]:

(1) Ferredoxin electron transfer following fusion and insertion of homologous proteins. During evolution, protein fusion and domain insertion can lead to new functions. To better understand the effects of these processes on Fds, we examined how the fusion and domain insertion of structurally-related proteins affects Fd electron transfer. We fused three different anti-GFP nanobodies to a Fd fragment and GFP to the complementary fragment. Using a cellular selection, we found that Fd activity differed between otherwise identical fusion configurations that used different nanobodies. Changes in the linker lengths did not affect ET. Domain insertion revealed similar variability. These findings reveal complexities of predicting protein function following topological mutations with involving protein paralogs.

(2) Studying putative ferredoxin peptide fossils using a cellular assay. Embedded within Fd sequence and structure is a symmetry that points to an ancient gene duplication event. To understand the nature of Fds prior to this duplication event, we use structure-guided protein design to revive a set of putative half-ferredoxins and their symmetric full-length counterparts. All half-Fds designs homodimerize and have structural, thermodynamic and electrochemical behaviors very similar to their cognate Fds. However, these half Fds behave differently when incorporated an in vivo electron transfer complementation assay. While they can couple with a Fd-NADP reductase and sulfite reductase (SIR), their ability to do so is highly sensitive to oxygen. Bioinformatics revealed that half-Fds are found in extant anaerobic microorganisms.

(3) Insertional profiling of an NADPH-dependent sulfite reductase. NADPH-dependent SIRs are oligomeric proteins, consisting of four flavoprotein (SIR-FP) and eight hemoprotein (SIR-HP) subunits. Unfortunately, we lack detailed structural information for the oligomeric protein. To better understand SIR sequence-structure-function, we evaluated the effect of peptide insertion in the SIR-HP by selecting a mutant library for activity. In the SIR-HP structure, peptide-insertion tolerance was inversely correlated with proximity to the cofactors and the region that mediates oligomerization with SIR-FP. Insertion was tolerated to a greater extent proximal to the interface where the SIR-FP Fld domain is thought to mediate ET to the SIR-HP. Domain insertion at these

interfaces also led to the creation of protein switches, illustrating how increased understanding of peptide insertion can be used to guide domain insertion.

Science objectives for 2025-2026:

We previously found that Fd-mediated e- transfer between defined partner proteins can be monitored via extracellular electron transfer (EET) using *Escherichia coli*. We also discovered that Flds can support electron transfer (ET) to Fd-dependent sulfite reductase (SIR) and used insertional mutagenesis to understand sequence-structure-function relationships in these elongated oxidoreductases. Surprisingly, a comparison of measured insertional tolerance with natural protein sequence variability revealed a striking correlation, identifying likely sites where sequence expansion can tune partner specificity. Looking forward, we plan to: (i) analyze how Fds and Flds with elongated structures vary in their e- cycling between defined partner proteins using cellular selections and EET; (ii) use biochemical analysis of Fds and Flds that present distinct ET efficiencies to understand if differences arise from variation in midpoint potentials, partner binding, and/or expression, and (iii) investigate whether carbon flux in consortia can be rapidly altered using synthetic catalytic RNA that cell-selectively switch on Fd/Fld and Fd/Fld-partner expression in undomesticated microbes. The last objective represents a novel strategy to accelerate studies of energy flow in consortia without the need for complex genetic modifications.

My scientific area(s) of expertise is/are: ferredoxins, flavodoxins, microbial genetics, protein electron carriers, oxidoreductases, redox cofactors, and synthetic biology

The ideal collaborator for my project would have expertise in: (i) computational docking, (ii) high-throughput electrochemistry, and (iii) machine learning.

Publications supported by this project [2022-2024]:

