Meeting Book for the Photosynthetic Systems Principal Investigators Meeting

Hilton Washington DC/Rockville Hotel & Executive Meeting Ctr, December 5 to 7, 2023

Foreword

This meeting book is a record of the biennial meeting of the principal investigators funded by Photosynthetic Systems, 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 natural photosynthesis, a process that exhibits great structural and chemical diversity across the biological world but that uniformly captures, converts, and stores solar energy with unmatched efficiency. The high caliber of the research in these abstracts reflects the talent, dedication, and industry of the principal investigators who make Photosynthetic Systems the vibrant and innovative science program that it is.

The purpose of the meeting is 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 Paul Hudson 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.

Stephen K. Herbert, Program Manager, Photosynthetic Systems, DOE BES
Katherine Brown, Program Manager, Physical Biosciences
B. Gail Mclean, Team Lead, Photochemistry and Biochemistry Team, DOE BES

Acknowledgement and Disclaimer

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2023 Photosynthetic Systems Principal Investigators Meeting

Hilton Washington DC/Rockville Hotel & Executive Meeting Ctr, December 5 – 7, 2023

AGENDA

Tuesday, December 5

7:30 – 8:45AM	*Continental Breakfast*
8:45 – 9:00AM	Welcome **Steve Herbert,** Program Manager, Photosynthetic Systems
	Section I: *Light Harvesting* ***Moderator:*** *Jennifer Ogilvie, University of Michigan*
09:00 –9:30AM	Quantum light studies of photosynthetic light harvesting at the single photon level **Birgitta Whaley**, University of California, Berkeley
09:30 – 10:00AM	Spectroscopic studies of protein-protein association in model membranes **Gabriela Schlau-Cohen**, Massachusetts Institute of Technology
10:00 – 10:30AM	Elucidating photoinduced processes of Photosystem I via multi-dimensional electronic and vibrational spectroscopies **Jessica Anna**, University of Pittsburgh
10:30 – 11:00AM	*Coffee Break*
11:00 – 11:30AM	Multidisciplinary tools for examining molecular details of photosynthetic light harvesting systems **Matt Francis**, Lawrence Berkeley National Lab
11:30 – 12:10PM	Regulation of photosynthesis **Masa Iwai, Graham Fleming, Kris Niyogi**, Lawrence Berkeley National Lab
	Section II: *Electron and Proton Transport* ***Moderator:*** *Christine Kirmaier, Washington University*
12:10 – 12:40PM	Structure-function studies of Photosystem II **Gary Brudvig**, Yale University
12:40 – 03:00PM	*Working Lunch and Afternoon Break*

03:00 – 3:30PM Metal ligands and hydrogen bonding networks of the O_2-evolving complex in Photosystem II
Rick Debus, University of California, Riverside

03:30 – 04:00PM Studies of reaction centers and biomimetic systems
Marilyn Gunner, City University of New York

04:00 –04:30PM Energy conversion in photosynthesis: capturing the sequence of events during the S_3 to S_0 transition in Photosystem II
Junko Yano, Lawrence Berkeley National Lab

04:30 – 05:00PM Structural dynamics in photosynthetic reaction centers
Jan Kern, Lawrence Berkeley National Lab

05:00 – 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.)

Wednesday, December 6

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

Section II: *Electron Transport, continued*
Moderator: David Mulder*, National Renewable Energy Lab*

08:30 – 09:00AM The Type I homodimeric reaction center in *Heliobacterium modesticaldum*
Kevin Redding, Arizona State University

09:00 – 9:30AM Dynamic molecular recognition underlying protein electron transfer studied by residue-specific vibrational spectroscopy
Megan Thielges, Indiana University

9:30 – 10:00AM Dynamics and consequences of PS I supercomplexes
Rachael Morgan-Kiss, Miami University of Ohio

10:00 – 10:30AM Modulation of PS I oligomeric form, spectral capacity, and electron flux in *Synechocystis* sp. PCC 6803
Cara Lubner, National Renewable Energy Lab

10:30 – 11:00AM *Coffee Break*

11:00 – 11:30AM Fundamental mechanisms for solar energy conversion in photosynthesis
Lisa Utschig, Argonne National Lab

11:30 – 12:00PM Fundamental research aimed at diverting excess reducing power in photosynthesis to orthogonal metabolic pathways
Alexey Silakov, Pennsylvania State University

12:00 – 12:30PM Photosynthetic energy capture, conversion, and storage: from fundamental mechanisms to modular engineering
Josh Vermaas, Plant Research Laboratory, Michigan State University.

12:30 – 03:00PM *Working Lunch and Afternoon Break*

Section III: *CO_2 capture and Fe management*
Moderator: *Asaph Cousins, Washington State University*

03:00 – 03:30PM Structure and function of cyanobacterial CO_2 uptake NDH-1 complexes
Rob Burnap, Oklahoma State University

03:30 – 04:00PM Characterizing Rubisco by phylogeny-informed mutagenesis
Maureen Hanson, Cornell University

04:00 – 04:30PM Exploring the functions of chloroplast triose phosphate transporters and other plastid activities in partitioning photoassimilate in *Chlamydomonas*
Matt Posewitz, Colorado School of Mines

04:30 – 05:00PM Molecular genetics of chloroplast Fe homeostasis
Sabeeha Merchant, University of California, Berkeley

05:00 – 07:30PM *Dinner on your own*

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.)

Thursday, December 7

7:30 – 8:30AM	*Continental Breakfast*

Section III: *Structural dynamics*
Moderator: *Rebecca Roston, University of Nebraska, Lincoln*

08:30 –9:00AM	Assembly and repair of Photosystem II, a membrane protein complex **Himadri Pakrasi**, Washington University
09:00 – 09:30AM	Unraveling the disassembly mechanisms of Photosystem II **Sujith Puthiyaveetil**, Purdue University
09:30 – 10:00AM	Control of linear electron transport by thylakoid membrane dynamics **Helmut Kirchhoff**, Washington State University
10:00 – 10:30AM	Signal transduction pathways for chloroplast quality control **Jesse Woodson**, Arizona State University
10:30 – 11:10AM	Diversification and function of bilin chromophores in oxygenic photosynthesis **Nathan Rockwell, Clark Lagarias**, University of California, Davis
11:10 – 11:30AM	*Coffee Break*
11:30 – 12:30PM	Photosynthetic Systems Program Update **Steve Herbert, Kate Brown,** DOE Program Managers **Gail McLean,** CSGB Division Director
12:30PM	*Meeting Ends*

Posters

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

1. Photosynthetic energy capture, conversion, and storage: from fundamental mechanisms to modular engineering
   Christoph Benning, Michigan State University

2. Structural and mechanistic studies of O_2-dependent and O_2-independent enzymes in chlorophyll biosynthesis
   Jennifer Bridwell-Rabb, University of Michigan

3. Spectroscopic studies of a Photosystem I hydrogenase chimera
   David Britt, University of California, Davis

4. Cryogenic electron tomography investigation of the structural dynamics of PS I supercomplexes in *Chlamydomonas priscuii*
   Wah Chiu, SLAC National Accelerator Laboratory

5. Photosynthetic reduction of carbon dioxide: Kinetic tradeoffs for Rubisco and phosphoenolpyruvate carboxylase
   Asaph Cousins, Washington State University

6. Probing enzymatic catalysis of carbon-carbon bond formation with photoexcited semiconductor nanocrystals
   Gordana Dukovic, University of Colorado, Boulder

7. Coherent dynamics for regulation of dissipation and energy transfer in photosynthetic systems (This poster will be presented Wednesday evening)
   Greg Engel, University of Chicago

8. Dynamics and consequences of PS I supercomplexes
   Petra Fromme, Arizona State University

9. Harnessing nature's solutions for enhanced biological CO_2 fixation
   Laura Gunn, Cornell University

10. Solar energy conversion in Photosystem I studied using time-resolved visible and infrared difference spectroscopy
    Gary Hastings, Georgia State University

11. Toward *in vivo* site-directed mutagenesis of LHCII functional domains in *Arabidopsis thaliana*
    Masa Iwai, Lawrence Berkeley National Lab

12. Characterization of a newly discovered phycobilisome linker protein
Cheryl Kerfeld, Michigan State University

13. Controlling electron transfer pathways in photosynthetic reaction centers
Christine Kirmaier, Washington University

14. Mechanism and control of cytochrome c biosynthesis
Robert Kranz, Washington University

15. (This poster combined with poster 13.)

16. Elucidating the principles that control electron and proton-coupled electron transfer in Photosystem II and Photosystem I
K. V. Lakshmi, Rensselaer Polytechnic Institute

17. Molecular mechanism of action of the cyanobacterial orange carotenoid protein and phycobilisome
Haijun Liu, St. Louis University

18. Optimizing photosystem performance through a bioengineered red sites library
Yuval Mazor, Arizona State University

19. Tuning of electron-transfer and catalysis in photosynthetic energy conversion
David Mulder, National Renewable Energy Lab

20. Modular synthesis of biohybrid assemblies for studying photosynthetic mechanisms and solar fuels catalysts
Karen Mulfort, Argonne National Lab

21. Multidimensional spectroscopies for probing coherence and charge separation in photosynthetic reaction centers
Jennifer Ogilvie, University of Michigan

22. Revealing nature's optimized quantum spin coherences in photosynthetic proteins
Oleg Poluektov, Argonne National Laboratory

23. Trafficking of metabolites and reductant between the chloroplast and other subcellular compartments
Matt Posewitz, Colorado School of Mines

24. Structural tuning of photosynthetic light harvesting
Michael Reppert, Purdue University

25. Photosynthetic membrane lipid transport through chloroplast membrane contact site homologs
Rebecca Roston, University of Nebraska, Lincoln

26. Intelligent mutagenesis of photosynthetic proteins: programming the function
Sergei Savikhin, Purdue University

27. A molecular thermostat for post-translational regulation of light harvesting complex biogenesis
Shu-ou Shan, Cal Tech

28. Energy conversion in photosynthesis: capturing the sequence of events during the S_3 to S_0 transition in Photosystem II
Phillipp Simon, Lawrence Berkeley National Laboratory

29. Photosystem I responses to differential electron flux in *Synechocystis* sp. PCC 6803
Sharon Smolinski, National Renewable Energy Laboratory

30. Probing limitations to C_4 carbon capture
David Stern, Boyce Thompson Institute

31. Mechanisms of protein transport across the chloroplast thylakoid membrane
Steven Theg, University of California, Davis

32. Assembly and Repair of the Photosystem II Reaction Center
David Vinyard, Louisiana State University

33. The green alga *Auxenochlorella protothecoides* as a chassis to study molecular players of non-photochemical quenching
Setsuko Wakao, Lawrence Berkeley National Lab

34. Electronic structure of the Mn cluster in Photosystem II using an XFEL
Vittal Yachandra, Lawrence Berkeley National Lab

35. Dynamics and consequences of PS I supercomplexes
Ru Zhang, Donald Danforth Center, St. Louis

Elucidating Photoinduced Processes of Photosystem I Via Multidimensional Electronic and Vibrational Spectroscopies

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Overall research goals:

The overarching goal of this project is to elucidate the mechanism of energy and electron transfer that governs the efficient charge separation in cyanobacterial photosystem I (PSI) complexes by applying ultrafast multidimensional spectroscopies in the visible and mid-IR spectral regions. Using multidimensional spectroscopies in the visible and mid-IR spectral regions we will alleviate spectral congestion allowing for a more direct determination of energy transfer pathways and ultrafast electron transfer processes. Focusing on different PSI complexes, including Chl f containing PSI complexes, PSI complexes with reaction center mutations, and WT PSI complexes, we are able to investigate different aspects of energy transfer and charge separation. We are making progress towards the overarching goal through three objectives: (1) mapping energy transfer pathways and timescales in FRL-PSI and engineered Chl f containing PSI complexes; (2) resolving the excitonic nature and vibrational motion of the red shifted Chl states; and (3) probing charge separation in PSI complexes through ultrafast spectroscopies using mid-IR vibrational probes.

Significant achievements (2021-2023):

Probing Charge Transfer in Photosystem I Complexes with Two-Dimensional Electronic Vibrational Spectroscopy: We have obtained two-dimensional electronic vibrational (2DEV) spectra of PSI complexes isolated from PCC 6803 (Figure 1 a). The 2DEV spectra resolve a chlorophyll cation band in the 1710-1730 cm^{-1} region. The cation band is elongated along the visible excitation axis at early t_2 waiting times, with amplitude in the Red Chl and reaction center Chl region. As the waiting time increases the cation band shifts towards the higher energy Bulk Chls. We interpreted the 2DEV spectra through extracting 2D Decay Associated Spectra (2D-DAS), and comparing the 2D-DAS from the 2DEV to that of the 2D-DAS from the 2DES spectra. We find that the Red Chls have a cationic contribution to their initial excited electronic states and the Red Chls transfer energy uphill to the Bulk Chls on the 3 ps timescale.

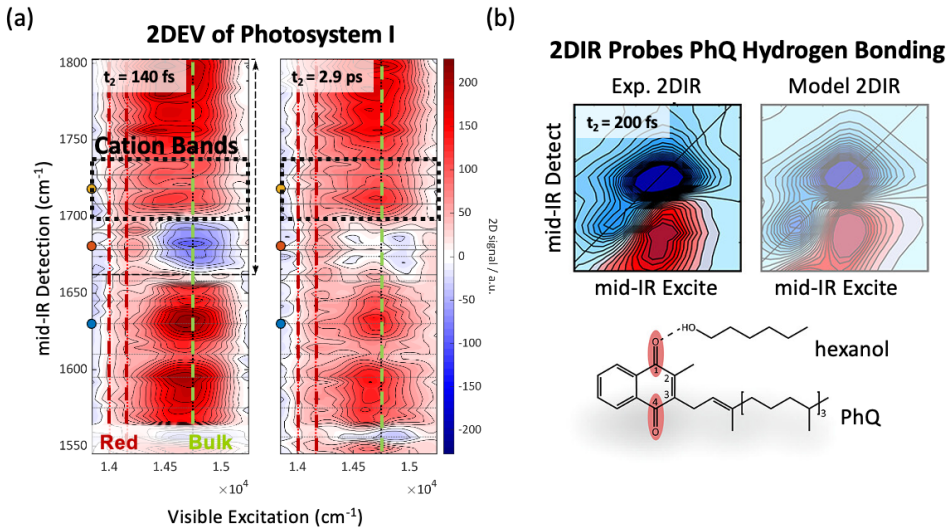

Figure 1: (a) Representative 2DEV spectra of PSI from PCC 6803. The cation bands lie within the dashed rectangles. (b) Early time experimental and simulated 2DIR of phylloquinone dissolved in hexanol.

Resolving the Impact of Hydrogen Bonding on Phylloquinone with Two-Dimensional Infrared Spectroscopy (2DIR): We have obtained 2DIR spectra of phylloquinone (PhQ) in hexanol, a hydrogen bonding solvent (Figure 1b). We found that hydrogen bonding acts to decouple the carbonyl groups of PhQ, leading to two peaks in the 2DIR spectra lying along the diagonal. At later waiting times we see a growth in the cross peak region arising from the making/breaking of hydrogen bonds. We interpreted the 2DIR results through MD simulations, DFT calculations, and spectral modeling. The results of our studies demonstrate how hydrogen bonding to PhQ presents in 2D vibrational spectroscopies and could help to interpret 2DEV, 2DIR, and T-2DIR measurements performed on PSI complexes.

Mapping Energy Transfer in Chl f Containing PSI Complexes: We applied two-dimensional electronic spectroscopy (2DES) to far red light PSI complexes (FRL-PSI) and white light PSI (WL-PSI) isolated from PCC 7521. With our current experimental setup we can probe the 750 nm Chl f states of the FRL-PSI complexes. We observed downhill energy transfer from the Bulk Chls to the 750 nm Chl f states as a growth in the cross peak region of the 2DES spectra. To further characterize the temporal evolution of the 2DES we applied a global analysis to the spectra. The 2D-DAS of the WL-PSI complex was consistent with our previously published work on WT PSI complexes from PCC 6803 and PCC 7002. The 2D-DAS of the FRL-PSI complexes resolve an additional 2D-DAS component that evolves with a 50 ps timescale. The position of the peak in the 2D-DAS indicates a decay of the 750 nm Chl f states, which we attribute to an additional trapping occurring on a longer timescale.

Science priorities for the next year (2023-2024):

- We will submit a manuscript to report the 2DEV spectra of photosystem I complexes and our finding that the initial Red Chl states have charge transfer character.
- We will obtain 2DEV spectra of PSI complexes with opened reaction centers. Our initial 2DEV studies were performed on PSI with closed reaction centers (P_{700} pre-oxidized to P_{700}^{+}). By investigating PSI complexes with opened reaction centers we will focus on characterizing electron transfer among the reaction center chlorophylls and the phylloquinone molecules.
- We will retake the 2DES of FRL-PSI complexes scanning to longer time delays to further resolve the electronic energy transfer processes involving the 750 nm Chl f state of FRL-PSI. We will then draft a manuscript to report the 2DES and 2D-DAS of the FRL-PSI complexes.

My major scientific area(s) of expertise is/are: My group has expertise using ultrafast multidimensional spectroscopies including two-dimensional electronic spectroscopy (2DES), two-dimensional infrared spectroscopy (2DIR), and two-dimensional electronic vibrational spectroscopy (2DEV) to study electronic energy transfer, electron transfer and charge transfer, and ultrafast dynamics of photosystem I complexes and isolated cofactors.

To take my project to the next level, my ideal collaborator would have expertise in: My ideal collaborator would have expertise in simulating electronic energy transfer and charge separation in photosystem I complexes where the charge transfer character of the red-shifted states can be incorporated into the model.

Publications supported by this project:

1. J. Lu, Y. Lee, J. M. Anna, "Extracting the Frequency-Dependent Dynamic Stokes Shift from Two-Dimensional Electronic Spectra with Prominent Vibrational Coherences", *J. Phys. Chem. B*, 2020, 124, 8857−8867. https://doi.org/10.1021/acs.jpcb.0c05522
2. P. Askelson, S. L. Meloni, A. M. Hoffnagle, J. M. Anna, "Resolving the Impact of Hydrogen Bonding on the Phylloquinone Cofactor through Two-Dimensional Infrared Spectroscopy" *J. Phys. Chem. B*, 2022, 126, 10120-10135. https://doi.org/10.1021/acs.jpcb.2c03556
3. J. D. Shipp, C. Li, M. Gorka, J. H. Golbeck, J. M. Anna, "Two-Dimensional Electronic Vibrational Spectroscopy Reveals Red Chlorophyll Chage Transfer State in Cynaobacterial Photosystem I" (manuscript in preparation)

Energy Transfer and Radiationless Decay in Light-Harvesting Proteins

Warren F. Beck, Principal Investigator

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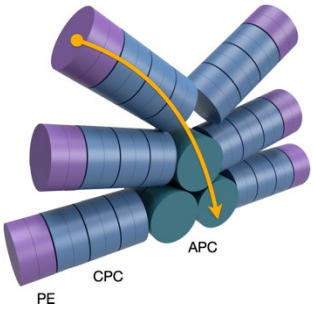

Overall research goals: This project seeks to determine the photophysical and structural mechanisms that mediate excitation energy transfer and photoprotection in photosynthetic light harvesting complexes. We are focusing in the current term on the mechanistic and dynamic importance of out-of-plane conformational and vibrational motions of bilin chromophores in the cyanobacterial phycobilisome.

Significant achievements in 2021–2023:

- Broadband multidimensional electronic spectroscopy (2DES/3DES) studies of excitation energy transfer mechanisms in intact phycobilisomes of *Fremyella diplosiphon*, with detection of vibronic coherences in the rods and dynamic localization processes in the core segments.
- Characterization of excitation energy trapping processes in complexes of the orange carotenoid protein (OCP) with intact phycobilisomes of *Synechocystis* sp. PCC 6803 using broadband 2DES/3DES measurements.
- Broadband 2DES/3DES measurements of exciton relaxation and dynamic exciton localization processes in trimers of allophycocyanin from *Arthrospira platensis*.