1. J. Bluford, L. Windham, A. Truong, J.J. Silberg, "Cellular strategies to study and engineer low potential protein electron carriers," Electron Transfer in Biomacromolecules (A. Furst, Ed.) De Gruyter Publishing (2024) [*in press*]
2. T. Coleman, T., J. Shin, J.J. Silberg, Y. Shamoo, J.T. Atkinson, "The biochemical impact of extracting an embedded adenylate kinase domain using circular permutation," *Biochemistry*, 63(5): 599-609 (2024) [DOI, 10.1021/acs.biochem.3c00605]
3. A.L. Goldman, E.M. Fulk, M. Osburn, L. Momper, C. Heider, J. Mulligan, C.A. Masiello, J.J. Silberg, "Microbial sensor variation across biogeochemical conditions in the terrestrial deep subsurface," *mSystems*, 9(1): e0096623 (2024) [DOI, 10.1128/msystems.00966-23]
4. A. Truong, D. Myerscough, I.J. Campbell, J.T. Atkinson, Silberg, J.J., "A cellular selection identifies elongated flavodoxins that support electron transfer to sulfite reductase," *Protein Science* 32(10), e4746 (2023) [DOI, 10.1002/pro.4746]
5. J.T. Atkinson, L. Sun, X. Zhang, G.N. Bennett, J.J. Silberg, C.A. Ajo-Franklin, "Real-time bioelectronic sensing of environmental contaminants," *Nature*, 610(7933): 548-553 (2022) [DOI, 10.1038/s41586-022-05356-y]
6. I.J. Campbell, J.T. Atkinson, M. Carpenter, D. Myerscough, C.A. Ajo-Franklin, J.J. Silberg, "Determinants of multiheme cytochrome extracellular electron transfer uncovered by systematic peptide insertion," *Biochem.,* 61: 1337-1350 (2022). [DOI, 10.1021/acs.biochem.2c00148]

Molecular Mechanism of Energy Transduction by Plant Membrane Proteins

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Tim Grant,, Co-PI
Mathew Blackburn, Postdoctoral Research Associate
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Overall research goals:

The plasma membrane is the point of contact between a cell and its external environment and plays a critical role in the growth and development of all organisms, including higher plants. Plants cannot move to escape from harmful environments and instead have evolved proteins, such as the plasma membrane proton pump (H^+-ATPase), to help minimize the negative impact. My laboratory has been developing and implementing mass spectrometric based proteomic technologies that allow us to reveal the molecular details of one unique coping mechanisms involving protein kinases and phosphatases that modify the inhibitory C-terminal domain (CTD). The main goal of this project is to determine how this regulatory phosphocode is translated into conformational changes of the pump which in turn control how short or tall a plant is, and its ability to bend in response to gravity and/or light. Although there is a catalytically similar proton pump in the yeast plasma membrane, the mechanism by which the CTD operates in these two species is completely different, as is their macromolecular 3D structure. Importantly, phylogenetic analyses indicate that this difference may have led to the terrestrialization of plants, i.e. their adaption to water and air, from oceanic ancestors.

More recently these studies have been expanded to include the full spectrum of high resolution physical bioscience tools (mass spectrometric based footprinting and cryoEM) available in the Sussman and Grant labs on campus at UW-Madison to study how the chemical energy of ATP is transduced into the electrochemical energy of a transmembrane proton gradient by the plasma membrane proton pump (abbreviated AHA, for ***A****rabidopsis* **H**+-**A**TPase). Specifically, we are determining a high resolution three dimensional structure of this protein, to determine how ATP hydrolysis is conformationally coupled to proton ejection and how its different states of regulatory phosphorylation in its C-terminal inhibitory domain, translate alter the protein's conformation and catalytic capabilities.

Significant achievements: 2021-2024

We have developed new methods for performing mass spectrometric based analyses of protein 3D structure including direct azidylation as well as a click enrichable method for detecting conformationally sensitive amino acid side chains, even in highly complex protein mixtures such as membranes or crude lysates (see publications below). Application of these technologies, as well as more recent cryoEM analyses have demonstrated that the plant's 104 amino acid long C-terminal inhibitory domain is in contact with many more subdomains of the catalytic region than a catalytically similar yeast enzyme with a shorter 38 amino acid long C-terminal domain. This plant specific CTD and its more complex interaction with the catalytic portion of the pump has important implications both for its more complex biological function and for its different macromolecular structure in vivo, compared to that in the single celled yeast.

Science objectives for 2024-2025

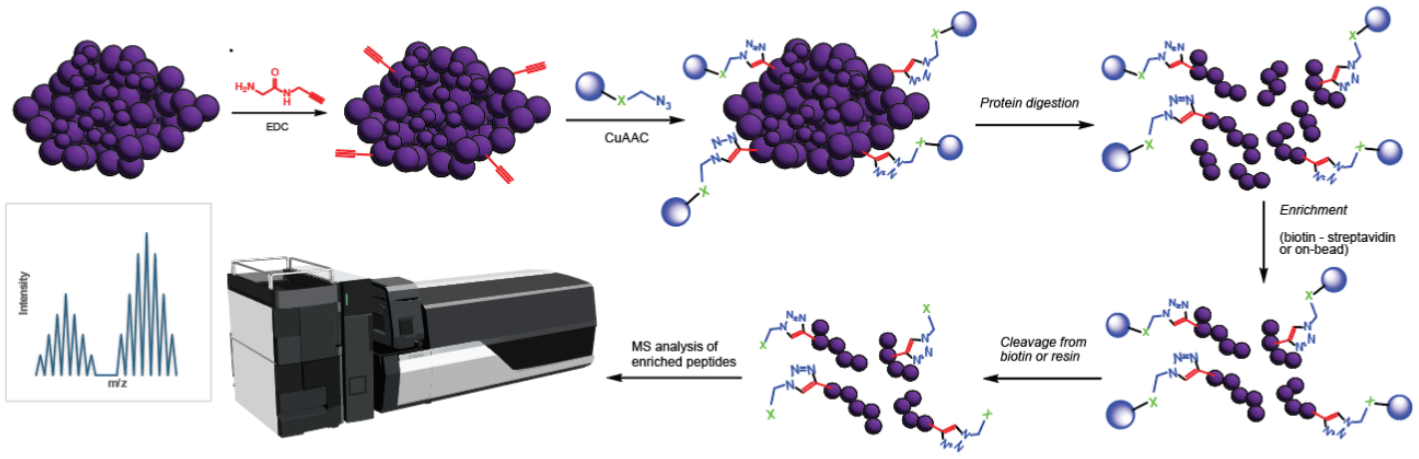
- We are determining a high resolution cryoEM structure of the plant's trimers and dimers in the inhibited and basal states using fresh sample (i.e. no freeze-thaw) in the presence of Post-Albers inhibitors to create more stable nondenatured conformers.
- We are examining a large number of site directed mutants to obtain conformational 'locks' to assist the cryOEM analysis and to provide genetic tests of our molecular models for how the plant enzyme is regulated.

My scientific area(s) of expertise is/are: Mass spectrometry, plant biology, protein chemistry, genomic technology development.

The ideal collaborator for my project would have expertise in: Membrane protein cryoEM experts like Huilin Li and Tim Grant.

Publications supported by this project 2021-2024:

- Blackburn, M. R., Nguyen, T.T., Patton, S.E., Bartosiak, J, and Sussman, M.R. Covalent labeling reveals 3D conformational changes involving the C-terminal regulatory domain of the *Arabidopsis* plasma membrane H+-ATPase. (FEBS Letters,submitted)
- Jamison D. Wolfer, Benjamin B. Minkoff, Heather L. Burch, and Michael R. Sussman. Enrichable Protein Footprinting for Whole Proteome 3D Structural Analysis (JASMS, submitted)
- Vilarrasa-Blasi J, Vellosillo T, Jinkerson RE, Fauser F, Xiang T, Minkoff BB, Wang L, Kniazev K, Guzman M, Osaki J, Barrett-Wilt GA, Sussman MR, Jonikas MC, Dinneny JR. Multi-omics analysis of green lineage osmotic stress pathways unveils crucial roles of different cellular compartments. Nature Communications. 2024 July 16;15(1):5988.
- Minkoff BB, Burch HL, Wolfer JD, Sussman MR. Radical-Mediated Covalent Azidylation of Hydrophobic Microdomains in Water-Soluble Proteins. ACS (American Chemical Society) Chemical Biology 2023 Aug 18;18(8):1786-1796.
- Blackburn MR, Minkoff BB, Sussman MR. Mass spectrometry-based technologies for probing the 3D world of plant proteins. Plant Physiology 2022 May 3;189(1):12-22.
- Conti BJ, Leicht AS, Kirchdoerfer RN, Sussman MR. Mass spectrometric based detection of protein nucleotidylation in the RNA polymerase of SARS-CoV-2. Communications Chemistry 2021;4:41.



Abstract image: Outline of the pipeline used for mass spectrometric based click enriched labeling of glutamate and aspartate containing amino acids in solvent accessible regions of proteins.

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

Breanna Urbanowicz, and Vivek Bharadwaj, Principal Investigators
Franklin Leach, Deepak Sharma, Lintao Bu, Michael Crowley, Co-PI(s) & Key Personnel
CCRC, The University of Georgia, Athens (BU, FL, DS); NREL Golden Co (VB, LB, MC)

Overall research goals:

The pectic fragment Rhamnogalacturonan II (RG-II) exists as a borate cross-linked dimer in the cell walls of all vascular plants. We have established that the glycosyl sequence of RG-II plays a critical role in controlling the rate of both dimer formation and the regiospecificity of the crosslinking reaction. We aim to study structural variants of RG-II to determine sequence-structure relationships that impact function using a suite of experimental techniques closely tied to computational molecular simulations. This will enable the elucidation of RG-II sidechain conformation and dynamics and provide crucial insight into their interactions with divalent cations and borate and how specific sugars of a sidechain contribute to dimer self-assembly.