Science objectives for 2023–2025:

- 2DES/3DES studies of vibrational and vibronic coherences in switching and nonswitching cyanobacteriochromes
- Studies of excitation energy transfer in complexes of the phycobilisome with the red carotenoid protein (RCP), the N-terminal domain of OCP

References to work supported by this project 2021-2023:

Sil, S.; Tilluck, R. W.; Mohan T M, N.; Leslie, C. H.; Rose, J. B.; Domínguez-Martín, M. A.; Lou, W.; Kerfeld, C. A.; Beck, W. F. Excitation Energy Transfer and Vibronic Coherence in Intact Phycobilisomes. *Nat. Chem.* **2022**, *14*, 1286–1294, DOI: 10.1038/s41557-022-01026-8.

Tilluck, R. W.; Ghosh, S.; Guberman-Pfeffer, M. J.; Roscioli, J. D.; Gurchiek, J. K.; LaFountain, A. M.; Frank, H. A.; Gascón, J. A.; Beck, W. F. Interexciton nonradiative relaxation pathways in the peridinin–chlorophyll protein. *Cell Reports Phys. Sci.* **2021**, *2*, 100380, DOI: 10.1016/j.xcrp.2021.100380.

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

Christoph Benning, Principal Investigator
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Overall research goals:

The Plant Research Laboratory brings together multiple labs as part of a larger collaborative effort to quantify and develop actionable models around light capture, downstream energy conversion, and storage in photosynthetic organisms. For organizational efficiency, the research is grouped into three themes. The first theme studies 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 is to integrate photosynthetic processes into a cellular context, such as managing photorespiration and carbon fluxes throughout metabolism.

Since this is a larger collaboration, the focus here is on the contribution by Christoph Benning. The Benning lab uses biochemistry, molecular biology, cell biology, and genetics to study the assembly of thylakoid membrane lipids and their role in electron transport processes. Non-BES work in the laboratory includes exploration lipid based abiotic stress signaling and the role of algal symbiont lipids in coral bleaching.

Significant achievements (2023-2026):

In plants, much of the photosynthetic infrastructure is housed in the densely packed membranes of the chloroplast, and the lipids that constitute them account for a majority of plant membrane lipids. The primary chloroplast glycolipid, monogalactosyldiacylglycerol (MGDG), is the product of diacylglycerol (DAG) galactosylation by the enzyme MGD1 at the intermembrane-facing leaflet of the chloroplast inner envelope. DAG is derived from the dephosphorylation of phosphatidic acid (PA), which in Arabidopsis was presumed to occur primarily at the inner envelope. PA is synthesized at both the chloroplast stroma and the endoplasmic reticulum (ER), and the points at which the two PA pools are dephosphorylated and converge to MGD1 remain unclear.
We have shown that:

- The chloroplast PA phosphatases LPPγ and LPPε1 are likely associated with the outer envelope membrane.
- In addition, the lppγ lppε1 double mutant retains the full capacity for conversion of plastid PA into MGDG, but is deficient in the conversion of phosphatidylcholine into MGDG, indicating that these outer envelope PA phosphatases are predominantly acting on ER-derived PA.
- Although LPPε2 is imported into interior chloroplast membranes, the lppε2 mutant is unaffected in the plastid pathway of MGDG biosynthesis.
- Together, these results have implications for the study of the *rbl10* mutant, which lacks an annotated rhomboid-like protease, and converts plastid PA to MGDG at a lower rate despite retaining PA phosphatase activity in mixed envelope fractions.
- Additionally, the *lppγ lppε1* double mutant exhibits growth defects and light sensitivity. Therefore, it is likely that PA or DAG may have a physiological role in the chloroplast outer envelope.

Science priorities for the next year (2023-2024):

- Potential metabolic roles and mechanisms of RBL10, LPPγ, LPPε1, and LPPε2 are currently being elucidated through truncation mutants, enzymatic assays, and genetic approaches including complementation studies and crosses to well-characterized lipid mutants.
- To investigate the role of PA and DAG as signaling components, a suppressor screen is currently underway in the lppγ lppε1 double mutant background.

My major scientific area(s) of expertise is/are: Plant Biochemistry, lipid metabolism in plants and algae, lipid analytics, plant and algal genetics.

To take my project to the next level, my ideal collaborator would have expertise in: structure determination, in particular for the complexes involved in photosynthesis and energy transfer.

Structural and Mechanistic Studies of O_2-dependent and O_2-independent Enzymes in Chlorophyll Biosynthesis

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Overall research goals:

The goals of this research proposal are to elucidate how photosynthetic organisms perform biosynthetic processes under variable amounts of light and O_2, both of which profoundly affect their growth and survival. Our approach is to study the structure-function relationships in the metalloenzymes that catalyze equivalent O_2-dependent and O_2-independent reactions in chlorophyll biosynthesis (Figure 1). Toward understanding how these enzymes harness the reactivity of transition metal ions to build and tailor the photosynthetic pigment scaffold in the presence or absence of O_2, we are using a combination of molecular biology, mechanistic enzymology, spectroscopy, and X-ray crystallography. This work will reveal the structure-function relationships in different classes of metalloenzymes involved in synthesizing chlorophyll pigments and will illuminate fundamental information regarding how diverse photosynthetic organisms build pigments to facilitate conversion of sunlight into chemical energy.

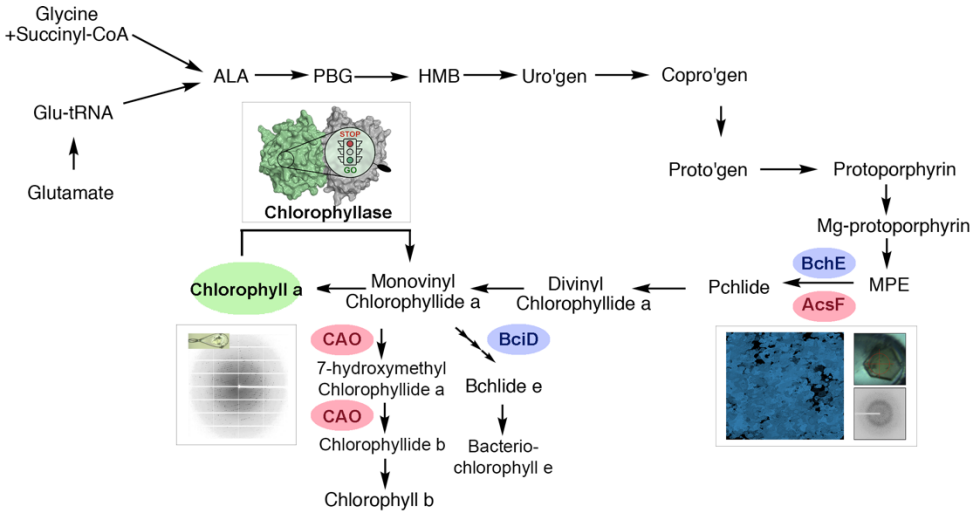

Figure 1. The biosynthesis of the chlorophyll, involves several steps that are catalyzed by parallel enzymes[1-2]. The enzymes involved in these steps are highlighted with red (O_2-dependent) and blue (O_2-independent) circles. This project is specifically focused on the O_2-dependent and O_2-independent enzymes used in building and tailoring the chlorophyll scaffold (AcsF/BchE and CAO/BciD). Recent ongoing structural and biochemical work is highlighted in the insets and involves enzymes that dephytylate or formylate chlorophyll, as well as other enzymes that are involved in building the chlorophyll scaffold.

Significant achievements

Toward the goals of this project, we have identified homologs of chlorophyllide a oxygenase (CAO) from *Prochlorothrix hollandica*, *Arabidopsis thaliana*, and *Chlamydomonas reinhardtii*, aerobic cyclization system Fe-containing subunit (AcsF), and BchE that can be isolated, purified, and crystallized. At this time, we are working to phase the structures of *P. hollandica* CAO (1.6-Å resolution)

and AcsF (1.7-Å resolution, Figure 1). For AcsF, using a combination of Fe- and S-anomalous data, we have calculated preliminary electron density maps, and developed purification protocols for SeMet labeled protein (Figure 1). Alongside these structural studies, we have created a sequence similarity network of CAO homologs and used this network to evaluate residue conservation. From this bioinformatic analysis, several well-conserved residues of interest were identified. To probe the importance of these residues to the mechanism and activity of CAO, we expressed, purified, and reconstituted a library of twelve CAO variants. We are currently optimizing our activity measurements on these variants using a newly identified non-native reductase that improves our product yields. We are also using a recently developed fluorescence assay to evaluate the ability of the CAO variants to bind the chlorophyllide *a* substrate. Furthermore, in studying CAO, a protocol was designed to express and purify chlorophyllase, an enzyme that produces the chlorophyllide *a* substrate of CAO via dephytylation of chlorophyll *a*. Although this transformation is chemically straightforward, until our recent work on this enzyme, many biochemical and molecular details regarding how chlorophyllase functions remained unknown. Using enzymology and structural biology experiments, we showed that the activity and stability of dimeric chlorophyllase depends on the presence of five disulfide bonds (Figure 1). Ongoing work in the laboratory is focused on a homolog of chlorophyllase from *A. thaliana* to assess whether the identified "redox switching" mechanism is an extensively used feature in plant chlorophyllase homologs.

Science priorities for the next year (2023-2024):

- Improve the anomalous electron density maps to solve the structure of AcsF
- Optimize initial crystals of BchE for structure solution
- Map structure-function relationships in AcsF using structural information
- Purify and crystallize SeMet-labeled *P. hollandica* CAO and CAO from *A. thaliana* and *C. reinhardtii*
- Complete activity and binding assays on the CAO variant library
- Complete studies on a chlorophyllase homolog (CLH2) from *A. thaliana*

My major scientific area(s) of expertise is/are: Metalloprotein Chemistry, X-ray Crystallography, Enzymology, Biophysical Techniques, Bioinformatics, and Structural Biology.

To take my project to the next level, my ideal collaborator would have expertise in: Our ideal collaborator would have (i) expertise in the genetic manipulation of photosynthetic organisms, (ii) the ability to synthesize labeled or derivative chlorophyll pigments, or (iii) have expertise in cell imaging.

Publications supported by this project

1. Bridwell-Rabb, J.; Li, B.; Drennan, C. L., *ACS Bio. Med. Chem. Au* 2022, *2* (3), 173-186.

2. Dill, Z.; Li, B.; Bridwell-Rabb, J., *Methods Enzymol* 2022, *669*, 91-116.

3. Li, B.; Jo, M.; Liu, J.; Tian, J.; Canfield, R.; Bridwell-Rabb, J., *Commun. Biol.* 2022, *5*, 275.

4. Knapp, M.; and Bridwell-Rabb, J., *Nat. Chem.* 2022, *14* (10), 1202.

5. Jo, M.; Knapp, M.; Boggs, D.G.; Brimberry, M.; Donnan, P.H.; Bridwell-Rabb, J., *J. Biol. Chem.* 2023, *299* (3), 102958.

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Spectroscopic Studies of a Photosystem I Hydrogenase Chimera

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Overall research goals:

Our goal is to explore photosynthetic hydrogen production, focused on [Fe-Fe] hydrogenase assembly and working with a Photosystem I – [Fe-Fe] hydrogenase chimera constructed by Kevin Redding's lab at ASU. One of the first goals is to characterize the interprotein interactions that lead to the biosynthesis of the catalytic H-cluster. In the prior proposal, we described the planned use of cryoEM to carry out this process, first on the isolated maturase enzymes along with the apo-hydrogenase HydA1. With that accomplished we can move to the H-cluster maturation within the PSI - hydrogenase chimera.

Significant achievements (2021-2023):

In order to carry out the cryoEM aspects of the project, we first had to build an anerobic cryoEM "grid preparation" device in order to prepare the cryoEM assembly intermediates within an O2-free glove box. That has been completed, and we are now testing sample spraying and freezing conditions that give good grid coverage and EM resolution. This is in collaboration with the James Letts laboratory at UC Davis. In addition, we added a new approach to study protein/protein interactions during hydrogenase assembly, by carrying out Native Mass Spectrometry with the Evan Williams laboratory at UC Berkeley. We have explored conditions needed to avoid oxidative damage and to obtain high resolution mass spectrometry data on the maturase proteins. This work is important to broaden our approach to understanding the maturation beyond the Fe-S cluster intermediates that we have characterized with EPR and other metal cluster focused spectroscopies. We can now work towards understanding what transient protein/protein interactions are used in the biosynthesis as performed by these maturase proteins (HydG, HydE, HydF, and HydA1)

We are excited about a fresh breakthrough in high frequency/field pulse EPR spectroscopy. We had previously built a 263 GHz pulse EPR spectrometer, but this requires a high power, high bandwidth amplifier to be very useful. Working with Neville Luhmann at UC Davis and some of his engineering associates, we have recently fabricated a 10 W pulse amplifier that operates at 263 GHz, with approximately 6 GHz instantaneous bandwidth. We are now integrating this unique amplifier into the pulse EPR spectrometer, in order to carry about high power pulse EPR at fields/frequencies almost 3 times greater than the current state of the art.

Science priorities for the next year (2023-2024):

- We will work to complete the cryoEM and native mass spec studies of Fe-Fe hydrogenase assembly and extend into applications with the PSI chimera
- We will return to studying integer spin states of the PSII OEC using pulse 263 GHz EPR, where the magnetic zeeman interaction is X30 than traditional x-band pulse EPR, thus minimizing the deleterious effects of the zero field splitting intrinsic to integer spin states such as S1 and S3.

My major scientific area(s) of expertise is/are: Mechanisms of metalloenzymes and radical enzymes, magnetic resonance spectroscopy.

To take my project to the next level, my ideal collaborators would have expertise in: hydrogenase function, coupling photosystems to hydrogenases, and/or mutagenesis and isotope labeling of Photosystem II.

Structure-Function Studies of Photosystem II

Gary W. Brudvig, Principal Investigator
Christopher J. Gisriel and Jieun Shin, Postdoctoral Research Associates; David A. Flesher, Graduate Student; Jimin Wang, Research Scientist; Victor S. Batista, M. R. Gunner, Donald A. Bryant, K.V. Lakshmi, David J. Vinyard, Jennifer P. Ogilvie, Roberta Croce and Richard J. Debus, Collaborators
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Overall research goals:

The specific aims of this project are to probe the structure and function of photosystem II (PSII) by: (i) investigating the S_3 to S_0 transition by using $^{16}O/^{18}O$ kinetic isotope effect (KIE) measurements, (ii) probing the substrate water delivery mechanism by studying the D1-S169A variant of *Synechocystis* sp. PCC 6803 (*Syn.* 6803), (iii) probing the oxidation of alternate substrates by the O_2-evolving complex (OEC), and (iv) determining the cryogenic electron microscopy (cryo-EM) structures of mutated PSII complexes from *Syn.* 6803 PSII, including D1-D61A, D1-D170E, D1-E189R, D2-K317A, D1-D342E, D1-S169A and D1-N181A.

Significant achievements (2022-2023):

(i) Determination of 1.9 Å cryo-EM structure of Syn. 6803 PSII. This is the first high resolution structure of PSII from a non-thermophilic organism, which allows a direct comparison with the structure of PSII from thermophilic cyanobacteria. It also enables a correlation of previous biochemical and biophysical data on site-directed mutated PSII to the structure of PSII from the same organism used for mutagenesis, as well as providing a platform for future structural studies of genetically altered PSII. From our structure, notable observations include altered water channels near the OEC when compared to structures of PSII from thermophilic cyanobacteria, as well as identification of the previously unknown PsbQ subunit binding site.

(ii) Cryo-EM structures of far-red light (FRL) acclimated PSI and PSII. We solved structures of both a monomeric PSII core complex at 2.25 Å resolution and a dimeric PSII complex at 2.6 Å resolution from FRL-acclimated *Synechococcus* sp. PCC 7335. In addition, we solved the structure of a PS I-ferredoxin complex from the same organism. These are the first structures of FRL-acclimated PSII and they reveal the previously unknown locations of the chlorophylls *d* and *f* in FRL-acclimated PSII.

(iii) Cryo-EM structure of D1-D170E mutated PSII. We solved a 2.1 Å structure of D1-D170E mutated PSII from *Syn.* 6803, which perturbs a bridging carboxylate ligand of the OEC and alters the properties of the OEC without fully abolishing water oxidation. The structure reveals that the mutation shifts the position of the OEC within the active site, without notably distorting the Mn_4CaO_5 cluster geometry, and perturbs the hydrogen-bonding network of structured waters near the OEC.

Science priorities for the next year (2023-2024):

Ongoing work involves the use of cryo-EM to determine structures of site-directed mutated *Syn.* 6803 PSII, including D1-E189R, D1-D61A and D2-K317A mutations, biophysical studies to probe the oxidation of alternate substrates and the role of second-shell amino-acid residues around the OEC, and computational modeling in collaboration with the Batista and Gunner groups to characterize the structure and function of the OEC.

Publications supported by this project (2022-2023):

1. "High-resolution Cryo-EM Structure of Photosystem II from the Mesophilic Cyanobacterium, *Synechocystis* sp. PCC 6803", Christopher J. Gisriel, Jimin Wang, Jinchan Liu, David A. Flesher, Krystle M. Reiss, Hao-Li Huang, Ke R. Yang, William H. Armstrong, M. R. Gunner, Victor S. Batista, Richard J. Debus & Gary W. Brudvig (2022) *Proc. Natl. Acad. Sci. U.S.A. 119*, e2116765118 (DOI: 10.1073/pnas.2116765118).
2. "Structure of a Photosystem I-Ferredoxin Complex from a Marine Cyanobacterium Provides Insights into Far-Red Light Photoacclimation", Christopher J. Gisriel, David A. Flesher, Gaozhong Shen, Jimin Wang, Ming-Yang Ho, Gary W. Brudvig & Donald A. Bryant (2022) *J. Biol. Chem. 298*, 101408 (DOI: 10.1016/j.jbc.2021.101408).
3. "Structure of a Monomeric Photosystem II Core Complex from a Cyanobacterium Acclimated to Far-Red Light Reveals Functions of Chlorophylls *d* and *f*", Christopher J. Gisriel, Gaozhong Shen, Ming-Yang Ho, Vasily Kurashov, David A. Flesher, Jimin Wang, William H. Armstrong, John H. Golbeck, M. R. Gunner, David J. Vinyard, Richard J. Debus, Gary W. Brudvig & Donald A. Bryant (2022) *J. Biol. Chem. 298*, 101424 (DOI: 10.1016/j.jbc.2021.101424).
4. "Binding of the Substrate Analog Methanol in the Oxygen-Evolving Complex of Photosystem II in the D1-N87A Genetic Variant of Cyanobacteria", Vidmantas Kalendra, Krystle M. Reiss, Gourab Banerjee, Ipsita Ghosh, Amgalanbaatar Baldansuren, Victor S. Batista, Gary W. Brudvig & K. V. Lakshmi (2022) *Faraday Discuss. 234*, 195-213 (DOI: 10.1039/d1fd00094b).
5. "Molecular Evolution of Far-Red Light-Acclimated Photosystem II", Christopher J. Gisriel, Tanai Cardona, Donald A. Bryant & Gary W. Brudvig (2022) *Microorganisms 10*, 1270 (DOI: 10.3390/microorganisms10071270).
6. "Glycerol Binding at the Narrow Channel of Photosystem II Stabilizes the Low-Spin S_2 State of the Oxygen-Evolving Complex", David A. Flesher, Jinchan Liu, Jessica M. Wiwczar, Krystle Reiss, Ke R. Yang, Jimin Wang, Mikhail Askerka, Christopher J. Gisriel, Victor S. Batista & Gary W. Brudvig (2022) *Photosynth. Res. 152*, 167-175 (DOI: 10.1007/s11120-022-00911-0).
7. "Comparison of PsbQ and Psb27 in Photosystem II Provides Insight into Their Roles", Christopher J. Gisriel & Gary W. Brudvig (2022) *Photosynth. Res. 152*, 177-191 (DOI: 10.1007/s11120-021-00888-2).
8. "How to Correct Relative Voxel Scale Factors for Calculations of Vector-Difference Fourier Maps in Cryo-EM", Jimin Wang, Jinchan Liu, Christopher J. Gisriel, Shenping Wu, Federica Maschietto, David A. Flesher, Elias Lolis, George P. Lisi, Gary W. Brudvig, Yong Xiong & Victor S. Batista (2022) *J. Struct. Biol. 214*, 107902 (DOI: 10.1016/j.jsb.2022.107902).
9. "Structure of a Dimeric Photosystem II Complex from a Cyanobacterium Acclimated to Far-Red Light", Christopher J. Gisriel, Gaozhong Shen, David A. Flesher, Vasily Kurashov, John H. Golbeck, Gary W. Brudvig, Muhamed Amin & Donald A. Bryant (2023) *J. Biol. Chem. 299*, 102815 (DOI: 10.1016/j.jbc.2022.102815).
10. "Helical Allophycocyanin Nanotubes Absorb Far-Red Light in a Thermophilic Cyanobacterium", Christopher J. Gisriel, Eduard Elias, Gaozhong Shen, Nathan T. Soulier, David A. Flesher, M. R. Gunner, Gary W. Brudvig, Roberta Croce & Donald A. Bryant (2023) *Science Adv. 9*, eadg0251 (DOI: 10.1126/sciadv.adg0251).
11. "Molecular Diversity and Evolution of Far-Red Light-Acclimated Photosystem I", Christopher J. Gisriel, Donald A. Bryant, Gary W. Brudvig & Tanai Cardona (2023) *Front. Plant Sci. 14*, 1289199 (DOI: 10.3389/fpls.2023.1289199).
12. "Two-Dimensional Electronic Spectroscopy of the Far-Red-Light Photosystem II Reaction Center", Yogita Silori, Rhiannon Willow, Hoang H. Nguyen, Gaozhong Shen, Yin Song, Christopher J. Gisriel, Gary W. Brudvig, Donald A. Bryant & Jennifer P. Ogilvie (2023) *J. Phys. Chem. Lett. 14*, 10300-10308 (DOI: 10.1021/acs.jpclett.3c02604).
13. "A Quantitative Assessment of (Bacterio)chlorophyll Assignments in the Cryo-EM Structure of the *Chloracidobacterium thermophilum* Reaction Center", Christopher J. Gisriel, David A. Flesher, Zhuoran Long, Jinchan Liu, Jimin Wang, Donald A. Bryant, Victor S. Batista & Gary W. Brudvig (2023) *Photosynth. Res.*, in press (DOI: 10.1007/s11120-023-01047-5).
14. "Structural Comparison of Allophycocyanin Variants Reveals the Molecular Basis for their Spectral Differences", Christopher J. Gisriel, Eduard Elias, Gaozhong Shen, Nathan T. Soulier, Gary W. Brudvig, Roberta Croce & Donald A. Bryant (2023) *Photosynth. Res.*, in press (DOI: 10.1007/s11120-023-01048-4).

Structure and Function of Cyanobacterial CO_2 uptake NDH-1 Complexes

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Overall research goals: This project aims to understand cyanobacterial **CO_2-concentrating mechanism (CCM)**, which efficiently supplies CO_2 to the photosynthetic mechanism. Essentially, it functions as a 'supercharger' for CO_2, concentrating it within the cell, thereby saturating the active sites of the CO_2-fixation enzyme, Rubisco, thereby increasing the efficiency of photosynthesis. The focus is the redox active **NDH-1 complexes**, particularly the specialized forms of the core complex, designated **NDH-1$_3$** and **NDH-1$_4$,** that function to directly hydrate CO_2 leading to the carbonation of the cytoplasm and loading of the carboxysome. *The most important aim of the project is to elucidate the mechanism coupling the core redox and H^+-pumping machinery of the NDH-1 complex to the Zn-centered CO_2 hydration reaction.*

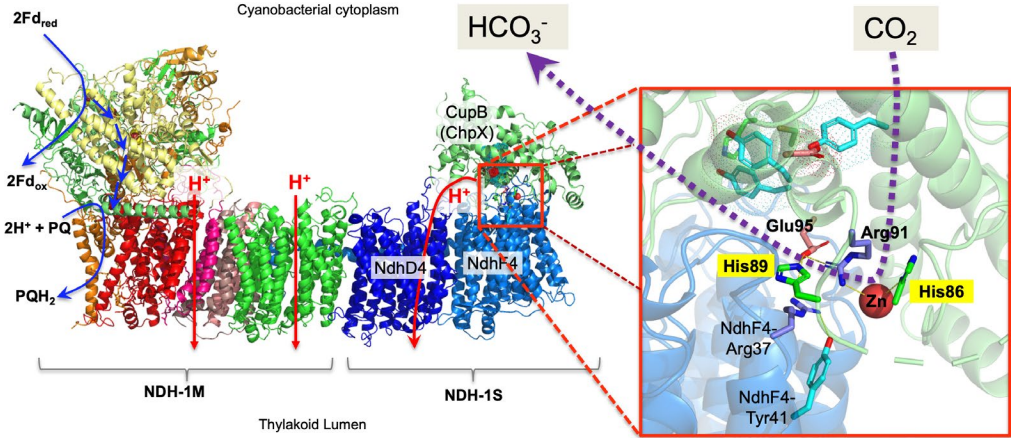

Figure 1. Homology model of the genetically amenable *Synechococcus* PCC7942 NDH-1$_4$ complex that catalyses high flux, CO_2-uptake through an energized carbonic anhydrase activity. The model is guiding site-directed mutagenesis and physiochemical analyses to understand proton-pumping and coupled CO_2-hydration mechanisms.

Significant achievements 2022-2024:

Mutagenesis of the CupB protein probing the unusual Zn-ligation environment indicates the assembly and/or stability of the CupB protein requires both Zn-ligation and ionic bond between the unusual arginine (CupB-R91) ligand and a second sphere glutamate residue (CupB-E95) as shown in **Fig. 1, right panel**. Mutagenesis of the CupB-H89, in contrast, indicates a proton handling role.

Significance: 1.) Provides independent support for the unorthodox ligation pattern assigned in the 3.2Å cryo-EM structure of the homologous CupA-containing, NDH-1$_3$ complex; 2.) results provide clues unique coupling of the anhydrase reaction to the energetics of the NDH-1 complex expected to involve unique active site chemistry a new model will be discussed.

Mutagenesis systems for the NdhD4 and NdhF4 subunits to probe proton pumping residues.

Significance: Our working hypothesis is that the CO_2 hydration reaction is coupled to H^+-pumping. The NdhD4 and NdhF4 subunits are likely involved in transporting protons from the CO_2 hydration site to the lumen, enabling the tests of that hypothesis.

New CRISPR, gene re-construction, and physiological selection techniques enable the expression of solely the NDH-1$_4$ CO_2 hydration complex in the cell without normal expression of the NDH-1$_{1/2}$ and NDH-1$_3$ complexes.

Significance: NDH-1 is present in the cell as a family of multiple isoforms (NDH-1$_{1/2}$, NDH-1$_3$, NDH-1$_4$). Strains that expressing only one isoform NDH-1$_4$ are essential to isolate its properties of CEF, CO_2 hydration and H^+-pumping.

Probing proton-pumping by NDH-1 complexes: We showed that NDH-1 is the dominant (90%) contributor to CEF using acridine orange experiments with Dr. Michael Vaughn at Spectrologix USA. Extending this cooperation, we are developing more straightforward difference-spectroscopic probing a electrochromic signal across the cyanobacterial thylakoids.

Significance: Enables wide range of non-invasive techniques to probe CEF and H^+-pumping, as well as using DIRK techniques that have found wide application in plant physiological studies and now can be extended to this project.

Science priorities for the next year (2023-2024):

1. Work out efficient procedures to purify NDH-1 complexes from *Synechococcus* sp. PCC7942 for structural analysis.
2. Build on recently initiated collaboration for computational analysis of NDH-1 structure/mechanism with Profs. Marilyn Gunner and Abhishek Singharoy.
3. Complete the physiological analysis of NdhD4 proton-pumping mutants including test accumulation and assembly of the NDH-1$_4$ polypeptides.

My major scientific area(s) of expertise are: molecular genetics, biophysics, bioinformatics

To take my project to the next level, my ideal collaborator would have expertise in: Computational analysis of the structure and dynamics of large protein complexes QMM/MM

Publications supported by this project (DOE-BES as sole financial support):

1. Burnap, R.L., (2023) ***Cyanobacterial bioenergetics in relation to cellular growth and productivity***, Burnap, R.L. (2023). In: Bühler, K., Lindberg, P. (eds) Cyanobacteria in Biotechnology. Advances in Biochemical Engineering/Biotechnology, vol 183. Springer, Cham. https://doi.org/10.1007/10_2022_215
2. Miller, N.T., G. Ajlani, and R.L. Burnap (2022) ***Cyclic electron flow coupled proton pumping in Synechocystis sp. PCC6803 is dependent upon NADPH oxidation by the soluble isoform of ferredoxin:NADP-oxidoreductase*** Microorganisms 10, doi:10.3390/microorganisms10050855
3. Burnap, R.L., ***Bioenergetics: To the dark side and back with cyanobacterial ATP synthase***. Curr Biol, 2022. **32**(1): p. R34-r36. https://doi.org/10.1016/j.cub.2021.11.028 (Commentary)
4. Artier, J., Walker, R.M., Miller, N.T., Woodger, F.J., Zhang, M., Price, G.D., and R.L. Burnap. ***Modelling and mutagenesis of amino acid residues critical for CO_2 hydration by specialized NDH-1 complexes in cyanobacteria*** Biochim Biophys Acta Bioenerg, 2021. 1863 (1):148503. doi:10.1016/j.bbabio.2021.148503
5. Miller, N.T., M.D. Vaughn, and R.L. Burnap, ***Electron flow through NDH-1 complexes is the major driver of cyclic electron flow-dependent proton pumping in cyanobacteria***. Biochim Biophys Acta Bioenerg, 2020. 1862(3): p. 148354. doi.org/10.1016/j.bbabio.2020.148354

Poster title: Cryogenic Electron Tomography investigation of the structural dynamics of PSI Supercomplexes in *Chlamydomonas priscuii.*

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Overall research goals:

One of the main goals of this research project is to characterize the diversity and variability of PSI Supercomplexes during long-term stress. We are developing cryogenic Electron Tomography (cryo-ET) workflow for efficient vitrification, cryogenic Focused Ion Beam-Scanning Electron Microscopy (cryoFIB-SEM) lamella preparation, cryo-ET data collection and data processing that will allow 3D visualization of PSI Supercomplexes in the thylakoids of *C. priscuii* chloroplasts adapted to long-term high-salt stress conditions.

Significant achievements (2022-2023):

We collected cryo-ET datasets on multiple ultrathin cryo-FIB-SEM prepared lamellae, and subjected all current datasets to novel computational methods of analysis that includes AI recognition and segmentation of features of interest. Supercomplexes were recognized through the prominent feature of associated ATP-synthase protruding from the thylakoid membrane, while PSI complexes are more challenging to identify due to their small size (24kD), embedment inside the thylakoid membrane, and unknown amount of adaptive changes in structural organizations under long-term stress conditions. It was evident that further datasets are needed to enable subvolume averaging with subnanometer resolution details. For this purpose, we received fresh cell cultures from PI Morgan-Kiss, and vitrified specimens using standard robotic plunge-freezing techniques, as well as High Pressure Freezing for advanced cryoFIB-milling application and cryo-data collection. In addition, biochemically isolated thylakoid membranes will be vitrified for cryo-ET data collection and structure determination. Such structure will be used to aid the identification of the location and the structure of supercomplexes *in situ*, which represent the native environment of PSI in the cell.

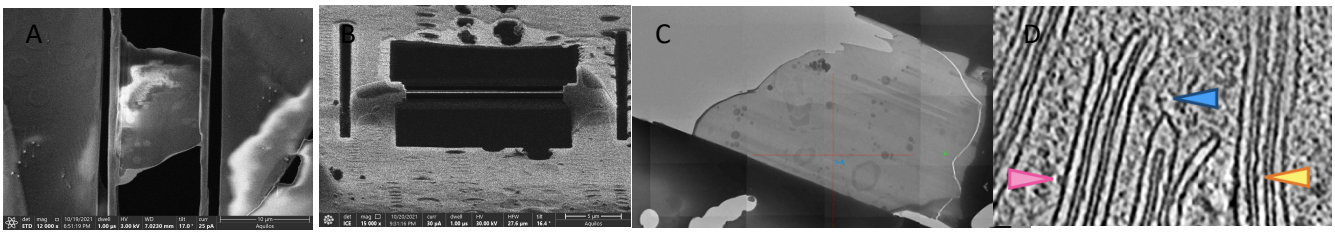

Figure 1: Cryo-ET workflow illustrated through cryoFIB-SEM lamellae as seen with (A) SEM, (B) FIB and (C) Krios/TEM. In (D) the location of PSI Supercomplexes are identified visually through the presence of ATP-synthase (blue arrow) PS1 (red arrow) and PSII (yellow arrow) in tiltseries collected from vitreous lamellae. Annotation is based on location, orientation and approximate size of proteins.

Science priorities for the next year (2023-2024):

We aim to conduct further lamellae preparation using basic and automated workflows, as well as more advanced 'waffle milling' techniques to accelerate the generation of higher numbers of ultrathin lamellae suitable for high resolution data collection. We will also vitrify and analyze isolated thylakoids for similar cryo-ET data collection. Data collection will be performed on-site at SLAC laboratory using a 300kV Krios™, and the latest Tomography 5 software from ThermoFisher. Additionally, we are employing the expertise of a computational specialist to characterize PSI Supercomplexes in current and

previous datasets. Our recent progress has been limited by the availability of *C. priscuii* cell cultures, which experienced a 6 month pause in growth, as described by Morgan-Kiss (PI).

Major scientific area(s) of expertise are: High resolution Cryo-Electron Tomography and AI-based Computation in various biological systems ranging from neuronal cells, coronavirus-infected cells to photosynthetic organisms relevant to therapeutic development and bioenergy prodcution research.

To take this project to the next level, my ideal collaborator would have expertise in: Culturing of photosynthetic organisms; isolation of thylakoid membranes; cryoFIB-SEM sample preparation; high resolution cryo-ET data processing.

Acknowledgements:
DOE BES grant FWP 100868
Dr. Arthur Grossman, Carnegie Institute for providing *C. reinhardtii* cell cultures.
Drs. Muyuan Chen and Michael Schmid at SLAC for data processing and 3-D visualization.

Photosynthetic Reduction of Carbon Dioxide:
Kinetic Trade-offs for Rubisco and Phospho*enol*pyruvate Carboxylase.

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Overview and Objectives

Nearly all photosynthetic organisms initiate the capture of atmospheric carbon with either ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) directly fixing CO_2 or by phosphoenolpyruvate carboxylase (PEPC) fixing carbon as bicarbonate (Fig. 1). However, these enzymes have kinetic tradeoffs that limit their catalytic efficiency. The Rubisco carboxylation rate is often competitively inhibited by oxygen and there is a potential kinetic trade-off between Rubisco's catalytic rate and affinity for CO_2. The fixation of bicarbonate by PEPC is insensitive to oxygen and has a greater carboxylation efficiency compared to Rubisco. However, there is a kinetic trade-off between PEPC's affinity for its substrates bicarbonate and phosphoenolpyruvate (PEP). To engineer an increase in the catalytic efficiency of Rubisco and PEPC to help meet globally increasing bioenergy demands will require a better understanding of these enzymes' kinetic trade-offs. *Therefore, it is important to understand the reaction mechanisms determining the kinetic trade-offs of Rubisco and PEPC to optimize their catalytic efficiencies so photosynthetic organisms can better utilize light energy to capture atmospheric carbon for energy storage.*

Overall research goals

Overall objectives of our research are to 1) resolve which reaction rates determine the kinetic trade-offs between Rubisco carboxylation and oxygenation reactions, and 2) determine trade-offs of PEPC's affinity for PEP and bicarbonate.

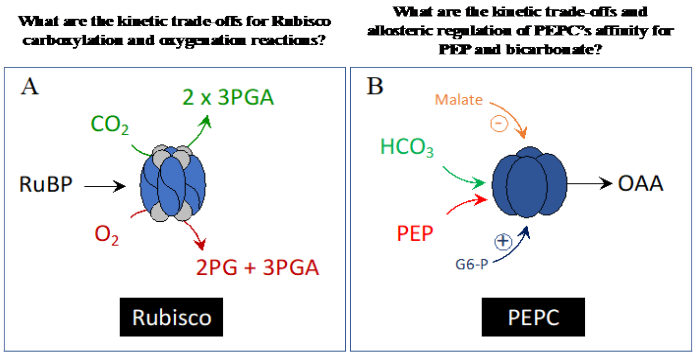

Figure 1. Rubisco and PEPC reactions. (A) Rubisco carboxylation of ribulose-1,5-bisphosphate (RuBP) with CO_2 is inhibited by O_2. Carboxylation produces 3-phosphoglycerate (3PGA), which is used to make sugar. Oxygenation generates 3PGA + 2-phosphoglycolate (2PG) but 2PG requires energy consumption by photorespiration to recycle carbon and nitrogen. (B) PEPC is insensitive to O_2 and uses bicarbonate (HCO_3^-) to carboxylate phospho*enol*pyruvate (PEP) into oxaloacetate (OAA). There is a kinetic trade-off in PEPC's affinity for its substrates, which is allosterically regulated by malate and G6-P (glucose 6-phosphate).

The *specific aims* are 1) Determine which rate constants drive the kinetic trade-off between Rubisco carboxylation and oxygenation reactions, and 2) Identify amino acid residues that determine the kinetic trade-off of PEPC's affinity for PEP and bicarbonate. These aims will be address by testing the *working hypotheses* that 1) Direct measurements of Rubisco CO_2 and O_2 kinetic parameters and their isotope effects will resolve kinetic trade-offs between the carboxylation and oxygenation reactions, and 2) Specific amino acid residues control kinetic trade-offs and their allosteric regulation of PEPC.

Significant achievements during 2022-2023

We have characterized PEPC isoforms from over 20 species and compared with non-photosynthetic isoforms. Several chimeric enzymes have been generated to determine how specific regions of the PEPC influence its kinetics and allosteric regulation. Thermal response of PEPC kinetics from temperate and high temperature adapted C_4 plants have been measured. We have acquired several isoforms of Rubisco form II enzymes that we are kinetically characterizing and optimizing the purification protocol so that we have enough enzyme to run the temperature response assays.

Objectives for the coming year

1. Publish Rubisco kinetic parameters and carbon isotope fractionation from *Oyrza sativa*.
2. Finish kinetic and isotope effect measurements of the form II Rubisco from *R. rubrum*.
3. Publish PEPC thermal kinetic response from the heat adapted C_4 plant.
4. Develop models to identify regions of thermal instability in non-heat adapted PEPC enzymes
5. Generate and kinetically characterize chimeric PEPC isoforms to test amino acid regions influencing kinetic trade-offs, allosteric regulation, and thermal responses.

My major scientific area(s) of expertise are: Leaf CO_2 exchange and assimilation, enzymology of carbon fixing enzymes, and leaf CO_2 and H_2O isotope exchange.

To take my project to the next level, my ideal collaborator would have expertise in: Enzyme structural chemist, modeling enzyme dynamics and thermal stability. We have started collaborations with Laura Gunn (structural chemist, Cornell University), James Evans (protein structural chemist, EMSL, PNNL), and Abhishel Singharoy (protein modeler, Arizona State University).