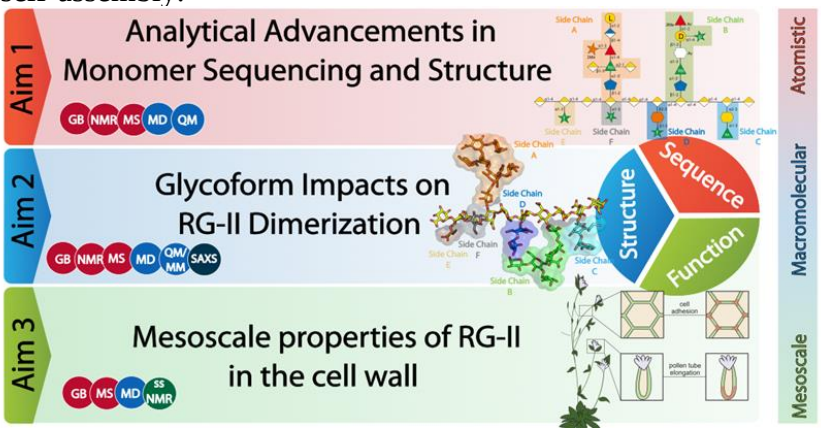


Figure 1. *Research overview highlighting specific aims, tools and techniques. The three specific aims are organized to probe and establish the sequence-structure-function paradigm for complex carbohydrates and aim to provide atomistic, macromolecular and mesoscale level insights into RG-II structure, dynamics and function.*

Significant achievements: 2021-2024:

- **Analytical roadmap for pectin isolation and analyses:** We developed and standardized workflow for the isolation and characterization of polysaccharide primary structure using RG-II as a model (Barnes et al., 2021)
- **Identification of mutants lacking chain C:** As part of an ongoing collaboration with Jenny Mortimer (LBNL, University of Adelaide), we identified applied new optimized methods to isolate and analyze pectin domains of the cell walls of callus mutants lacking a putative RG-II specific glycosyltransferase (Zhang et al., 2024). RG-II isolated from the *rckt1* mutant is monomeric and led to the hypothesis that RG-II dimerization is perturbed due to altered glycosyl sequence. To test this *in vitro*, we generated a ΔC glycoform and showed that dimerization issignificantly hindered relative to its native counterpart demonstrating that the chain C of RG-II is required for normal dimer formation both in vitro and in vivo.
- **Experimentally-validated molecular model of the RG-II monomer:** We developed forcefield parameters for the monosaccharides and glycosidic linkages that were not already present in the CHARMM molecular-mechanics forcefield to enable molecular dynamics simulations RG-II in fully solvated environments. In concert with solution-based NMR techniques, our replica-exchange molecular dynamics (REMD) simulations enabled the evaluation and validation of the 'most representative' sidechain arrangements from a set of 8 distinct structural variants to present the first 3-D structure for RG-II. (Koj et al. Under Review)
- **RG-II specific glycoprobes:** Building upon our toolbox of *B. theta* glycoside hydrolases, we generated regiospecific probes targeting sidechains A and B that are able to target and bind RG-II *in muro*. MicroScale

Thermophoresis (MST) has been used to quantify the binding affinity of each of our probes to RG-II monomer and dimer (Thorne et al., 2024 in prep).

- **Identification of RG-II glycoforms that modulate dimerization:** We have successfully created a diverse library of over 20 structurally distinct RG II molecules by selectively cleaving natural molecules recombinant enzymes, while preserving the core structure. *In vitro* dimer self-assembly assays have revealed that these modifications have unique effects and can modulate the rate of dimer formation (Sharma et al., 2024 in prep)

Science objectives for 2024-2027

- Complete analysis of RG-II glycoform library and develop MS polymer sequencing techniques
- Bridge sequence-structure relationships with molecular models
- Establish sequence-structure relationships in complex polysaccharides, explore dimer structure and formation mechanisms
- Application of RG-II glycoprobes and molecular localization by MS imaging of the cell wall
- Solid state NMR & MS to inform mesoscale computational and molecular science models of RG-II

My scientific area(s) of expertise is/are: Cell wall structure and function, polysaccharide chemistry biochemistry, molecular biology (CCRC), molecular modelling (quantum mechanics and molecular dynamics) of complex carbohydrates, biopolymers and enzymes (NREL).

The ideal collaborator for my project would have expertise in: (i) Solid state NMR spectroscopy. We believe that recent developments in high-resolution ssNMR of cell walls will provide information on the architecture and properties of walls that lack the borate crosslink. (ii) Data-driven machine learning methods to analyze large datasets generated from molecular simulations to develop robust structure-property-function relationships.