Publications in 2022-2023 supported by the award:

1. Wessendorf, R.L., and **Cousins, A.B.** Insights into kinetic trade-off and allosteric regulation of phosphoenolpyruvate carboxylase. In revision, *Photosynthesis Research.*
2. DiMario, R.J., Kophs, A.N., Apalla, A.J.A., Schnable, J.N., **Cousins, A.B.** (2023) Multiple highly expressed phosphoenolpyruvate carboxylase genes have divergent enzyme kinetic properties in two C_4 grasses. *Annals of Botany* https://doi.org/10.1093/aob/mcad116
3. Rai, A.K., DiMario, R.J., Kasili, R.W., Groszmann, M., **Cousins, A.B.,** Donze, D. Moroney, J.V. (2022) A Rapid Method for Detecting Normal or Modified Plant and Algal Carbonic Anhydrase Activity Using *Saccharomyces cerevisiae*. *Plants* 11: 1882.
4. Varsha, P., DiMario R., Koteyeva N., **Cousins A.B.** (2022) Mesophyll conductance response to short-term changes in pCO_2 is related to leaf anatomy and biochemistry in diverse C_4 grasses. *New Phytologist* 236:1281-1295
5. DiMario R., Giuliani, R., Ubierna, N., Slack A., **Cousins A.B.**, Studer, A. (2022) Lack of leaf carbonic anhydrase activity eliminates the C_4 carbon concentrating mechanism requiring direct diffusion of CO_2 into bundle sheath cells. *Plant Cell & Environment* 45:1382-1397
6. Crawford J.D., **Cousins A.B.** (2022) Limitation of C_4 photosynthesis by low CA activity increases with temperature but does not influence mesophyll CO_2 conductance. *Journal of Exp Botany* 73:927-938.

In prep

1. Ryan L. Wessendorf and **Cousins A.B.** Kinetic trade-off PEPC does not extrapolate across C_4 photosynthetic isozymes.

Metal Ligands and Hydrogen Bond Networks of the O_2-evolving Complex in Photosystem II

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Overall research goals: To identify the two substrate H_2O molecules that form the O-O bond, to identify the dominant substrate access and proton egress pathways that link the Mn_4CaO_5 cluster with the thylakoid lumen, to characterize dynamic changes in the H-bond networks that comprise the substrate access and proton egress pathways, and to obtain high resolution 3-D cryo-EM structures of intact Photosystem II core complexes containing mutations of specific amino acid residues to extend interpretations based on spectroscopic data.

Significant achievements, 2021-2023: On the basis of FTIR and EPR studies (the latter performed in collaboration with R. D. Britt and coworkers at UC Davis and P. Oyala at Caltech), we showed that the independent mutation of two metal-bridging Mn_4CaO_5 carboxylate ligands, creating D1-D170E and D1-D342E, plus constructing the double mutant D170E/D342E, alter the equilibria between different forms of the cluster's S_1 and S_2 oxidation states so that illumination of dark-adapted PSII (in the S_1 state) generates high spin forms of the S_2 state instead of the low spin form that displays the $g = 2$ multiline EPR signal in wild-type. These mutations also perturb the network of H-bonds that surrounds the Mn_4CaO_5 cluster in much the same manner as other mutations that have been constructed in this network. Because the D1-D170E, D1-D342E, and D170E/D342E mutations do not eliminate O_2 evolution, these findings are relevant to understanding the mechanism of O_2 formation in terms of catalytically active/inactive conformations of the Mn_4CaO_5 cluster's individual oxidation states.

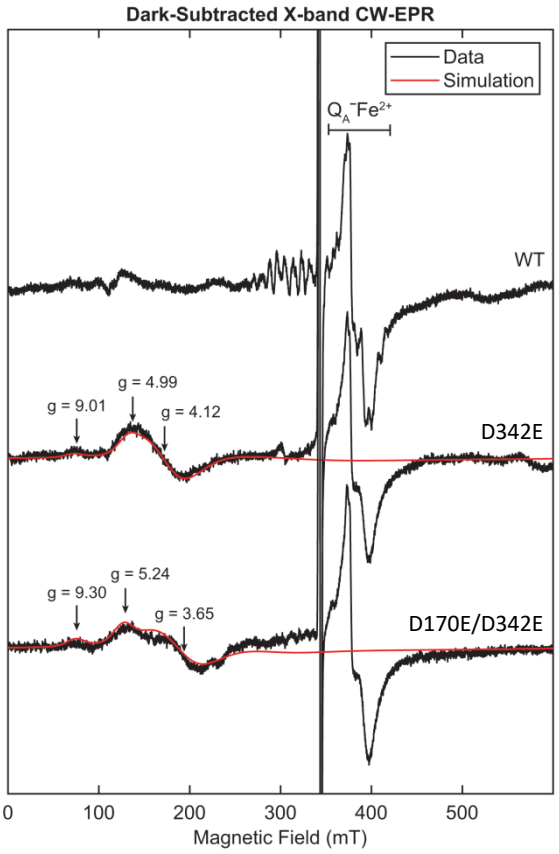

Fig. 1. S_2-*minus*-S_1 EPR spectra (black) and simulations (red) of D342E and D170E/D342E PSII core complexes in comparison to wild-type. Samples contained 10 mM formate to prevent oxidation of the non-heme Fe during sample manipulation/dark-adaptation.

On the basis of cryo-EM analyses conducted in collaboration with G. W. Brudvig and workers at Yale University, we determined the structure at 2.14 Å of intact *Synechocystis* sp. PCC 6803 PSII core complexes containing the D1-D170E mutation. The structure shows that the mutation shifts the Mn_4CaO_5 cluster's position without significantly distorting its geometry, and perturbs the network of H-bonds that surround the Mn_4CaO_5 cluster.

On the basis of time-resolved IR and polarographic measurements conducted in collaboration with H. Dau and coworkers at Freie Universität Berlin, we showed that the D1- N298A

mutation (1) substantially slows *steady-state* O_2 evolution *but does not slow the rate that O_2 is released after a flash* and (2) slows the S_3 to (S_4) to S_0 transition so that it is biphasic in a wavenumber-dependent fashion, with a fast component that tracks with rapid O_2 release and a slow component that is slower by more than an order of magnitude. We propose that the slow component represents the insertion of a new substrate H_2O into the Mn_4CaO_5 cluster *after* O_2 release, thereby regenerating the S_0 state. Because D1-N298 links the "water wheel" (W26-W30) to other H_2O molecules in the O1 network of H bonds that connects the Mn_4CaO_5 cluster with the lumen, we propose that the newly inserted substrate H_2O originates from the O1 network. This is the first example of a mutation that slows regeneration of the S_0 state after O_2 formation/release.

On the basis of FTIR studies, we found that mutation of residues 10-12 Å from Mn_4CaO_5 cluster in the O1 network (e.g., CP43-V410, I411, T412), cause the same perturbations of the H-bond network surrounding the cluster as mutations near the cluster. In contrast, mutation of a residue deep in the O4 network (D1-N338) does not cause these perturbations. Evidently, structural coupling of H-bond networks to the Mn_4CaO_5 cluster extends deep into the O1 network but not into the O4 network, possibly reflecting the greater mobility of H_2O in former.

Science Priorities for 2023-2024:

- Complete the analysis of our existing cryo-EM data sets of D2-K317A, D1-D342E, D61A, and E189R, acquire data sets of D1-N298, D1-S169A, and a new data set of D2-K317A in the absence of Cl ions (D2-K317 is implicated in binding Cl), all with the aim of producing high resolution 3D structures of PSII containing mutations.
- Extend our time-resolved IR studies to include mutations of other residues in the O1 network (e.g., CP43-V410) and residues in the D1-E65/D1-R334/D2-E312 triad.
- Extend our earlier studies of substrate water exchange kinetics to include mutations of residues located in the O4 and O1 H-bond networks (e.g., D1-N87A and CP43-V410A), and to mutations in the D1-65/D1-R334/D2-E312 triad.
- Complete our FTIR studies of mutations constructed in the O4 and O1 H-bond networks.
- Further characterize the perturbations to the electronic structures of the Mn_4CaO_5 cluster produced by the D1-D170E and D1-D342E mutations.

Major scientific areas of expertise: O_2 formation by PSII, FTIR spectroscopy, cryo-EM

Ideal Collaborators: I already collaborate with them: Brudvig (cryo-EM), Britt (EPR), Dau (time-resolved IR); Messinger (Membrane-inlet mass spectrometry), Yano/Yachandra (XAS).

Publications supported by the award in last two years:

1. Debus, R. J. (2021) "Alteration of the O_2-Producing Mn_4Ca Cluster in Photosystem II by the Mutation of a Metal Ligand," *Biochemistry* 60, 3841-3855 (https://pubs.acs.org/doi/full/10.1021/acs.biochem.1c00504).
2. Gisriel, C. J., Wang J., Liu, J., Flesher, D. A., Reiss, K. M., Huang, H.-L., Yang, K. R., Armstrong, W.H., Gunner, M. R., Batista, V. S., Debus, R.J., Brudvig, G. W. (2022) "High-resolution cryo-electron microscopy structure of photosystem II from the mesophilic cyanobacterium, *Synechocystis* sp. PCC 6803," *Proc. Natl. Acad. Sci. (U S A)* 119(1), e2116765118 (https://www.pnas.org/doi/10.1073/pnas.2116765118)
3. Chakarawet, K., Debus, R. J., Britt, R. D. (2023) "Mutation of a metal ligand stabilizes the high-spin form of the S_2 state in the O_2-producing Mn_4CaO_5 cluster of Photosystem II," *Photosynth. Res.* 156, 309–314 (https://doi.org/10.1007/s11120-023-00998-z).

Probing enzymatic catalysis of carbon-carbon bond formation with photoexcited semiconductor nanocrystals

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Overall research goals:

The overarching long-term goal of this project is to learn how to drive and probe redox enzyme catalysis by tuning the remarkable light-harvesting properties of colloidal semiconductor nanocrystals (NCs) to act as light-triggered interfacial electron donors. This approach is inspired by photosynthesis, which couples light absorption to enzyme catalysis via electron transfer steps. In the NC-enzyme architectures, NCs absorb light and photoexcited electrons transfer to enzymes, thereby driving enzyme catalysis with light. This project focuses on adapting the structural properties of semiconductor NCs towards productive interfaces with redox enzymes, controlling the electron pathways involved in delivery of electrons to enzyme active sites, and probing the responses of enzymes to electron delivery from NCs. We aim to provide the conceptual framework and design principles for synthetically tailoring the light absorbers to inject electrons in a manner synergistic with the catalytic cycle of the enzyme to enable probing of catalytic mechanisms. The impact of this work will be twofold. We will develop new tools for probing the behavior of poorly understood enzymes with complicated and fundamentally important catalytic mechanisms and we will learn how to drive the normally dark enzyme reactions with sunlight.

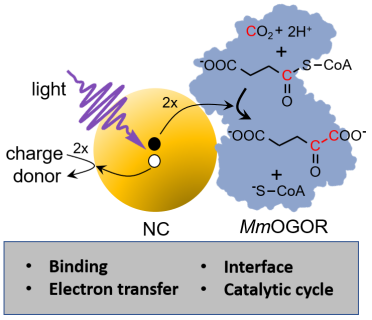

Fig. 1*. Schematic summary of the proposed research.*

In this project, we direct the light-harvesting properties of NCs towards understanding the behavior of enzymes from the 2-oxoacid:ferredoxin oxidoreductase (OFOR) family. OFORs reduce CO_2 and make new C-C bonds (the reductive direction) and/or oxidize 2-oxoacids and reduce ferredoxins (Fds) (oxidative direction). Some of the key questions about OFORs are: what is the molecular basis of the reductive and oxidative catalysis direction in nature? What is the role of Fd partners in controlling the catalysis? What are the kinetics of the many steps involved in catalysis? NCs as light-triggered electron donors can help answer these questions.

In this first 3-year funding period, we will establish the conceptual and experimental framework for using photoexcited NCs to study OFOR catalysis, compare our results with work on Fd-driven enzymes, and identify specific ways in which our methods will enable new discoveries about OFOR catalysis. We will start with 2-oxoglutarate oxidoreductase (OGOR) from *Magnetococcus marinus*, supplied by our collaborators in the Elliott group at Boston University. We will focus on understanding of how the structural parameters of NCs, such as composition, size, shape, and surface chemistry, impact their binding interactions with OGOR and the interfacial electron transfer processes. We will also focus on the kinetics of the overall photochemical 2-oxoglutarate generation. The **specific objectives** are to:

1. *Measure the kinetics of ET from CdSe and CdS quantum dots (QDs) of different diameters to MmOGOR using ultrafast transient absorption (TA) spectroscopy.*
2. *Refine the kinetic models for interpretations of TA spectroscopy measurements of ET from QDs to MmOGOR.*
3. *Characterize the binding interactions between QDs and MmOGOR*
4. *Examine how surface-capping ligands can be used to control the interactions between NCs and MmOGOR and demonstrate simultaneous injection of multiple electrons into MmOGOR.*

5. *Combine experimental measurements with simulations to extract rate constants that are otherwise difficult to measure*.

Significant achievements (2023-2026):

- We have identified some experimental parameters that are particularly important for the NC-OGOR binding and colloidal stability of our samples. Because we rely upon electrostatic attractions to bind these two components and electrostatic repulsions to keep the hybrid structures colloidally stable, identifying the right solution environments is a critical development for this project.
- We have observed significant differences in electron transfer (ET) behavior from CdSe QDs of different diameters to OGOR. This is consistent with our hypothesis that the structure of OGOR makes its binding the NCs particularly sensitive to size and shape and it suggests that we will be able to manipulate ET transfer behavior with NC design.

Science priorities for the next year (2023-2024):

- Develop the kinetic models for analyzing the measurements of ET kinetics by TA spectroscopy
- Determine how ET rate constants and NC photophysics vary with QD diameter
- Explore methods for characterizing NC-OGOR binding
- Develop simulations for analyzing the kinetics of the photochemical carbon-carbon bond formation in the NC-OGOR systems

My major scientific area(s) of expertise is/are: Nanocrystal synthesis and characterization; characterization of excited state dynamics by ultrafast spectroscopy; coupling of nanocrystals with redox enzymes; electron microscopy.

To take my project to the next level, my ideal collaborator would have expertise in: Binding studies – experimental and/or computational; enzymes for oxidation reactions.

Publications supported by this project 2023-2026:

N/A (new grant)

Coherent Dynamics for Regulation of Dissipation and Energy Transfer in Photosynthetic Systems

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Overall research goals:

For photosynthetic organisms to thrive, they must harvest light while protecting themselves from damage during periods of high light or other stresses. These dual requirements for light harvesting efficiency and photoprotection may at first seem at odds: Efficient light harvesting requires strong couplings between chromophores to drive fast transfer and outcompete radiative losses. Photoprotection demands facile and reversible disruption of these same couplings to drive quenching. Thus, the coupling between chromophores must be strong and robust for fast, reliable transfer but also weak enough to allow redirection of the energy. Yet, photosynthetic light harvesting antennae manage to accomplish both processes creating robust yet switchable dynamics. Vibronic coupling mechanisms can resolve this paradox.

There are only a few ways to drive photoprotection: 1) couple a trap state or quencher strongly so that it outcompetes light harvesting pathways, 2) break a coupling within the complex to slow the light harvesting pathways, or 3) create a bottleneck where excitons interact and annihilate. All three mechanisms are operative in biology, but the first two mechanisms provide control over a greater dynamic range of light conditions because they work with individual excitations rather than simultaneous excitation. What we seek to understand is how to reliably outcompete or break strong couplings between chromophores in a switchable fashion.

We use ultrafast spectroscopy to investigate the microscopic physical mechanisms that provide robust yet switchable control of light harvesting. While the proteins, quenchers, and biological signaling differ dramatically across photosynthetic organisms, the underlying physics of coupling, internal conversion, fluorescence, and annihilation does not. This allows comparison of the control strategy and design principles across complexes and organisms. In this work, we will examine phycobilisomes from cyanobacteria and the Fenna-Matthews-Olson Complex from green sulfur bacteria. We will observe how biology "turns the knobs" using feedback mechanisms that regulate and direct photosynthetic light harvesting. **In particular, we will investigate mechanisms of coherent energy transport that exploit vibronic coupling — resonances between vibrations and electronic energy gaps — as such mechanisms particularly suited for robust transport yet sensitive regulatory control. By identifying patterns of vibronic coupling in photosynthetic antenna complex, we seek to understand how this coupling can be used as a design principle in excitonic systems.**

Significant achievements (2022-2023):

We now have data from c-phycocyanin and allophycocyanin within an intact phycobilisome showing different patterns of vibronic coupling and we have extended coherent modified redfield theory to allow off-diagonal system-bath couplings to model the dynamics. We have been able to extract experimental spectral densities for both APC and CPC (shown above), and we see important differences in the vibronic structure between complexes. In short, we see that the vibronic mixing serves both to facilitate energy transfer as well as relaxation within the vibrational manifold as a rate far faster than the ground state vibrational lifetime. This work has not yet been submitted.

Science priorities for the next year (2023-2024):

- Finish analysis of vibronic coupling in the phycobilisome and publish both the theoretical method advancements and the experimental data.
- Address coupling of OCP to the phycobilisome
- Isolate and acquire data on IsiA and Bacteriorhodopsin

My major scientific area(s) of expertise is/are: Ultrafast spectroscopy, quantum sensing, quantum dynamics.

To take my project to the next level, my ideal collaborator would have expertise in: We benefit immensely from extant close collaborations with biologists who can help use to identify samples, design controls, and provide biological context for our measurements.

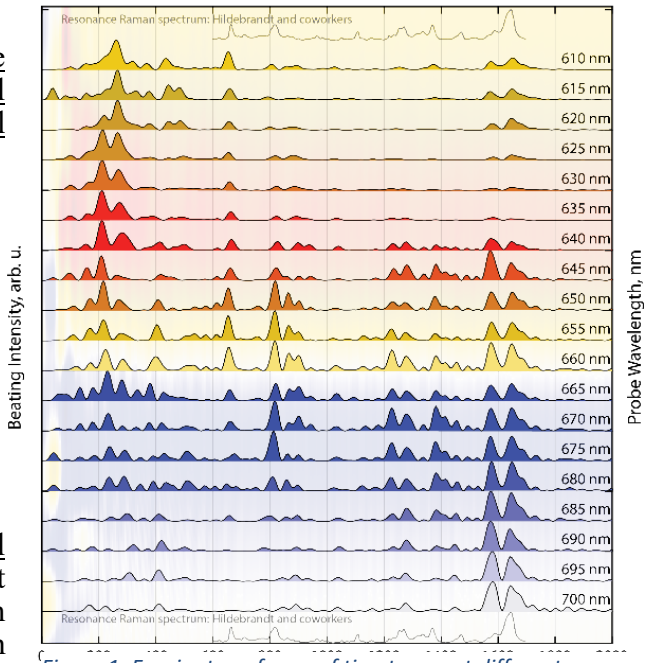

Figure 1: Fourier transforms of timetraces at different wavelengths. The x- axis shows the vibrational beating frequency. Intensity is normalized to maximum at each wavelength. The resonance Raman spectrum of C-phycocyanin is shown for comparison from work by Hildebrandt and coworkers. This data allows isolation of spectral densities for APC and CPC

Publications supported by this project :

1 Higgins, J. S., Allodi, M. A., Lloyd, L. T., Otto, J. P., Sohail, S. H., Saer, R. G., Wood, R. E., Massey, S. C., Ting, P.-C. & Blankenship, R. E. Redox conditions correlated with vibronic coupling modulate quantum beats in photosynthetic pigment–protein complexes. *Proceedings of the National Academy of Sciences* **118**, e2112817118 (2021).

2 Higgins, J. S., Lloyd, L. T., Sohail, S. H., Allodi, M. A., Otto, J. P., Saer, R. G., Wood, R. E., Massey, S. C., Ting, P.-C. & Blankenship, R. E. Photosynthesis tunes quantum-mechanical mixing of electronic and vibrational states to steer exciton energy transfer. *Proceedings of the National Academy of Sciences* **118**, e2018240118 (2021).