Publications supported by this project [2021-current]: *Co-contribution

1. Barnes, W.J., Koj, S., Black, I.M., Aracher-Hartmann, S.A., *Urbanowicz, B.R., *Pena, M-J, *O'Neill, M.A. (2021) Protocols for isolating and characterizing polysaccharides from plant cell walls: a case study using rhamnogalacturonan II. *Biotechnol Biofuels* 14 142
2. Wang, G., DiTusa, S.F., Oh, D.H., Herrmann, A.D., Mendoza-Cozatl, D.G., O'Neill, M.A., Smith, A.P. and Dassanayake, M., (2021). Cross species multi-omics reveals cell wall sequestration and elevated global transcript abundance as mechanisms of boron tolerance in plants. *New Phytologist*, *230*(5), pp.1985-2000
3. Ding, A., Tang, X., Yang, D., Wang, M., Ren, A., Xu, Z., Hu, R., Zhou, G., O'Neill, M. and Kong, Y., (2021). ERF4 and MYB52 transcription factors play antagonistic roles in regulating homogalacturonan de-methylesterification in Arabidopsis seed coat mucilage. *The Plant Cell*, *33*(2), pp.381-403.
4. Bharadwaj,V.S., Westawker, L.P., Crowley, M..F. (2022) Towards Elucidating Structure–Spectra Relationships in Rhamnogalacturonan II: Computational Protocols for Accurate 13C and 1H Shifts for Apiose and Its Borate Esters. *Front Mol Biosci*. 8: 756219.
5. Thorne, K., Urbanowicz, B..R.*, Hahn, M.G* "Plant Cell Wall Glycan-Directed Monoclonal Antibodies" (2023). *In: Geitmann A (ed) Plant Cell Walls – Research Milestones and Conceptual Insights.* CRC Press, Taylor & Francis Group
6. Koj, S.; Bu, L.; Crowley, M. F.; Sharma, D.; Urbanowicz, B. R.; O'Neill, M. A.; Pena, M. J.; Bharadwaj, V. S., (2024) Probing Sequence-Structure Paradigms in Complex Carbohydrates – A Case Study on Rhamnogalacturonan-II (Under Consideration at *PNAS*)
7. Zhang, Y., Sharma, D., Liang, Y., Downs, N., Dolman, F., Thorne, K., Pereira, J. H., Adams, P., Scheller, H. V., O'Neill, M., Urbanowicz, B.R.*, & Mortimer, J. C.* (2024) Putative rhamnogalacturonan-II glycosyltransferase identified through callus gene editing which bypasses embryo lethality. *Plant Physiology*, kiae259, DOI: 10.1093/plphys/kiae259

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

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Overall research goals:

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

Significant achievements: [2023-2024]:

We are utilizing solid-state NMR (ssNMR) to investigate ^{13}C-labeled Arabidopsis inflorescence stems in collaboration with Dr. Daniel J. Cosgrove. Our research focuses on compositional changes in lignin and carbohydrates and their physical interactions across the stems, including two lignin mutants provided by Dr. Chang-Jun Liu. A comprehensive dataset has been collected and analyzed by postdoc Peng Xiao, and manuscript preparation is underway. Our findings reveal that lignin-carbohydrate packing is significantly influenced by lignin's chemical structure, composition, and plant developmental stages. This is also the first time for us to observe direct lignin-pectin interactions in younger stem tissues using ssNMR approach, shedding light on the early-stage lignification process.

Additionally, Peng Xiao is analyzing eight ^{13}C-labeled pine and spruce samples (leaf, root, needle, and stem) to investigate glucomannan structure and its role in stabilizing the lignin-carbohydrate interface within secondary cell walls of softwoods.

In collaboration with Dr. Jamie Barros-Rios, we are characterizing Brachypodium samples (wild-type and a lignin mutant) labeled with ^{13}C using glucose, tyrosine, and phenylalanine. Led by graduate student Priya Sahu, this work aims to provide novel insights into lignin biosynthesis in grasses.

We are also exploring new collaborations, including studies on RG-II structure with Drs. Breeanna Urbanowicz and Malcolm O'Neill, and optimizing low-cost ^{13}C-labeling protocols for ssNMR characterization with Dr. Ahmed Faik. Graduate student Debkumar Debnath leads these projects, with one manuscript submitted for the low-cost ^{13}C-labeling protocol, and promising NMR data collected on RG-II revealing the impact of boron chelation on cell wall structure.

Technically, we are exploring three technical frontiers. First, we are optimizing Dynamic Nuclear Polarization (DNP) for rapid screening of unlabeled lignocellulosic samples, achieving success with

single-hour analysis time as recently demonstrated on cordgrass and the soil material derived from these plants. This is also the first time where we succeeded in examining lignin-polysaccharide interactions using unlabeled samples. A manuscript is under review and available on ChemRxiv (DOI: 10.26434/chemrxiv-2023-p4rvg). The technical capability has clearly been improving over the past few years but there is still a long way to go; we hope such improvement can eventually allow us to examine plant materials without the need for isotopic enrichment, thus benefiting our plant research community. Second, in addition to the ^{13}C that we heavily rely on for plant analysis, we are now exploring the use of ^{1}H for such purpose in the solid-state, which is a nucleus more sensitive to structural changes of carbohydrates and lignin. Third, we are also extending these techniques to other photosynthetic organisms, including green microalgae.

Science objectives for 2024-2025:

First, we would like to wrap up three current projects on Arabidopsis inflorescence, pine and spruce, and Brachypodium to identify the universal physical principles that regulate lignin-carbohydrate interactions in plant secondary cell walls across plant species. This should result in three major manuscripts, which will be submitted for peer review. Second, we would like to start to develop methods to search for lignin-carbohydrate interactions *in muro* using DNP and proton-detection approaches.

My scientific area(s) of expertise is/are: Solid-state NMR and DNP analysis of biomolecules (carbohydrate, lipid, protein, lignin) for understanding their structure, dynamics, and interactions.

The ideal collaborator for my project would have expertise in: plant biology, modeling, natural and synthetic polymers, microbiology, algae.

Publications supported by this project [2023-2026]:

1. Fernando, L, D.; Zhao, W.; Gautam, I.; Ankur, A.; Wang, T. Polysaccharide assemblies in fungal and plant cell walls explored by solid-state NMR. Structure **31** 1375-1385 (2023).
2. Poulhazan, A.; Arnold, A.A.; Mentink-Vigier, F.; Muszynski, A.; Azadi, P.; Halim, A.; Vakhrushev, S.Y.; Joshi, H.J.; Wang, T.*; Warschawski, D.E.*; Marcotte, I.* Molecular-level architecture of Chlamydomonas reinhardtii's glycoprotein-rich cell wall. Nature Communications **15**, 986 (2024)

Structure, Biochemistry, and Physiological Roles of Multiheme Cytochrome Nanowires

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Overall research goals:

Our research aims to elucidate structure-function-physiology relationships of novel bacterial cytochrome structures from and beyond the model organism *Geobacter sulfurreducens*. This will involve defining the nanowire electron transport pathway, comparing biochemical and electron transport properties of multiheme cytochrome nanowires, and developing pipelines for identifying novel multiheme cytochrome nanowires across different bacterial species.

Significant achievements: [2023-]:

The Wang lab focused on identifying novel conductive nanowires from environmental bacteria using bioinformatics and AlphaFold3. We also cultured various bacteria and made exciting discoveries about extracellular cytochrome nanowires and flagellar filaments. In addition, we studied cyanobacteria's extracellular filaments and found they are not conductive. Finally, we examined various cytochrome nanowires under cryo-EM and identified novel types of nanowires.

Science objectives for 2024-2025:

As originally proposed, during the next reporting period, our lab will perform bioinformatic analysis on bacterial cytochromes to identify putative extracellular nanowires, characterize targets identified through cryo-EM studies, and prepare for cryo-ET studies on extracellular nanowires already identified.

My scientific area(s) of expertise is/are: Structure Biology, Cryo-EM, helical filament.

The ideal collaborator for my project would have expertise in: Microbiology.

Publications supported by this project 2022-2024 (co-PI, since 2023):

1. P- Structural diversity and clustering of bacterial flagellar outer domains (2024)
2. P- Microbial Nanowires: type IV pili or cytochrome filaments? (2023)
3. P- Electron transfer beyond the outer membrane: putting electrons to rest (2023)
4. P- Cryo-EM structure of an extracellular Geobacter OmcE cytochrome filament reveals tetrahaem packing (2022)
5. P- Structure of Geobacter OmcZ filaments suggest extracellular cytochromes polymers evolved independently multiple times (2022)

Elucidating the Biochemical Mechanisms Controlling Secondary Wall Biosynthesis in Plants

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Overall research goals:

The major goal of this 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. Depending on plant species, xylan side chains may include 2-*O*- and/or 3-*O*-linked arabinofuranose (Ara*f*), 3-*O*-linked Ara*f* substituted at *O*-2 with another Ara*f* [Ara*f*-(1->2)-Ara*f*] or Xyl [Xyl-(1->2)-Ara*f*], and 2-*O*-linked glucuronic acid (GlcA)/4-*O*-methylglucuronic acid (MeGlcA) residues. The biochemical mechanisms controlling the substitutions of xylan with these various side chains 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, the results of which will shed light on the biochemical mechanisms controlling cell wall biosynthesis in general.