3 Onizhuk, M., Sohoni, S., Galli, G. & Engel, G. S. Spatial patterns of light-harvesting antenna complex arrangements tune the transfer-to-trap efficiency of excitons in purple bacteria. *The Journal of Physical Chemistry Letters* **12**, 6967-6973 (2021).

4 Rolczynski, B. S., Yeh, S.-H., Navotnaya, P., Lloyd, L. T., Ginzburg, A. R., Zheng, H., Allodi, M. A., Otto, J. P., Ashraf, K. & Gardiner, A. T. Time-domain line-shape analysis from 2d spectroscopy to precisely determine hamiltonian parameters for a photosynthetic complex. *The Journal of Physical Chemistry B* **125**, 2812-2820 (2021).

5 Higgins, J. S., Dardia, A. R., Ndife, C. J., Lloyd, L. T., Bain, E. M. & Engel, G. S. Leveraging Dynamical Symmetries in Two-Dimensional Electronic Spectra to Extract Population Transfer Pathways. *The Journal of Physical Chemistry A* **126**, 3594-3603 (2022).

6 Navotnaya, P., Sohoni, S., Lloyd, L. T., Abdulhadi, S. M., Ting, P.-C., Higgins, J. S. & Engel, G. S. Annihilation of Excess Excitations along Phycocyanin Rods Precedes Downhill Flow to Allophycocyanin Cores in the Phycobilisome of Synechococcus elongatus PCC 7942. *The Journal of Physical Chemistry B* **126**, 23-29 (2022).

7 Sohoni, S., Lloyd, L. T., Hitchcock, A., MacGregor-Chatwin, C., Iwanicki, A., Ghosh, I., Shen, Q., Hunter, C. N. & Engel, G. S. Phycobilisome's Exciton Transfer Efficiency Relies on an Energetic Funnel Driven by Chromophore–Linker Protein Interactions. *Journal of the American Chemical Society* (2023).

Multidisciplinary Tools for Examining the Molecular Details of Photosynthetic Light Harvesting Systems

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Overall research goals:

Determining the key molecular features that facilitate energy transduction in photosynthetic systems is paramount to our future ability to harness and mimic them in the context of photocatalytic materials and optical sensors. A full and detailed understanding of photosynthetic light harvesting has remained elusive, however, due to our inability to determine the individual contributions of the many functional components that must work in concert to yield higher-level function. Additionally, the effects of protein dynamics and conformational disorder on the performance of these systems are poorly understood and often overlooked. In this project, we are applying an integrated set of biological, chemical, spectroscopic, and computational tools to examine how these features work together in the context of phycobilisomes and thylakoid membranes. By producing analogs of natural systems with isolated chromophores, we are determining how the molecular details of the individual chromophore binding pockets influence energy transfer in phycocyanin and allophycocyanin pigment proteins. We are also using bioconjugation chemistry to generate well-defined protein-protein contacts, with the goal of measuring the rates of energy transfer between individual assemblies. Finally, we are examining the ways in which proteins and lipids interact to influence the structure and dynamics of thylakoid membranes. The strength of this project lies in its tight feedback loop of biosynthesis, spectroscopy, and multiscale modeling, which come together to characterize how pigment compositions, geometries, ranges of motion, and surrounding environments are tuned by nature to achieve optimal function in these complex, multicomponent systems.

Significant achievements (2021-2023):

- We have developed an efficient protein expression system to produce phycobilisome proteins in *E. coli* hosts. This greatly accelerates the generation of new protein mutants and protein libraries.
- Using this system, we have produced systematic sets of phycocyanin and allophycocyanin assemblies that lack chromophores in key positions. This has allowed us to characterize the influences of individual chromophore environments in more detail than was previously possible.
- We have produced specific phycobiliprotein mutants to test specific hypotheses about how the protein environments modulate chromophore properties.
- New residue scanning expression systems have been established to produce fitness landscape libraries of phycobilisome proteins. This will allow the effects of all possible single point mutations to be evaluated in a rapid fashion. We have also developed high throughput screening assays that can evaluate the shifts in chromophore properties that occur with each mutation.
- New protein chemistry has been developed to couple large assemblies with specific geometric relationships. This has been used to study TMV-based synthetic light harvesting systems, and is currently being applied to phycobilisome assemblies.
- We have run MD simulations to examine how protein environments restrict chromophore movements and thereby control the dynamics of light harvesting systems.
- Charge detection mass spectrometry has been used to characterize large protein assemblies, including artificial light harvesting systems build from tobacco mosaic virus coat proteins.

Science priorities for the next year (2023-2024):

- We will continue to characterize mutants of phycobilisome proteins, with the goal of determining the molecular features of the surrounding protein environments that facilitate energy transfer.
- Fitness landscapes and high-throughput fluorescence analysis techniques will be used to examine the effects of remote mutations on chromophore properties.
- Phycobilisome components will be linked through specific locations, and the rates of energy transfer will be measured between individual assemblies.

- Computational modeling will be used to understand how protein environments tune the spectroscopic properties of individual chromophores, and to understand how large-scale protein fluctuations can influence energy transfer dynamics.

My major scientific area(s) of expertise is/are: Protein modification chemistry, organic synthesis, chromophore chemistry, DFT calculations, and MD simulations, .

To take my project to the next level, my ideal collaborator would have expertise in: SAXS data analysis for protein complexes and cryo-TEM for structural analysis.

Publications supported by this project :

1. Bischoff, A. J.; Hamerlynck, L. M.; Li, A. J.; Roberts, T. D.; Ginsberg, N. S.; Francis, M. B. "Protein-Based Model for Energy Transfer between Photosynthetic Light-Harvesting Complexes Is Constructed Using a Direct Protein–Protein Conjugation Strategy" *J Am Chem Soc* **2023**, *145*, 15827-15837. DOI: 10.1021/jacs.3c02577.

2. Dai, J.; Wilhelm, K. B.; Bischoff, A. J.; Pereira, J. H.; Dedeo, M. T.; García-Almedina, D. M.; Adams, P. D.; Groves, J. T.; Francis, M. B. "A Membrane-Associated Light-Harvesting Model is Enabled by Functionalized Assemblies of Gene-Doubled TMV Proteins" *Small* **2023**, *19*, 2207805. https://doi.org/10.1002/smll.202207805.

3. Bischoff, A. J.; Harper, C. C.; Williams, E. R.; Francis, M. B. "Characterizing Heterogeneous Mixtures of Assembled States of the Tobacco Mosaic Virus Using Charge Detection Mass Spectrometry" *J Am Chem Soc* **2022**, *144*, 23368-23378. DOI: 10.1021/jacs.2c09160.

4. Harper, C. C.; Miller, Z. M.; Lee, H.; Bischoff, A. J.; Francis, M. B.; Schaffer, D. V.; Williams, E. R. "Effects of Molecular Size on Resolution in Charge Detection Mass Spectrometry" *Analytical Chem* **2022**, *94*, 11703-11712. DOI: 10.1021/acs.analchem.2c02572.

5. Hamerlynck, L. M.; Bischoff, A. J.; Rogers, J. R.; Roberts, T. D.; Dai, J.; Geissler, P. L.; Francis, M. B.; Ginsberg, N. S. "Static Disorder has Dynamic Impact on Energy Transport in Biomimetic Light-Harvesting Complexes" *J Phys Chem B* **2022**, *126*, 7981-7991. DOI: 10.1021/acs.jpcb.2c06614.

6. McKeon, C. A.; Hamed, S. M.; Bruneval, F.; Neaton, J. B. "An optimally tuned range-separated hybrid starting point for ab initio GW plus Bethe–Salpeter equation calculations of molecules" *J Chem Phys* **2022**, *157*, 74103. DOI: 10.1063/5.0097582.

7. Dai, J.; Knott, G. J.; Fu, W.; Lin, T. W.; Furst, A. L.; Britt, R. D.; Francis, M. B. "Protein-Embedded Metalloporphyrin Arrays Templated by Circularly Permuted Tobacco Mosaic Virus Coat Proteins " *ACS Nano* **2021**, *15*, 8110-8119. DOI: 10.1021/acsnano.0c07165.

8. Song, C.; Neaton, J.B.; Martínez, T. J. "Reduced Scaling Formulation of CASPT2 Analytical Gradients Using the Supporting Subspace Method" *J Chem Phys* **2021**, *154*, 014103. DOI: 10.1063/5.0035233.

9. Song, C.; Martínez, T. J.; Neaton, J.B. "An Automatic Differentiation and Diagrammatic Notation Approach for Developing Analytical Gradients of Tensor Hyper-Contracted Electronic Structure Methods" *J Chem Phys* **2021**, *155*, 24108. DOI: 10.1063/5.0055914.

10. Ramsey, A. V.; Bishoff, A. J.; Francis, M. B. "Enzyme Activated Gold Nanoparticles for Versatile Site-Selective Bioconjugation" *J Am Chem Soc* **2021**, *143*, 7342-7350. DOI: 10.1021/jacs.0c11678.

Collaborative Project: Dynamics and consequences of PSI Supercomplexes

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Xing Wang, Wah Chiu, Rachael Morgan-Kiss, Ru Zhang, Co-PI(s)
Associate Researcher: Jay-How Yang, graduate students: Najia Nihum undergraduate students: Jackson Carrion and Joseph Boyer
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Overall research goals:

The overall principal research goal is to understand how PSI plasticity contributes to the consequences of CEF during short- and long-term stress acclimation. There are three challenge objective goals under this principal goal. 1. To determine the localization and structure of the PSI super-complex in the *C. priscuii* (formerly *C. sp.* UWO241). 2. To determine the roles of PSI-super-complexes and PSI-megacomplexes and CEF in stress response over broad time scales. 3. To dissect the underlying consequences of sustained high CEF on supporting robust growth and photosynthesis under environmental stress. The three-challenge objective goal will be accomplished through the following tasks: 1. To determine the ultrastructure of the *C. priscuii* PSI super-complex using Cryo-EM and localize the UWO241 high-salt PSI supercomplex in relation to other thylakoid complexes using Cryo-ET (Dr. Fromme at Arizona State University, Dr. Chiu at SLAC National Accelerator Laboratory). 2. To monitor presence of PSI super- and mega-complexes and CEF activation/function during stress acclimation and to characterize proteomes of the PSI super-complex (Dr. Morgan-Kiss and Dr. Wang at Miami University, Dr. Zhang at Danforth Plant Science Center). Also, to investigate excitation energy transfer of the PSI super-complex (Dr. Fromme at Arizona State University). 3. To determine the contribution of CEF to cellular energy charge status and the consequences of high CEF in driving high flux ATP-consuming pathways (Dr. Morgan-Kiss and Dr. Wang at Miami University).

Significant achievements (2022-2023):

- During the first year of our renewal project, PI Petra Fromme and her research scientist, Dr. Yang, and the graduate students have made progress on the following major activities:

1. Stabilization of the Chl. Priscii PSI supercomplex for CryoEM grid preparation, data collection, and data analysis:

In 2022 and 2023, we freshly prepared samples using the original method where the PSI super-complex was solubilized in low yields in 1% n-dodecyl-α-D-maltoside (α-DDM) that were further stabilized by lipids. Samples were prepared at ASU and the final steps Dr. Yang performed at SLAC to prepare the supercomplex on site for cryo-EM analysis. From the cryo-EM grids that were screened, nice sample particle density in a thin film of ice were observed and overnight data were collected, resulting in 1166 micrographs collected with the pixel size of 0.86Å; the estimate resolution was between 5.7Å and 5.9Å. Around 9000 micrographs with the pixel size of 0.86Å and an estimate resolution from 3.0Å to 4.3Å were collected overnight in 2023. After initial auto-particle-picking, a total number of >1.7 billion particles were picked. The data-processing is in progress. With this dramatic increase in particle number from 0.6 to 1.7 billion, the estimated resolution could be extended to 3Å. Data processing is in progress.

2. The ASU Algae Farm of *C. priscuii* (UWO241):

One of the challenges of the project in the past was that the cells were grown at U of Miami and the cells and thylakoids had to be shipped to ASU for the isolation of the PSI supercomplex. Therefore we designed a prototype culture tank at ASU to establish the culturing of the very sensitive Chl priscii cells at ASU. We has setup an “algae farm” for temperature controlled growth where cells are illuminated by LED light. We already started cultures immediately after receiving the subculture from the team of Dr. Morgan-Kiss. This new culture supply will benefit our structure studies of the PSI super-complex and also provides a “backup” culture of cells for the whole collaboration.

3. Leica Automatic Grid Plunger (Leica EM GP2) installation:

The ASU team has previously relied on a vitrobot plunge freezing systems that was shared with the whole Cryo-EM community of researchers at ASU, leading to frequent misalignments of the system so that reproducible grid freezing was a challenge. In 2023 the Biodesign Center for Applied Structural Discovery purchased and installed a new Leica EM-GP2 plunge freezer at Arizona State University. This instrument now allows us to plunge- freeze samples on-site without a need to travel to the SLAC cryo-EM facility after sucrose gradient ultracentrifugation of the samples. In addition, we also purchased the cryo-EM grid clipping station and the cryo-EM grid puck for storage and shipping. We anticipate that incorporating the new capability of these instruments will dramatically further improve our progress as we can now prepare high quality grids at ASU and analyze them by Cryo-EM at the high resolution Cryo-EM facility at SLAC.

4. Optimizing the purification condition of PSI super-complex from Chlamydomonas priscii:

Dr. Yang was working closely with the team of Dr. Morgan-Kiss at Miami University to optimize the purification condition. He screened the detergent concentration and solubilization time. There was a green band on the bottom of the sucrose gradient, which is a candidate of an even larger PSI mega-complexes, which we are currently investigating by Cryo-EM.

5. Progress on ultrafast spectroscopy of the PSI supercomplex.

Fluorescence emission spectra and kinetics were further analyzed using streak camera techniques. Detailed data analysis has further progressed leading to the following conclusions: (i) The PSI Chlamy Priscuii supercomplex contains a huge fully coupled antenna system (ii) It contains fewer long wavelength chlorophylls than any of the other PSI systems (iii) The antenna is so large that it takes 6 times longer for the excitation energy to be trapped (fastest charge separation takes 235ps vs 40-44ps in all other PSI systems (iv) the unique feature was detected that most of the excitation energy takes even longer 1790 ps to be trapped. (v) All chlorophylls in this huge PSI supercomplex are as tightly coupled as in cyanobacterial trimeric PSI as indicated by the lack of long lived fluorescence.

Science priorities for the next year (2023-2024):
ASU has started to grow the culture of the *C. priscuii* which we plan to use as fresh supply for our structural and functional studies of the supercomplex avoiding the challeges of shipping and freezing. This will allow us to make fast progress on the ptimizing the purification protocol of the supercomplex from *C. priscuii* and also study the influence of environmental factors (light, temperature nutrients) on the structure of the supercomplex. Also, the team of ASU will also optimize the sample preparation of cryo-EM grids, including variety of cryo-EM grids (carbon and gold grids), blotting time and blotting force using the new Gp2 plunge freezer. To push resolution beyond 3Å and determine the cryo-EM structure of the PSI super-complex at high-resolution, we will need to collect more than 4000 high-quality micrographs with a density of ~500 particles per micrography. Thus, new sections of grid screening and data collection will continue with appropriate samples isolated with the optimized protocol as well as data processing. We also plan to investigate the MEGA complex that we recently isolated under extremely mild detergent extraction conditions by Cryo-EM and functionally characterize the complex by streak Camera Fluorescence Spectroscopy.

My major scientific area(s) of expertise is/are: structural biology, protein crystallography, femtosecond X-ray crystallography, vryo-EM.

To take my project to the next level, my ideal collaborator would have expertise in: High resolution native mass spectroscopy

Publications in preparation that are supported by this project :(*, undergraduate student; **, graduate student; **bold**, PI or Co-PI on this project).

Journal Article (in preparation):

Yang, J-H, J. Carrion**, N Nihum*, J Goyer,** Morgan-Kiss, R and **Fromme,P** " Isolation and Stabilization of a Photosystem I supercomplex from the Antarctic green algae *Chlamydomonas priscuii* for structural studies", 2023, in preparation for submission to *"Protein Isolation and Purification"*

Journal Article (in preparation):

Yang,J-H., Nijhum*, NQ,Carrion**, J, Lin, S, Morgan-Kiss,R. and **Fromme,P** " A large, tightly coupled antenna system in Photosystem I supercomplexes from the Antarctic green algae *Chlamydomonas priscuii* revealed by fluorescence spectroscopy" ,2023, in preparation for submission to "*Photosynthesis Research*"

This results from this work were also presented in form of a poster at the 2023 Photosynthesis Gordon Research Conference

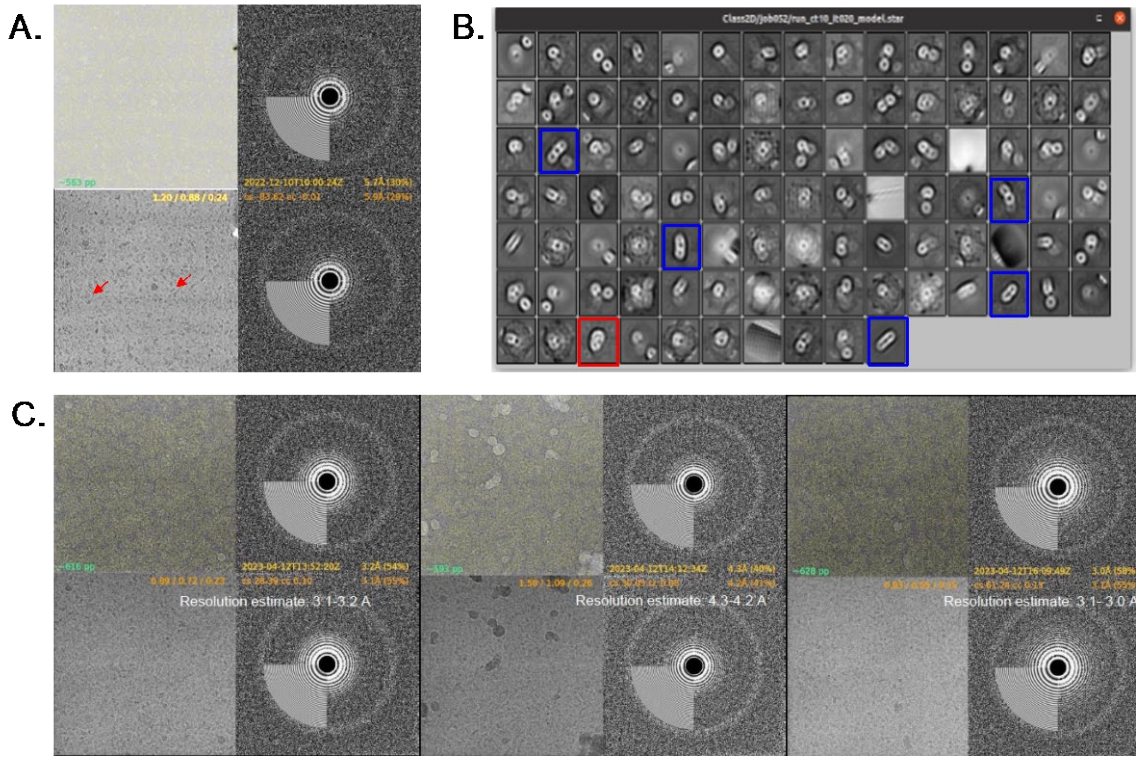

Figure 1. Image information and 2D classification of the collected Cryo-EM micrographs in 2022 and in 2023.