Significant achievements: [2022-2025]:

1. Uncovering glycosyltransferases responsible for 2-*O*-arabinopyranose (Ara*p*) or 2-*O*-galactopyranose (Gal) substitutions of GlcA/MeGlcA side chains of xylan. Xylan in dicots is often decorated with GlcA/MeGlcA side chains, which may be further substituted at *O*-2 with arabinopyranose (Ara*p*) or galactopyranose (Gal) residues in some plant species. We have discovered that a subclade of glycosyltransferases (XAPTs) residing in the MUR3 clade of family GT47 are 2-*O*-arabinopyranosyltransferases and 2-*O*-galactosyltransferases catalyzing 2-*O*-Ara*p* and 2-*O*-Gal substitutions of xylan GlcA side chains, which expands our understanding of glycosyltransferases involved in xylan substitutions.

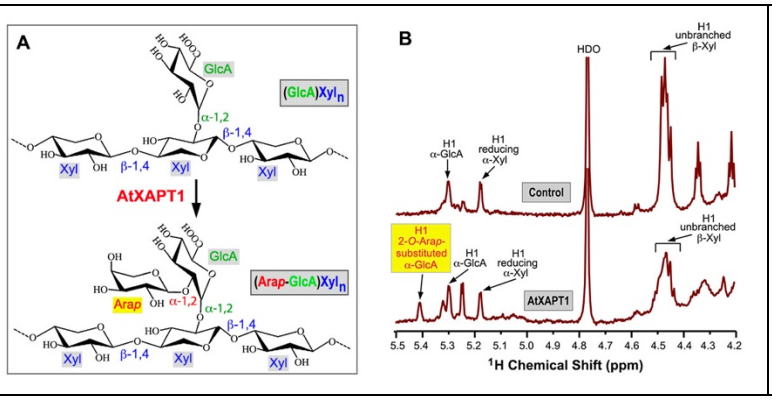

Fig. 1 An Arabidopsis MUR3-clade GT47 member catalyzes 2-*O*-Ara*p* transfer onto GlcA side chains of xylan.

(A) Diagram of the structural units of the acceptor and the reaction product of AtXAPT1. Note that AtXAPT1 catalyzes the addition of Ara*p* onto *O*-2 of GlcA side chains of xylan.

(B) ^{1}H NMR spectra of the reaction products of AtXAPT1 incubated with (GlcA)Xyl$_6$ and UDP-Ara*p*. Note that the reaction products of AtXAPT1 exhibited a new resonance peak at 5.41 ppm corresponding to GlcA substituted at *O*-2 with Ara*p*.

2. We found that rice xylan synthases required short xylooligomers as acceptors for their activities and that the intrinsic biochemical properties of xylan glucuronyltransferases and arabinosyltransferases contribute to the distinctive substitution pattern of rice xylan. Our findings provide new insights into the biochemical mechanisms underlying xylan backbone elongation and substitutions in grass species.
3. We carried out biochemical studies of two rice GT61 clade B members and found that one of them was capable of transferring both Xyl and Ara*f* residues from UDP-Xyl and UDP-Ara*f*, respectively, onto xylan, whereas the other specifically catalyzed Xyl transfer onto xylan, which expands our knowledge of roles of the GT61 family in grass xylan synthesis.

4. The reducing end of xylan from gymnosperms and dicots contains a unique tetrasaccharide sequence consisting of β-D-Xyl*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*A-(1→4)-D-Xyl*p*, the synthesis of which requires four different glycosyltransferase activities. We have discovered a member of family GT106 as a new player in the synthesis of the unique reducing end sequence of xylan.
5. We biochemically characterized a suite of Arabidopsis DUF231/TBL proteins for their roles in pectin acetylation and identified 10 of them as acetyltransferases involved in the acetylation of the pectin polysaccharides HG and RG-I, which deepens our understanding of the biochemical mechanisms controlling the acetylation of plant cell wall polysaccharides.
6. Family GT61 glycosyltransferases in gymnosperms are phylogenetically separated from the grass-expanded GT61 members that are known to mediate xylan substitutions. We have demonstrated that gymnosperm GT61 members exhibit various glycosyltransferase activities toward xylan, including xylan 3-*O*-arabinosyltransferases, 2-*O*-arabinosyltransferases and 2-*O*-xylosyltransferases. Our findings provide new insight into the involvement of family GT61 glycosyltransferases in xylan substitutions in gymnosperms.
7. We have discovered that a group of grass GT61 members are novel xylosyltransferases catalyzing the addition of Xyl onto *O*-2 of Ara*f* side chains of xylan to form the Xyl-Ara*f* disaccharide side chains, which enriches our understanding of genes involved in xylan biosynthesis.

Science objectives for 2024-2025:

We are currently in the process of biochemical characterization of many un-characterized GT61 members for their roles in xylan substitutions and deciphering how nucleotide sugar donor specificity is determined. We will also continue our efforts on identification and biochemical characterization of additional glycosyltransferases involved in xylan synthesis. These research objectives will further our understanding of glycosyltransferases responsible for the synthesis of xylan, the second most abundant polysaccharide in plant biomass.

My scientific area(s) of expertise is/are: Biochemistry and molecular biology

The ideal collaborator for my project would have expertise in: Protein and carbohydrate structures

Publications supported by this project [2022-2025]:

1. Zhong, R., Zhou, D., Phillips, D.R., Adams, E.R., Chen, L., Rose, J.P., Wang, B.-C. and Ye, Z.-H. (2024). Plant cell wall polysaccharide *O*-acetyltransferases. Plants 13, 2304.
2. Zhong, R., Zhou, D., Phillips, D.R., Adams, E.R., Chen, L., Rose, J.P., Wang, B.-C. and Ye, Z.-H. (2024). Identification of glycosyltransferases mediating 2-*O*-arabinopyranosyl and 2-*O*-galactosyl substitutions of glucuronosyl side chains of xylan. Plant J. DOI:10.1111/tpj.16983
3. Haghighat, M., Zhong, R., and Ye, Z.-H. (2024). WUSCHEL-RELATED HOMEOBOX genes are critical for normal vascular tissue organization and wood formation in poplar. Plant Sci. 346, 112138.
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5. Zhong, R., Zhou, D., Phillips, D.R., Adams, E.R., Chen, L., Rose, J.P., Wang, B.-C. and Ye, Z.-H. (2024). A rice GT61 glycosyltransferase possesses dual activities mediating 2-*O*-xylosyl and 2-*O*-arabinosyl substitutions of xylan. Planta 259, 115
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Exploring the Role of TOR kinase in the Regulation of Central Metabolism and Lipid Synthesis

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Overall research goals:

The overall research goals of this research program are to understand how carbon signals interact with and modulate the activity of plant master metabolic regulator Target of Rapamycin (TOR), and how activated TOR regulates lipid synthesis.

Significant achievements for 2022-2024:

So far, we have discovered that TOR signaling primarily functions in promoting lipid synthesis. We have also discovered that trehalose 6-phosphate (T6P), a proxy signal molecule of sucrose availability, is a potent direct activator of TOR.

Science objectives for 2024-2025:

1. We are carrying out immunoprecipitation of TOR to screen for TOR interacting proteins during TOR activation.
2. We try to produce and purify recombinant TOR with protein expression system in mammalian cells
3. Structure of T6P-TOR complex structure will be resolved by cryo-EM.

My scientific area(s) of expertise is/are: Pant molecular biology/genetics, plant primary metabolism (sugar and lipid metabolism), signaling transduction, protein structural biology.

The ideal collaborator for my project would have expertise in: Plant signaling transduction, protein structural biology.

Publications supported by this project 2022-2024:

1. Molecular mechanism of trehalose 6-phosphate inhibition of the plant metabolic sensor kinase SnRK1. Blanford JK1, **Zhai Z**1, Baer M1, Guo G, Liu H, Liu Q, Raugei S, Shanklin J. ***Science Advances***. 2024 May 17. doi: 10.1126/sciadv.adn0895. 1, Contributed equally
2. GRIK phosphorylates and activates KIN10 which also promotes its degradation. Sun J, Liu H, Blanford JK, Cai Y, **Zhai Z***, Shanklin J*. ***Frontiers in Plant Science***. 2024 Mar 25;15:1375471. doi: 10.3389/fpls.2024.1375471. *, Corresponding author
3. Target of Rapamycin kinase is a positive regulator of plant fatty acid and lipid synthesis.
   Liu H, Blanford J, Shi H, Schwender J, Shanklin J*, Zhai Z*. **Plant Physiology**. 2024. *, Corresponding author. Submitted
4. Trehalose-6-phosphate is an activator of TARGET OF RAPAMYCIN in plant.
   Liu H, Blanford J, Anaokar S, Shi H, Schwender J, Shanklin J*, Zhai Z*. **Science**. 2024. *, Corresponding author. Submitting