A. a number of 1166 images was collected overnight with the pixel size of 0.86Å and the estimate resolution was between 5.7Å and 5.9Å in December 2022. The potential candidate of the PSI supercomplex were shown as red arrow. B. a total number of picked particles of 1166 micrographs were around 622,000, which were classified into 100 classes. 6 of 100 classes were selected as candidates of potential PSI supercomplexes (framed by red and blue). After filtering and auto-particle-picking, a total number of 165 particles was picked in the red class, and a total number of 100,326 particles was picked in the blue class. C. ~9000 micrographs with the pixel size of 0.86Å and the estimate resolution from 3.0Å to 4.3Å were collected overnight in April 2023, and a total number of more than 1.7 billion particles were auto-picked. The data-processing is in progress.

Exploring the Functions of Chloroplast TRIOSE PHOSPHATE TRANSPORTERs and Other Plastid Activities in Partitioning Photoassimilate in Chlamydomonas

Weichao Huang (黄伟超)[1], Anagha Krishnan[2]†, Anastasija Plett[3], Michelle Meagher[4], Nicole Linka[3], Yongsheng Wang[1,5], Bijie Ren[1], Justin Findinier[1], Petra Redekop[1], Neda Fakhimi[1], Rick G. Kim[1], Devin A. Karns[2], Nanette Boyle[4], Matthew C. Posewitz[2], Arthur R. Grossman[1]

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ABSTRACT

The regulation of fixed carbon export from chloroplasts is essential for controlling growth and maintenance of the cell's physiological processes, including photosynthesis and respiration. We show that two of the chloroplast triose phosphate/phosphate translocators, CreTPT2 and CreTPT3, of the green alga *Chlamydomonas reinhardtii* (Chlamydomonas) have similar substrate specificities and exhibit differential expression over the diel cycle. The Cre*TPT3* gene is highly expressed, with the protein product localized to the chloroplast envelope. CreTPT3 displays its highest activities when using DHAP and 3-PGA as substrates, and strains with null mutations in this gene exhibit a severe, pleiotropic phenotype; the mutants grow more slowly than wild-type cells even in low light and show aberrant photosynthetic activities and metabolite profiles, with organelle-specific accumulation of H_2O_2. Null mutants for the Cre*TPT2* gene are also compromised for growth and are sensitive to high light, but the phenotype of the *tpt2* mutant is much less severe than that of the *tpt3*. In contrast, the *tpt1* mutant has little phenotype in the light but appears to be unable to grow in the dark. Our current results demonstrate that CreTPT3 is a dominant conduit on the chloroplast envelope for the transport of photoassimilate to other subcellular locations and functions to move fixed carbon and reductant out of the chloroplast, which is essential for preventing the cells from experiencing oxidative stress and accumulating reactive oxygen species. Our results also indicate subfunctionalizations of CreTPT transporters in Chlamydomonas, enabling management of the distribution of photoassimilate under dynamic environmental conditions. Other aspects of carbon partitioning in cells that we are examining include metabolite transport by oxaloacetate-malate transporters, the impact of strains compromised for the production of NADP (strains defective for NAD kinases) on the physiology of cells, and the integration of mitochondrial and chloroplast function, and especially the role of mitochondria in acquiring and assimilating inorganic carbon when CO_2 levels decline.

Acknowledgements: This work was supported by a grant from the US Department of Energy, Office of Science, Basic Energy Systems (DE-SC0019417 to ARG), which was used as the sole support for WH. AK and MCP were supported solely by a grant from the US Department of Energy, Office of Science, Basic Energy Systems (DE-SC0019341 to MP). NB and MM were supported by US Department of Energy, Office of Science, Office of Biological and Environmental Research (BER), (DE-SC0018301 to NB). NL and AP were supported by the

German Research Foundations (DFG Grant LI1781/2-2). BR was supported by the National Science Foundation (Award Number 1645164). RGK and JF were supported by the Department of Plant Biology of the Carnegie Institution for Science. PR was supported by Human Frontier Science Program (HFSP) RGP0046/2018 (to ARG).

Title of grant: Using ancient enzymes for modern photosynthesis
Title of Poster: Harnessing nature's solutions for enhanced biological CO_2 fixation

Laura Gunn, Principal Investigator
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Overall research goals:

The CO_2-fixing enzyme Ribulose-1,5-carboxylase/oxygenase (Rubisco) represents the major point of carbon entry into the biosphere. Rubisco has a slow catalytic rate and exhibits poor substrate specificity such that Rubisco catalysis often limits the growth rate of photosynthetic organisms, including crop species. Rubisco's catalytic limitations are exacerbated under elevated temperature, which is especially concerning because the average global temperature is projected to increase by up to 5.7ºC by the year 2100. One approach to equipping Rubisco to perform better under increasingly challenging environmental conditions is to take advantage of the wealth of Rubisco functional diversity that has evolved in different lineages under different selection pressures. Accordingly, the Gunn lab uses computational biology, structural biology (x-ray crystallography, cryo-electron microscopy, small angle x-ray scattering), synthetic biology (SynBio), and biochemical approaches to develop the prerequisite mechanistic information and tools to adapt more efficient CO_2-fixing strategies from diverse organisms to synthetic biology systems and vascular plants.

Presented at this meeting will be an overview of the lab's research directions, including prior successes and ongoing work. These directions include the DOE CAREER-funded grant to understand the mechanisms underlying the adaptation of Solanaceae Rubisco from a hot and high CO_2 environment (ancestrally-reconstructed sequences from 20-25 million years ago) to a relatively cool and low CO_2 environment (current era before recent climate change). Understanding the mechanism behind Rubisco kinetic and thermal adaptation to modern day can be applied in reverse: how can we adapt current Rubiscos to a warmer climate? This project has three specific aims: (i) determine the kinetic and temperature contributions of independent amino acid substitutions in high-performing ancestrally reconstructed Solanaceae Rubiscos, (ii) model the Photosynthetic Carbon Assimilation (PCA) of reconstructed Rubiscos in a C3 chloroplast in response to varying CO_2 partial pressures and temperatures, and (iii) test whether the biochemical alterations to CO_2-reduction conferred by specific amino acid substitutions in reconstructed Rubiscos translate to increased plant growth and photosynthesis *in planta*.

Other research directions presented include: developing Rubisco SynBio systems, harnessing (the superior) Rubisco kinetic properties from rhodophytes, providing fundamental insights into Rubisco biogenesis including the biogenesis requirements of divergent plant lineages and characterizing and engineering CO_2-concentrating mechanisms from divergent plant lineages.

Significant achievements (2023-2028)

Award granted July
-Currently rotating prospective graduate students to begin the project in 2024
-Set up computational environment and analysis pipeline for estimating the stability effect of amino acid substitutions in Rubisco, using FoldX.
-Optimized protocol for melting curves using Sypro Orange in 384 well plates in a quantitative-PCR machine – for both purified Rubisco, and from *E. coli* soluble protein.

Science priorities for the next year (2023-2024):

- Select two graduate students to work on the project

- Obtain CryoEM structure of a specific tobacco Rubisco variant
- Select residues to probe from previously reported ancestrally reconstructed Rubisco sequences
- Construct new Rubisco variants to probe the individual and combinatorial effects of substitutions in ancestrally reconstructed Rubisco
- Computationally predict the stability effects of selected substitutions on tobacco Rubisco, and similarly explore the entire Rubisco energy landscape (*in silico* saturating mutagenesis)

My major scientific area(s) of expertise is/are: Structural Biology (predominantly X-ray crystallography and CryoEM), Biochemistry, Protein engineering, Synthetic Biology.

To take my project to the next level, my ideal collaborator would have expertise in: Molecular evolution, Establishing new transformation capabilities in crop species

Publications supported by this project: N/A. New award

Studies of Photosynthetic Reaction Centers and Biomimetic Systems

Marilyn Gunner, Principal Investigator
Victor Batista, Co-PI(s)
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Overall research goals:

Click here to enter text. Gunner and Batista work in collaboration with Brudvig and Wang (Yale), Debus (UC Riverside), Kaur (Brock), Lakshmi (RPI), Armstrong (BU), Amin (NIH), Kirmaier and Holten (Wash U), Kern (LBL) and Guadagno (U Wy).

• Pathways for proton transfers. In Photosystem II (PSII) the Oxygen Evolving Center (OEC) is buried ≈15Å from the lumen. There are multiple channels on the lumen side of the protein. We are tracing and evaluating the hydrogen bonded pathways in PSII from multiple species.

•Barriers for key S-state transitions. We determine the impact of water orientation around the OEC on the energy for water exchange, insertion of substrate water into the catalyst and O-O bond formation.

•Control of the direction of electron transfer in type II reaction centers All reaction centers have two symmetric paths for electron transfer. In PSII and type II bacterial reaction centers the electron chooses one path. We ask if the relative electron affinity of symmetrically arranged cofactors controls this choice.

•Determine the criteria for binding different chlorophyll (Chl) types. The light harvesting protein LHCII binds Chl a and b. Cyanobacteria adapted for far-red light bind Chl a, d and f. Simulations try to identify the determinants for choosing the correct Chl type in each binding site with an aim to modify Chl composition.

•Develop computational tools to enhance studies of photosynthetic proteins. Gunner has developed MCCE to evaluate the electrochemical midpoint potential and the distribution of protonation states and hydrogen bonds. Unique analysis provides the correlation of protonation states and hydrogen bonding in the Boltzmann ensemble. The Batista lab has developed enhancements of QM/MM and other methodologies. New MD force fields that allow for exchange of OEC water ligands are being developed.

Significant achievements ([2021-2023])

[From 2021-2023 13 papers were published, one is being revised and one submitted.

• Proton transfer paths were mapped from the OEC in *T. vulcanus* PSII and to Q_A and Q_B in bRCs. The broad channel is found to be he proton transfer path and Q_A is isolated so it cannot bind protons (2,5,12).

• The S-state transitions wer studied with methanol bound in the N87A mutant and with glycerol bound (6,9).

• Quantum delocalization traps a proton between the OEC ligand W1 and D61 to keep a proton from being released on formation of the S_2 state (10).

• The interactions within a Chl binding site is insufficient to fully control the type of Chl that is bound (1).

•Cryo-EM structures were determined of the mesophilic cyanobacterium, Synechocystis sp. PCC 6803, of a far-red light acclimatized PSII and of a helical allophycocyanin (3,7,8, 13).

•Analysis shows heterogeneity of PSII structures and the degree of reduction of the Mn center in the XFEL structures (4,11)

Science priorities for the next year (2023-2024):

[•Waters poised to be substrata and those that are prepared to receive the product protons will be found in each S-state. QM/MM and DFT calculations of the barriers to the proton transfer and bond making with different water configurations to study water exchange, water insertion into the OEC in the S_3 state, and O-O bond formation.

•Regions further from the OEC differ between different species. The proton pathways in *T. vulcanus* will be compared with the pathways in Pea, Spinach and from the mesophilic cyanobacterium, Synechocystis sp. PCC 6803. The hydrogen bonded networks will be found within MD trajectories and with MCCE.

•Kirmaire and Holten have made mutants to coax the electron down the inactive path in bacterial RCs and Kern is solving mutant structures. MCCE calculations will determine the relative electron affinity of the cofactors on the active and inactive branches in wild-type protein and mutated proteins.

My major scientific area(s) of expertise is/are: Computational studies use MD, QM/MM and DFT. Gunner uses MCCE to obtain redox and protonation states and hydrogen bond networks. Batista develops new QM/MM tools and MD force fields. Amin uses Machine Learning. Experimentalist collaborators include Debus (FTIR), Lakshmi (EPR) Brudvig, Gisriel, Wang (PSII biochemistry and structure determination).

To take my project to the next level, my ideal collaborator would have expertise in: Computer programing to move MCCE from a program for specialists to be a standard computational tool.

Publications supported by this project [Enter Publications Supported by This Grant/2021-2023] :

1) Ranepura, G., Mao, J, Vermaas,J.V., Wang,J. Gisriel,C.J., Wei,R. Ortiz-Soto,J. Md. Raihan Uddin,M.R., Amin,M., Brudvig, G.W. and Gunner, M.R. (accepted for publication) Computing the relative affinity of chlorophylls *a* and *b* in light-harvesting complex II, *J. Phys. Chem B.*

2) Wei, R. J., Khaniya, U., Mao, J., Liu, J., Batista, V. S., and Gunner, M. R. (2023) Tools for analyzing protonation states and for tracing proton transfer pathways with examples from the *Rb. sphaeroides* photosynthetic reaction centers, *Photosynth Res 156*, 101-112.

3) Gisriel, C. J., Elias, E., Shen, G., Soulier, N. T., Flesher, D. A., Gunner, M. R., Brudvig, G. W., Croce, R., and Bryant, D. A. (2023) Helical allophycocyanin nanotubes absorb far-red light in a thermophilic cyanobacterium, *Sci Adv 9*, eadg0251.

4) Amin, M. (2023) Predicting the oxidation states of Mn ions in the oxygen-evolving complex of photosystem II using supervised and unsupervised machine learning, *Photosynth Res 156*, 89-100.

5) Wei, R. J., Zhang, Y., Mao, J., Kaur, D., Khaniya, U., and Gunner, M. R. (2022) Comparison of proton transfer paths to the Q_A and Q_B sites of the *Rb. sphaeroides* photosynthetic reaction centers, *Photosynth Res,*52, 153-165.

6) Kalendra, V., Reiss, K. M., Banerjee, G., Ghosh, I., Baldansuren, A., Batista, V. S., Brudvig, G. W., and Lakshmi, K. V. (2022) Binding of the substrate analog methanol in the oxygen-evolving complex of photosystem II in the D1-N87A genetic variant of cyanobacteria, *Faraday Discuss 234*, 195-213.

7) Gisriel, C. J., Wang, J., Liu, J., Flesher, D. A., Reiss, K. M., Huang, H. L., Yang, K. R., Armstrong, W. H., Gunner, M. R., Batista, V. S., Debus, R. J., and Brudvig, G. W. (2022) High-resolution cryo-electron microscopy structure of photosystem II from the mesophilic cyanobacterium, Synechocystis sp. PCC 6803, *PNAS 119*. e2116765118

8) Gisriel, C. J., Shen, G., Ho, M. Y., Kurashov, V., Flesher, D. A., Wang, J., Armstrong, W. H., Golbeck, J. H., Gunner, M. R., Vinyard, D. J., Debus, R. J., Brudvig, G. W., and Bryant, D. A. (2022) Structure of a monomeric photosystem II core complex from a cyanobacterium acclimated to far-red light reveals the functions of chlorophylls d and f, *J Biol Chem 298*, 101424.

9) Flesher, D. A., Liu, J., Wiwczar, J. M., Reiss, K., Yang, K. R., Wang, J., Askerka, M., Gisriel, C. J., Batista, V. S., and Brudvig, G. W. (2022) Glycerol binding at the narrow channel of photosystem II stabilizes the low-spin S(2) state of the oxygen-evolving complex, *Photosynth Res 152*, 167-175.

10) Yang, K. R., Lakshmi, K. V., Brudvig, G. W., and Batista, V. S. (2021) Is Deprotonation of the Oxygen-Evolving Complex of Photosystem II during the S_1--> S_2 Transition Suppressed by Proton Quantum Delocalization?, *J Am Chem Soc 143*, 8324-8332.

11) Wang, J., Gisriel, C. J., Reiss, K., Huang, H. L., Armstrong, W. H., Brudvig, G. W., and Batista, V. S. (2021) Heterogeneous Composition of Oxygen-Evolving Complexes in Crystal Structures of Dark-Adapted Photosystem II, *Biochemistry 60*, 3374-3384.

12) Kaur, D., Zhang, Y., Reiss, K. M., Mandal, M., Brudvig, G. W., Batista, V. S., and Gunner, M. R. (2021) Proton exit pathways surrounding the Oxygen Evolving Complex of Photosystem II, *Biochim Biophys Acta Bioenerg*, 148446.

13) Gisriel, C. J., Huang, H. L., Reiss, K. M., Flesher, D. A., Batista, V. S., Bryant, D. A., Brudvig, G. W., and Wang, J. (2021) Quantitative assessment of chlorophyll types in cryo-EM maps of photosystem I acclimated to far-red light, *BBA Adv 1*, 100019.

Characterizing Rubisco by Phylogeny-Informed Mutagenesis
DOE DE-SC0020142

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Overall research goals:

The current C_3 Rubisco enzymes are clearly adapted to CO_2 levels much lower than the present and future levels of > 400 ppm because preindustrial CO_2 levels were mostly below 300 ppm for the last 20 Myrs. Compared to the C_3 tobacco Rubisco, some ancestral Solanaceae Rubisco versions are likely to have been better adapted to higher CO_2 concentrations and could potentially provide higher carbon fixation rates at the current and future CO_2 levels. Our goal was to identify variants of Rubisco enzymes that have higher k_{cat} and equal or lower $S_{(C/O)}$.. Following identification of promising candidates through expression of Rubisco in *E. coli* and kinetic measurements, we would determine whether the kinetic measurements of plant-synthesized ancestral Rubisco enzymes are also superior to current-day Rubisco when expressed in tobacco. Replacing endogenous Rubisco with ancestral Rubisco will require production of a line that lacks all expression of nuclear-encoded Rubisco small subunits.

Significant achievements (2020-2023):

In order to have adequate large (LSU) and small subunit (SSU) sequences to use to predict ancestral Rubisco, we developed a semi-automated computational procedure named "START" (Selected Transcript Assembly from RNA-Seq data using Trinity) to expand the available sequences of both LSU and SSU in the family Solanaceae. The resulting computational efficiency allowed us to assemble sequences of both Rubisco subunits from the public RNA-Seq data of all available Solanaceae species within a week using a personal computer. Due to the technical limitation of the *de novo* assembly strategy employed by the Trinity program in distinguishing transcripts from a multigene family, we found that many of the assembled sequences of the small subunits are chimeric sequences. In order to readily identify such erroneously assembled transcripts, START runs Trinity multiple times for each RNA-Seq dataset and generates starting base read coverage for each assembled transcript. The program has been made available on Github.

We were able to obtain sequences of 151 unique small subunits representing 22 genera (vs. only 38 sequences in five genera from NCBI and Solgenomics). We were able to increase LSU sequences so that we had a total of 104 unique large subunits representing 36 genera. Using Bayesian (MrBayes) and Maximum Likelihood (RAxML) approaches, a phylogenetic tree was produced from which ancestral Rubisco variants could be predicted.

We produced clones for 98 predicted ancestral Rubisco variants using site-directed mutagenesis. Using our two-vector *E. coli* expression system, which was modified from the Hayer-Hartl group's original three-vector system, we expressed the 98 enzymes in *E. coli*. As an initial screening, we measured the RuBP carboxylation rates at a saturated [CO_2] using the soluble extracts of *E. coli* cultures. We found that about half of the variants had higher RuBP carboxylation rates compared to the reference enzyme made up of wild-type LSU and SSU-T2.

41 ancestral variants were selected and the RuBP carboxylation rates were measured at 25 °C under six different [CO_2] levels to obtain their Michaelis-Menten constants ($K_{M,air}$) and catalytic turnover numbers (k_{cat}). The majority of those predicted ancestral Rubisco variants possess higher k_{cat} than the native Rubisco from tobacco and the wild-type Rubisco with SSU-S1/T1/T2 produced in *E. coli*. More importantly, most of those ancestral Rubisco variants displayed catalytic efficiencies ($k_{cat}/K_{M,air}$) superior to the wild-type enzymes.

Six of the superior ancestral variants were selected for testing at 30 °C and some were found to perform better at 30 °C than at 25 °C. Furthermore, the specificity factor of these six variants were similar to that of native tobacco Rubisco and the wild-type LSU with S1 or T1 in *E. coli.*

In order to be able to examine the ancestral enzymes properties in tobacco, CRISPR/Cas9 mutagenesis was used to knock out all 11 functional tobacco *RbcS* genes in a plant line expression cyanobacterial RbcL and RbcS from the chloroplast genome. When tobacco *RbcL* was transformed into the line to replaced the cyanobacterial genes, the resultant plants are yellow and non-photosynthetic, as expected. We have now obtained green chloroplast transformants expressing one Nico and one Sola ancestral Rubisco.

Science priorities for the next year (2023-2024):

During a no-cost extension, we will perform kinetic measurements on ancestral enzymes expressed in plants. The levels of Rubisco achieved through chloroplast transformation vs. a combination of chloroplast and nuclear expression will be determined. We will prepare a manuscript describing the tobacco *RbcS* knockout line and the expression of the ancestral enzymes.

My major scientific area(s) of expertise is/are: C3 photosynthesis, gene expression in plant organelles; organelle cell biology

To take my project to the next level, my ideal collaborator would have expertise in: Rubisco structure and enzymology.

Publications supported by this project:

Lin MT, Salihovic H, Clark FK, Hanson MR. 2022. Improving the efficiency of Rubisco by resurrecting its ancestors in the family Solanaceae. Science Advances 8(15):eabm6871. doi: 10.1126/sciadv.abm6871.

Lin MT, Orr DJ, Worrall D, Parry MAJ, Carmo-Silva E, Hanson MR. 2021. A procedure to introduce point mutations into the Rubisco large subunit gene in wild-type plants. Plant J. 106(3):876-887 doi: 10.1111/tpj.15196

Hanson, MR. PL Conklin, A Sattarzadeh. 2021. Fluorescent labeling and confocal microcopy of plastids and stromules Methods Mol Biol 2317:109-132. doi: 10.1007/978-1-0716-1472-3_5.

Hanson, MR and PL Conklin. 2020 Stromules, functional extensions of plastids within the plant cell Current Opinion Plant Biology 58-25-32. doi: 10.1016/j.pbi.2020.10.005

Lin MT, Stone WD, Chaudhari V, Hanson MR. 2020. Small subunits can determine enzyme kinetics of tobacco Rubisco expressed in *Escherichia coli*. Nature Plants 6(10):1289-1299. doi: 10.1038/s41477-020-00761-5.

Solar Energy Conversion in Photosystem I Studied Using Time-resolved Visible and Infrared Difference Spectroscopy

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Overall research goals:

The goal of this project is to gain a molecular-level understanding of the factors that modulate the functional properties of quinones that occupy the A_1 binding site in photosystem I.

To address this goal, we use time-resolved infrared difference spectroscopy (TR IR DS) to study native and modified PSI. PSI will be modified either by incorporating different quinones into the A_1 binding site, or by using site directed mutagenesis to disrupt specific pigment-protein interactions. Much of the time-resolved work is undertaken on PSI samples at 77 K. This is done to specifically probe the $P700^+A_1^-$ radical pair state. Extending upon this goal we also study PSI samples in which the chlorophyll pigments that make up P700 are modified, and how this impacts the bioenergetics of the $P700^+A_1^-$ radical pair state.

Nanosecond TR IR DS was used to study $P700^+A_1^-$ to $P700^+F_x^-$ forward electron transfer at room temperature (295 K).

To more fully understand the TR IR DS, QM:MM computational methods are developed and used to simulate the difference spectra.

Significant achievements ([2022-2023]):

[Beyond the five publications reported below describing our most recent work, we have:

1. In collaboration with Dennis Nurnberg in Berlin, we have produced nanosecond time resolved infrared difference spectra at 295 K, and microsecond time resolved step scan FTIR difference spectra at 77 K, for PSI samples from *Chroococcidiopsis thermalis* PCC 7203 (*C. thermalis*) cells grown under white light and far-red light. This data, when compared to corresponding data (that we also obtained) for native PSI samples from *Synechocystis* sp. 6803 (S6803) and *Thermosynechococcus vestitus* BP-1 (TV), as well as that obtained for PSI from *Fischerella thermalis* cells grown under white light and far-red light, allow us to draw some very interesting and unexpected conclusions on the nature of P700.
2. We have found that P700 in PSI from *C. thermalis* cells grown under white light is highly abnormal and unlike that found in any other cyanobacterial strain. In contrast, P700 in PSI from cells grown under far-red light is quite normal and comparable to that found in PSI from other cyanobacterial strains grown under far-red light. It is likely that P700 is a monomeric chlorophyll-a species in PSI from cells grown under white light. Probably P700 is just the P_B pigment. The P_A pigment is not part of P700 and may not even be a chlorophyll-a' species. These conclusions were completely unexpected.
3. In PSI from white light grown cells from *C. thermalis*, although P700 is modified, we found that the phylloquinone species in the A_1 binding site is structurally similar to that found in other cyanobacterial strains.
4. In PSI from *C. thermalis* cells grown under far-red light two microsecond phases are observed, potentially suggesting two phases of electron transfer at 77 K. This was not expected.
5. From studies of PSI samples from *S6803* with mutations near the A_{-1} pigment (on both the B and the A branch) we found that the A_{-1} pigment is an integral part of P700. We also found that the bioenergetics in these A_{-1} mutants are considerably altered compared to native PSI.

6. We developed a method to produce flash induced absorption changes in the 1900-1770 cm^{-1} region, and showed we could use this to very accurate probe $P700^{+}A_1^{-}$ radical pair recombination at 77 K.
7. In collaboration with Jan Kern, we have been able to produce polarized ($P700^{+}$ - P700) FTIR difference spectra using PSI micro-crystals. Similar work has also been undertaken on purple bacterial reaction centers. The polarization patterns open a whole new area for understanding the nature of bands in the FTIR difference spectra and how they can be related to precise molecular structural details of the pigments in the protein complex.
8. Light induced FTIR difference spectra have been obtained for a series of purple bacterial reaction centers with a plethora of mutations designed to promote electron transfer down the inactive branch. This work is in collaboration with Phil Liable and Christine Kirmaier.

Science priorities for the next year (2023-2024):

- Incorporate a series of benzoquinone and anthraquinone derivatives into the A_1 binding site in PSI. Assess incorporation levels and study using nano-microsecond time-resolved infrared and visible difference spectroscopy at both 295 and 77 K.
- Undertake QM:MM vibrational frequency calculations for these benzoquinone and anthraquinone derivatives incorporated into the A_1 binding site in PSI.
- Use observed bioenergetics to assess what electron transfer theories may be applicable to PSI.
- Further nano-microsecond time resolved infrared spectroscopy work on PSI from white light and far-red light grown cells from *C. thermalis.*
- Undertake femto-nanosecond time resolved measurements on PSI samples with different quinones incorporated into the A_1 binding site.

My major scientific areas of expertise are: Vibrational spectroscopy. FTIR difference spectroscopy. Time-resolved spectroscopy of Photosystem I. Density functional theory based vibrational frequency calculations.

To take my project to the next level, my ideal collaborator would have expertise in: Topic area: Quantum chemical calculations of photosynthetic pigments in proteins. Victor Batista has relevant expertise. Topic area: Calculation of the EPR parameters of non-native quinones incorporated into the A_1 binding site. KV Lakshmi, Jens Niklas or Oleg Poluektov have relevant expertise. Topic Area: Spectroscopy of PSI microcrystals. Jan Kern has relevant expertise. Topic Area: Quantum cascade laser-based nanosecond time resolved infrared difference spectroscopy. Brian Dyer, Emory University has expertise. Topic Area: Photosystem I Site directed mutagenesis. Wu Xu has expertise.

Publications supported by this project 2022-2023:

1. Mausle, S., Agarwala, N., Eichmann, V., Dau, H., Nurnberg, D. and **Hastings, G.** (2023). Nanosecond Time-Resolved Infrared Spectroscopy for the Study of Electron Transfer Photosystem I. *Photosynthesis Research*. https://doi.org/10.1007/s11120-023-01035-9
2. Neva Agarwala and **Gary Hastings** (2023). Time-resolved FTIR Difference Spectroscopy for the Study of Photosystem I with High Potential Naphthoquinones Incorporated into the A_1 Binding Site 2. Identification of Neutral State Quinone Bands. *Photosynthesis Research*. https://doi.org/10.1007/s11120-023-01036-8
3. Leyla Rohani, Hari Lamichhane and **Gary Hastings.** (2023). Calculated Vibrational Properties of Pigments in Protein Binding Sites 2: Semiquinones in Photosynthetic Proteins. *Spectrochimica Acta A. Molecular and Biomolecular Spectroscopy*, Vol. 295, 122518. DOI: 10.1016/j.saa.2023.122518
4. Neva Agarwala, Hiroki Makita and **Gary Hastings** (2023). Time-Resolved FTIR Difference Spectroscopy for the Study of Photosystem I with High Potential Naphthoquinones Incorporated into the A_1 Binding Site. *Biochim Biophys Acta, Bioenergetics*. 1864, 1, 148918. https://doi.org/10.1016/j.bbabio.2022.148918
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Toward *in vivo* site-directed mutagenesis of LHCII functional domains in *Arabidopsis thaliana*

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Trimeric light-harvesting complexes for photosystem II (LHCII) are the most abundant photosynthetic antenna proteins in nature and thus the most impactful for global primary productivity. Despite decades of study, little is known about the specific functional domains of LHCII protein structures *in vivo*. This is due to their multigene family and redundancy, and the site-directed mutagenesis of one LHCII gene will generally not provide clear phenotypes. There are nine LHCII genes in *Arabidopsis thaliana* (*LHCB1.1–1.5*, *2.1–2.3*, and *3*), some of which are tandemly duplicated. Although *Arabidopsis* mutant lines for each individual LHCII gene are available, these complete loss-of-function mutants do not provide information about the function of specific protein domains. One could generate a full knock-out mutant, lacking all the *LHCB* genes, and then transform it with mutagenized *LHCB* genes. However, this strategy will likely introduce positional effects by random insertion of transgenes as well as different protein expression patterns by exogenous promoters. To avoid these uncertainties, we used a multiplexed CRISPR-Cas9 gene-editing approach to generate LHCII partial knock-out lines, containing only one *LHCB* gene: *solo-LHCB1*, *solo-LHCB22*, and *solo-LHCB3*. Using these *solo-LHCB* lines, we will utilize prime editing and/or base editing CRISPR-Cas systems, which allow introduction of specific amino acid substitutions, insertions, and deletions, for site-directed mutagenesis of *LHCB* genes at their native chromosomal loci. These lines will be invaluable to elucidate specific roles of functional domains of LHCII in the regulation of energy transfer, non-photochemical quenching, protein-protein interactions, protein folding, and thylakoid stacking *in vivo*.

Structural Mechanisms Governing Photosynthetic Energy Flow in Cyanobacteria

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Overall research goals:

All photosynthetic organisms require mechanisms to balance energy flow to the photochemical reaction centers as a response to fluctuating environmental conditions. In cyanobacteria, the structure and positioning of the phycobilisome (PBS) antenna provides an example of a macroscale control mechanism—at the level of the PBS and its association with one or both photosynthetic reaction centers—that governs energy flow. When the amount of captured light energy in the PBS exceeds what can be used for photochemistry, the rapid conversion of excess excitation energy into heat before it causes harm is known as non-photochemical quenching. This is mediated by a water-soluble photoreceptor that binds a single carotenoid molecule, the Orange Carotenoid Protein (OCP) which has also been proposed to influence interactions among the PBS and the Photosystems. We have recently determined the structures of the PBS from *Synechocystis* sp. PCC6803 in both a light harvesting and OCP-bound state, and we are continuing to improve the resolution of the models to sub-2.0 Å resolution, which will provide unprecedented structural detail, including the environments and orientations of pigments in the PBS and the OCP. Identification of the OCP binding site and the discovery of two proteins that we propose mediate positioning of the PBS provide the foundation for goals of this project--gaining an understanding of the structural basis of PBS attachment to the membrane/photosystems. Moreover, our data suggests that regulation by phosphorylation occurs for both the OCP and the PBS attachment proteins. We expect that, as outcomes of determining the high-resolution structure of the OCP-PBS complex and the mechanistic details of PBS positioning, our model system will become a key platform for fundamental research on energy transfer in pigment-protein complexes. It will provide an inspiration for synthetic biologists and materials scientists to design new sustainable technologies for harnessing the clean and abundant energy in sunlight.

Significant achievements (2022-2023

- We have characterized the function of the first of the two newly discovered PBS linker proteins. This was recently reported in Espinoza-Corral R, Iwai M, Zavřel T, Lechno-Yossef S, Sutter M, Červený J, Niyogi KK, Kerfeld CA (2023). Phycobilisome protein ApcG interacts with photosystem II and regulates energy transfer in Synechocystis. Plant Physiol. PMID: 37972281, 2023.

- We have completed the high-resolution cryo-EM structure of the OCP-PBS complex. This structure supersedes the one reported in Dominguez-Martin, Sauer et al., *Nature* (2022) both in resolution and completeness. By taking advantage of recent technological developments in cryo-EM data acquisition and processing we have obtained an OCP-PBS model with a resolution of 2.1 Å in the PBS core, 1.8 Å in the rods with some regions reaching 1.6 Å resolution. This resolution is one of the highest recorded and surpassed by only three biologically relevant samples in the public EM database. The visible details include more than 7000 water molecules and even hydrogen atoms can be discerned at this resolution. Collectively, the improvements in the model and the volume of data combined with new software developments allowed us to describe previously unknown motions within the PBS that may play a role in regulating light harvesting and, its photoprotective counterpart, non-photochemical quenching. These intrinsic

motions provide an example of breakthroughs in capturing conformational motions and structural dynamics from Cryo-EM data.

Moreover, the new atomic representation of the protein matrix, solvation and the embedded pigments is of unprecedented accuracy and, for the first time, provided the foundation for multiscale quantum chemical calculations of the quenching mechanism. Our calculations represent the most precise description to-date of the control exerted by the protein and the solvent on the excited states of the embedded pigments, enabling a 34kD protein--the OCP --to serve as an energy sink for the 6.2 MDa PBS.

By identifying key protein residues within OCP-PBS we clarified how the carotenoid embedded in the OCP can become an efficient acceptor of excitation energy from the phycocyanobilins of the PBS. Our energy transfer model therefore explains the quenching efficiency of the OCP and offers a detailed understanding of the atomic determinants of light harvesting regulation and antenna architecture in cyanobacteria.

- This work is described in "*Structural and quantum chemical basis for OCP-mediated quenching of phycobilisomes,*" currently in revision at Science Advances.

Science priorities for the next year (2023-2024):

- Continue structural and functional analysis of second newly identified PBS linker protein. We are also beginning additional structural studies with different PBS and different quenchers.

My major scientific area(s) of expertise is/are: Structural and synthetic biology; bioinformatics, cyanobacterial photosynthesis.

To take my project to the next level, my ideal collaborator would have expertise in: additional cryo-EM methods and capacity, including tomography .

Publications supported by this project Click here to enter text.

1. Espinoza-Corral, R.D., Iwai, M., Zavřel, T., Lechno-Yossef, S., Sutter, M., Červeny, J., Niyogi, K.K. and Kerfeld, C.A. The phycobilisome linker protein ApcG interacts with photosystem II and regulates energy transfer to photosystem I in Synechocystis sp. PCC 6803. Plant Physiology, 2023.
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4. Sil, S., Tilluck, R.W., Mohan, N T., Leslie, C.H., Rose, J.B., Domínguez-Martín, M.A. Lou, W., Kerfeld, C.A and Beck, W.F. Coherent Excitation Energy Transfer in the Rods of Intact Phycobilisomes. Nature Chemistry 14:1286-1294, 2022.
5. Khan, T., Kuznetsova, V., Dominguez-Martin, M.A., Kerfeld, C.A. and Polívka, T. UV excitation of carotenoid binding proteins OCP and HCP: Excited-state dynamics and product formation. ChemPhotoChem 6: e202100279, 2022.

Structural Dynamics in Photosynthetic Reaction Centers

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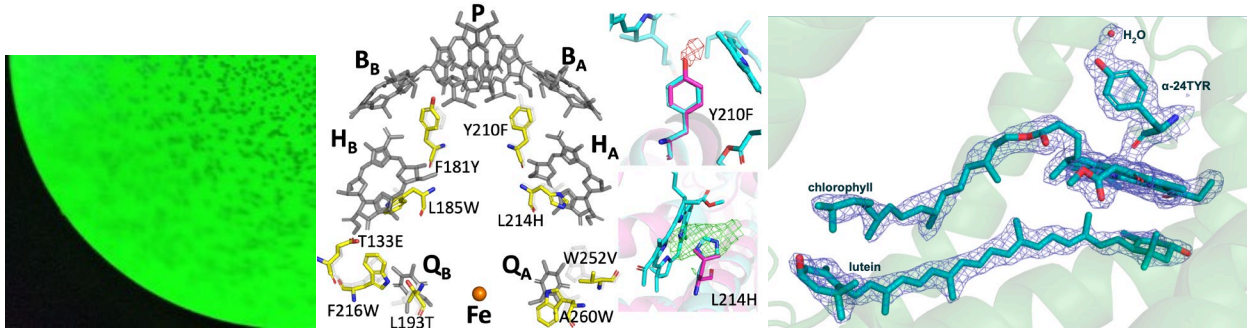
Overall research goals:

One of the fundamental questions regarding light driven charge transfer in natural photosynthetic systems is how the charge-separated states are stabilized, and what is the rationale for the directionality of charge transfer in photosynthetic reaction centers. Time resolved pump-probe X-ray diffraction (XRD) studies at room temperature using femtosecond X-ray pulses from X-ray free electron lasers (XFEL) are used in this project to structurally probe the dynamics of photosynthetic systems in the sub-picosecond to microseconds time scale. The aim of these studies is to provide details of geometric structural changes of the protein environment, which facilitate steering electron transfer along a specific branch of co-factors within the highly symmetric reaction centers, leading to the formation of stable charge-separated states. We are utilizing a range of photosynthetic complexes, including photosystem I (PSI) , the purple bacterial reaction center (BRC) and plant light harvesting complex II (LHCII) and selected point mutants to study specific light induced states. These results will be beneficial for designing artificial photosynthetic systems, where the question of efficient stabilization of a charge-separated state is of critical importance.

Significant achievements (Current Funding, 2021-2023):

- Room temperature dark state and ascorbate reduced structure of thermophilic PSI from *T. vestitus*.
- Room temperature structure of a mutant (obtained from Laible/Kirmaier/Holten) that routes ET along the B branch of the BRC from *Rhodobacter sphaeroides.*
- Room temperature structure of spinach LHCII (Iwai/Niyogi) and first data collected in ps time range after light excitation.
- Time resolved diffraction data collected for PSI, BRC wt and BRC mutant at different delay times and laser excitation power settings.
- Established protocols for *Synechocystis* PSI purification, crystallization, and cryo-EM sample preparation.



Left: Microcrystals of PSI from *Synechocystis*. **Middle**: Point mutations in *R. sphaeroides* BRC and observed difference density vs. wild type for two residues in the mutant RT structure. **Right**: Detail of the spinach LHCII RT structure showing electron density obtained for a Chl a and a lutein molecule as well as a water in the vicinity of Tyr a-24.

Science priorities for the next year (2023-2024):

- Evaluation of the already obtained time resolved data for the BRC and PSI.
- Using the structural results to better understand the effect of the individual mutations in BRC on the redox properties and kinetics of ET (collaboration with Holten/Kirmaier/Laible/Gunner groups).
- Obtain structural information for point mutants in *Synechocystis* PSI (EM and XRD) (collaboration with Xu group).
- Collect additional time resolved diffraction data for PSI and BRC to improve resolution and for additional time points along the charge separation path (collaboration with Holten/Kirmaier/Laible).
- Establish XRD protocols for *Synechocystis* PSI point mutants and subsequent time resolved XRD.
- Connect transient structural data with TR vibrational/electronic spectroscopy results (collaboration with Fleming group) and calculations about phonon/ET coupling (collaboration with Whaley group).
- Understand how ultrafast and slower structural dynamics in LHCII are coupled to excitation transfer and quenching (collaboration with Iwai/Niyogi group).

My major scientific area(s) of expertise is/are: membrane protein biochemistry, X-ray crystallography, time resolved XFEL methods (diffraction and spectroscopy), polarized spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: 1) Computation/ simulation of ultrafast structural relaxation/reorganization processes in reaction centers and LHCII; 2) Analysis methods for diffraction data that takes into account the presence of multiple conformations/populations in each data set, that can evaluate several data sets/time points together in a joint analysis, maybe using ML approaches.

Publications supported by this project [2021-2023] :

1. S. M. Keable, A. Kölsch, P. Simon, et al., "Room temperature XFEL crystallography reveals asymmetry in the vicinity of the two phylloquinones in Photosystem I'" Scientific Reports 11, 21787 (2021). [DOI: 10.1038/s41598-021-00236-3]
2. M. M. Chestnut, S. Milikisiyants, R. Chatterjee, J. Kern, A. I. Smirnov, "Electronic Structure of the Primary Electron Donor $P700^{+\bullet}$ in Photosystem I Studied by Multifrequency HYSCORE Spectroscopy at X- and Q-Band." J. Phys. Chem. B 125, 36-48 (2021). [DOI: 10.1021/acs.jpcb.0c09000]
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4. D. Shevela, J. F. Kern, G. Govindjee, J. Whitmarsh, J. Messinger, "Photosystem II." In: Encyclopedia of Life Sciences (ELS), Vol. 2: 1-20. John Wiley & Sons, Ltd: Chichester. (2021). [DOI: 10.1002/9780470015902.a0029372]
5. G.E. Borgstahl, W.B. O'Dell, M. Egli, J. Kern, A.Y. Kovalevsky, J.Y.Y. Lin, D.A.A. Myles, M.A. Wilson, W. Zhang, P. Zwart, L. Coates, "EWALD: A Macromolecular Diffractometer for the Second Target Station.", Rev. Sci. Instrum. 93, 064103 (2022). [DOI: 10.1063/5.0090810]
6. H. Makita, M. Zhang, J. Yano, J. Kern, "Room temperature crystallography and X-ray spectroscopy of metalloenzymes.", Methods Enzymol. 688, 307-348 (2023). [DOI: 10.1016/bs.mie.2023.07.009]
7. H. Makita, P.S. Simon, J. Kern, J. Yano, V.K. Yachandra, "Combining On-line Spectroscopy with Synchrotron and XFEL Crystallography", Current Opinion Struct. Biol. 80 102604 (2023). [DOI: 10.1016/j.sbi.2023.102604]
8. D. Shevela, J.F. Kern, Govindjee, J. Messinger, Solar energy conversion by photosystem II: principles and structures, Photosynth Res. 156, 279-307 (2023). [DOI: 10.1007/s11120-022-00991-y]
9. J.K. Bindra, J. Niklas, Y. Jeong, A.W. Jasper, M. Kretschmar, J. Kern, L.M. Utschig, O.G. Poluetkov, "Coherences of Photoinduced Electron Spin Qubit Pair States in Photosystem I." J. Phys. Chem B (2023). [DOI: 10.1021/acs.jpcb.3c06658]

Control of linear electron transport by thylakoid membrane dynamics

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Karen Davies, Co-PI(s)
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Overall research goals:

Our long-term goal is to understand how structural dynamics of photosynthetic thylakoid membranes in plants triggered by environmental fluctuations control the efficiency of energy conversion, its regulation, and the maintenance of the photosynthetic apparatus. The specific aim of our current grant is to determine how structural reorganizations of thylakoid membranes control the light-induced redistribution of (cytochrome (cyt) b_6f complexes between stacked and unstacked thylakoid domains and to examine the consequences of this reorganization for electron transport. Furthermore, we aim to employ state-of-the-art cryo-electron tomography to visualize protein organization dynamics in grana with molecular resolution as a basis to understand light-triggered changes in the PQ-diffusion efficiency.

The specific research objectives of the project are:

Aim 1: Determine the fraction of cytochrome b_6f complexes involved in linear electron transport. Our *working hypothesis* the cyt b_6f complexes in stacked and unstacked thylakoid domains are functionally indistinguishable. We postulate that in dark-adapted plants, only the grana-hosted cyt b_6f fraction is involved in linear electron transport (LET). We further postulate that in light-adapted plants, an addition fraction of cyt b_6f complexes localized in unstacked thylakoid membranes contribute to LET.

Aim 2: Measuring light-induced lateral cyt b_6f complex redistributions between stacked and unstacked thylakoid domains and the implications for electron transport. Our *working hypothesis* for aim 2 is that depending on light intensity, cyt b_6f complexes redistribute from stacked grana to unstacked thylakoid regions as was found for green algae and that this redistribution controls the fraction of complexes involved in LEF and CEF.

Aim 3: Employing cryo-electron tomography for mapping cyt b_6f complex and PSII organization dynamics in stacked grana as basis to understand PQ-dependent electron transport. Our *working hypothesis* for aim 3 is that in crowded stacked grana, small PQ-diffusion domains bounded by PSII, LHCII, and cyt b_6f complexes restrict long-range PQ diffusion in dark and low light adapted plants. At higher light intensities, however, we postulate that protein rearrangements in stacked thylakoid membranes allows facilitated PQ-diffusion out of grana to reach additional cyt b_6f complexes in distant unstacked domains.

Significant achievements (2020-2023):

- Established a methodical pipeline for imaging and analyzing the thylakoid ultrastructure by electron microscopy for dark- and light-adapted intact leaf discs.
- Established a complete set of biochemical and biophysical tools for the quantification of all energy converting building blocks in thylakoid membranes.
- Determine light-induced ultrastructural changes of the entire thylakoid membrane network.
- Quantification of light-induced lateral cyt b_6f (and other protein complex) redistribution between stacked and unstacked thylakoid domains.

- Uncovered light-induced stimulation of electron transport rate from cyt b_6f complex to photosystem I and examined the mechanistic reasons for this stimulation
- Correlation between ultrastructural thylakoid alterations and changes in electron transport rates.
- Generation of cryo-electron tomograms from isolated grana membranes and intact thylakoid membranes.
- Single particle analysis of photosystem II complexes in stacked grana organized either in semi-crystalline arrays or in randomly organized membrane regions
- First identification of cyt b_6f complexes in electron tomograms.

Science priorities for the next year (2023-2024):

- Extend our studies on overall thylakoid membrane dynamics to kinase and phosphatase mutants.
- Further examination of the relationship between ultrastructural dynamics and linear electron transport for wildtype plants.
- Establishing a functional test to measure electron transport from photosystem II to isolated plastocyanin.
- Generate mid-resolution maps for photosystem II in random and semi-crystalline arrays and back projection of photosystem II structures in grana membrane to establish supramolecular protein maps for both dark- and light adapted states.

My major scientific area(s) of expertise is/are: Structure-function relationship for thylakoid membranes; analyzing structural dynamics of thylakoid membranes from the nanometer to micrometer length scales by biochemical, light, and electron microscopic techniques; computer modeling of photosynthetic light reactions.

To take my project to the next level, my ideal collaborator would have expertise in: Cryo electron microscopy on intact thylakoid membranes or membrane fragments, coarse grain computer modeling, molecular dynamics simulations, generate combinations of multiple knock out plants.

Publications supported by this project (2020-2023) :

1. V. Svoboda, H.M.O. Oung, H. Koochak, R. Yarbrough, S.D. Mckenzie, S. Puthiyaveetil, H. Kirchhoff (2023) Quantification of energy-converting protein complexes in plant thylakoid membranes. Biochim. Biophys. Acta 1864, 148945, doi.org/10.1016/j.bbabio.2022.148945
2. H. Kirchhoff, L. Vance (2023) Evaluation of lipids for the study of photosynthetic membranes. In: Methods in Molecular Biology. (Editor, S. Covshoff), Springer Press, in press.
3. H.M.O. Oung, R. Mukhopadhyay, V. Svoboda, D. Charuvi, Z. Reich, H. Kirchhoff (2022) Differential response of the photosynthetic machinery to dehydration in older and younger resurrection plants. J. Exp. Bot. 73, 1566-1580, doi: 10.1093/jxb/erab485
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8. M. Li, R. Mukhopadhyay, V. Svoboda, H.M.O. Oung, D.L. Mullendore, H. Kirchhoff (2020)

Measuring the dynamic response of the thylakoid architecture in plant leaves by electron microscopy. Plant Direct 4, e00280, doi.org/10.1002/pld3.280

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10. S. Tietz, M. Leuenberger, R. Höhner, A.H. Olson, G.R. Fleming, H. Kirchhoff (2020) A proteoliposome-based system reveals how lipids control photosynthetic light harvesting. J. Biol. Chem. 295, 1857-1866, doi./10.1074/jbc.RA119.011707

Controlling Electron Transfer Pathways in Photosynthetic Reaction Centers

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Overall research goals: Photosynthetic reaction centers (RCs) convert light energy into chemical energy via a series of electron transfer (ET) reactions that give transmembrane charge separation with near unity yield. Of symmetry-related sets of A- and B-branch cofactors, only the A pathway is active in native bacterial RCs (Fig. 1A). Differences in the free energies of the A- versus B-side charge-separated states, and in electronic couplings between cofactors, underlie differences in the rate constants for forward ET and charge recombination on the two pathways (Fig. 1B).

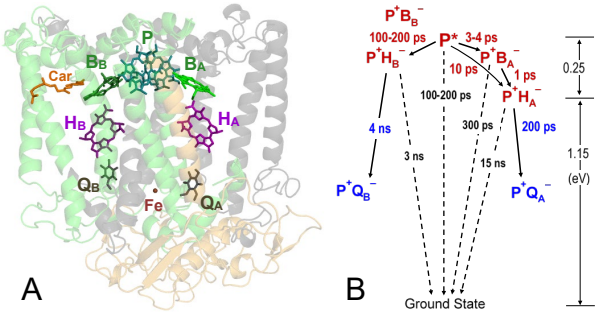

Fig. 1. (A) *R. sphaeroides* RC (PDB:1PCR). (B) Model free energy diagram and time constants for primary (red) and secondary (blue) ET processes: forward ET (solid) and charge recombination (dashed).

The goal of this project is to engineer a RC that performs transmembrane charge separation efficiently and exclusively via the B-side cofactors. Such a RC – and, equally, the steps toward it – provide a fundamental understanding of how ET between the A-side cofactors is optimized and ET between the B-side cofactors is inhibited. Protein-cofactor interactions in RCs from the purple photosynthetic bacteria are manipulated using a semi-directed, molecular-evolution approach. In this way, productive substitutions are found that would not have been chosen using more traditional site-directed approaches. Photochemistry is analyzed using time-resolved spectroscopic studies spanning <1 ps to >10 s. Selective pressure for photosynthetic growth is employed to identify variants that are photocompetent using B-side ET solely. The principles realized afford deep insights into RC function and can aid design of biomimetic systems for solar energy conversion.

Significant achievements 9/2021 – 9/2023:

(1) Primary ET was compared at 295 K and 77 K for RCs from thirteen *R. sphaeroides* mutants and wild-type. Overall P* decay, and $P^+H_B^-$ formation, is ~2-fold faster at 77 K than at 295 K. The $P^+H_B^-$ yield ranges from <5% to ~90%, and is unchanged (for a given mutant) at 77 K and 295 K. At least half of the $P^+H_B^-$ yield is produced by "slow" (~20–50 ps) P* → $P^+H_B^-$ one-step superexchange ET. Thus, our studies show that the dominance of rapid two-step ET is not a prerequisite for a near-quantitative (≥90%) yield of unidirectional charge separation.

(2) Twelve pairs of *R. sphaeroides* and *R. capsulatus* mutant were studied. *R. sphaeroides* RCs have larger $P^+H_B^-$ yields (up to ~90%) than *R. capsulatus* analogs (up to ~60%). Mutations near B_B primarily affect the amount of fast (<5 ps) P*→ $P^+B_B^-$ → $P^+H_B^-$ two-step ET. Mutations near H_B mainly influence the amount of slower (20–50 ps) P* → $P^+H_B^-$ one-step superexchange ET. The studies demonstrate cooperative amino-acid control of rates, yields, and mechanisms of ET. Insightful differences and commonalities between species are revealed. This work provides a host of targets for analysis by theory and artificial-intelligence methods.

(3) Parallel efforts have focused on enhancing $P^+H_B^- \rightarrow P^+Q_B^-$ secondary ET. This includes equipping the protein channel between H_B and Q_B with up to two non-native tryptophans in concert with a threonine to optimize electronic coupling between H_B and Q_B and Q_B binding/positioning. The collective work has afforded several mutants that remarkably combine a $P^* \rightarrow P^+H_B^-$ yield of ~70%, and a $P^+H_B^- \rightarrow P^+Q_B^-$ yield of ~90%, giving an overall $P^+Q_B^-$ yield of ~65%.

(4) During the course of this project (2009–2023), ~800 mutants from *R. capsulatus* and ~350 from *R. sphaeroides* have been designed, made, and RCs characterized. Dozens with enhanced yields of B-side ET have been subjected to a revertant-selection process. One with a ~60% $P^+Q_B^-$ yield gives a phenotypic revertant that incorporates an additional substitution that supports photosynthetic growth of the organism using only B-side cofactors. Many aspects of this remarkable finding are currently being dissected within our own and outside teams.

(5) Collaborations are ongoing with Drs. Jan Kern (static and time-resolved x-ray diffraction), Marilyn Gunner (electrostatics calculations) and Gary Hastings (FTIR) to explore structure-function relationships and effects of mutations on energetics and ET yields.

Science priorities for next year (2023–2024):

- Analyze revertant(s) that are photocompetent via B-side ET; find minimum mutation set(s).
- Characterize ET rates and yields in isolated RCs of spontaneous revertants and synthetic analogs.
- Complete work on $P^+H_B^- \rightarrow P^+Q_B^-$ ET in a dozen pairs of *R. sphaeroides/capsulatus* mutants.
- Continue collaborative studies with Drs. Jan Kern, Marilyn Gunner and Gary Hastings.

Our major scientific area(s) of expertise is/are: Molecular biology, protein biochemistry, and directed molecular evolution (ANL), ultrafast and slower optical spectroscopy (WU)

To take my project to the next level, my ideal collaborator would have expertise in:
In addition to ongoing collaborations noted above, exciting new collaborations would be to (1) use artificial intelligence methods to explore our vast library of mutants and associated results on charge separation events, and (2) characterize the proton-coupled ET and light-harvesting events involving $P^+Q_B^-$ that are integral to photosynthetic growth solely via the B-side cofactors.

References to work supported by this project over the past 3 years:

1. N. C. M. Magdaong, J. C. Buhrmaster, K. M. Faries, H. Liu, G. A. Tira, J. S. Lindsey, D. K. Hanson, D. Holten, P. D. Laible, and C. Kirmaier, "In Situ, Protein-Mediated Generation of a Photochemically Active Chlorophyll Analog in a Mutant Bacterial Photosynthetic Reaction Center." Biochemistry *60*, 1260–1275 (2021).
2. J. B. Weaver, C-Y. Lin, K. M. Faries, I. Mathews, S. Russi, D. Holten, C. Kirmaier, and Steven G. Boxer, "Photosynthetic Reaction Center Variants Made Via Genetic Code Expansion Indicate Tyrosine at M210 Tunes the Mechanism for Primary Electron Transfer." Proceedings of the National Academy of Science U.S.A. *118*, e211643911 (2021).
3. V. R. Policht, A. Niedringhaus, C. Spitzfaden, P. D. Laible, D. F. Bocian, C. Kirmaier, D. Holten, T. Mančal, and J. P. Ogilvie, "Hidden Vibronic and Excitonic Structure and Vibronic Coherence Transfer in the Bacterial Reaction Center, Science Advances." *8*, eabk0953 (2022).
4. N.C. M. Magdaong, K. M. Faries, J. C. Buhrmaster, G. A. Tira, R. M. Wyllie, C. E. Kohout, D. K. Hanson, P. D. Laible, D. Holten, and C. Kirmaier, "High Yield of B-side Electron Transfer at 77 K in the Photosynthetic Reaction Center Protein from *Rhodobacter sphaeroides*." J. Phys. Chem. B *126*, 8940–8956 (2022).

Mechanisms and control of cytochrome c biosynthesis.

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Overall research goals:

Aim 1 will determine the CXXCH binding site on CcsBA. Cryo-EM structural approaches will be used (**Aim1A)**, as well as disulfide crosslinking **(Aim1B)** of CXXCH to the CcsBA active site. We will obtain new CcsBA structures with our CXXCH peptide substrates. Note that the Aim1B crosslinking could lead to a stable CXXCH-bound CcsBA, which would optimize cryo-EM, if CXXCH peptide-bound CcsBA molecules are rare. We will use cryo-EM images/densities on CcsBA to model CcmF/H for the structural basis of holoCcmE access into CcmF **(Aim1C**). We will also search for conserved residues in CcmF/H that like CcsBA could form the CXXCH binding site. This aim will address how the hundreds of different cyt c proteins used in electron transfer chains in prokaryotes and plants are all recognized by these two synthases.

Aim 2 will investigate mechanisms at the active sites that lead to thioether attachments. For CcmF/H, the Kranz group has proposed that a stable, stoichiometric heme in CcmF functions to reduce (to Fe^{+2}) the incoming heme from CcmE. Fe^{+2} heme is needed to form thioethers once CXXCH binds in the proper stereochemical position at the active site. Thus CcmF/H is a heme reductase as well as a cyt c synthase. This reductase relay will be explored **(Aim2A).** Besides the previously proposed studies on tryptophans as electron conduits (W214/251) in the channel, we will also analyze quinone analogs that potentially inhibit CcmF reduction, thus using a second approach to test the above hypothesis. Both CcmF/H and CcsBA use two periplasmic histidines (called P-His1 and P-His2) to ligand the external heme substrate. We hypothesize that the histidine of CXXCH replaces P-His2 (switching ligands) at the active site, this ligand switching will be investigated in CcsBA (Aim2B).

Aim 3 will investigate release of CXXCH-attached heme from the active site. We use already mutated conserved residues in CcsBA. It is likely that release mechanisms such as thioether-based heme distortion, and key conserved residues at the active site, are common to both synthases.

Significant achievements

DOE funding started in May, 2023 so the following achievements and manuscript are since that date:

Achievements: Our studies concern the two prokaryotic cytochrome c synthases, CcsBA (System II) and CcmF/H (System I). These have been termed membrane molecular machines: CcsBA is a heme transporter and cyt c synthase, while CcmF/H is a heme reductase and cyt c synthase. We recently produced and used CryoEM densities of two conformations of CcsBA (from *Helicobacter hepaticus*) for detailed analyses of conserved domains and mechanisms within this large family, from chloroplasts to Gram negative and positive bacteria (see Fig1, marked in "red"). Two conserved pockets, for the first

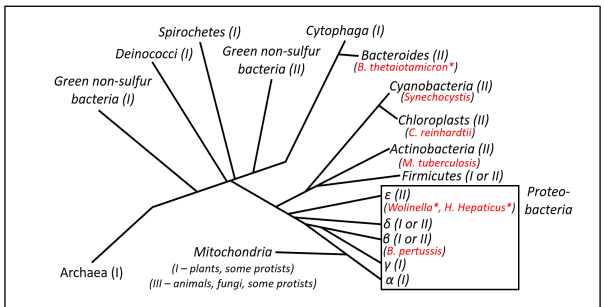

Fig1. Distribution of System I (Ccm) or II (Ccs).

and second cysteines of CXXCH, explain stereochemical heme attachment. In addition to other universal features, a conserved periplasmic beta stranded structure, called the Beta cap, protects the active site when external heme is not present. Structural features are present that suggest direct interaction with ferrochelatase for heme delivery to CcsBA.

For CcmF/H, we provide evidence that CcmF/H receives Fe^{+3} heme from holoCcmE via a periplasmic entry point in CcmF, whereby heme is inserted directly into a conserved WWD /P-His domain from above. We used our structural densities of heme at the active site (of CcsBA), in addition to other results, that suggest this "periplasmic model" for heme trafficking. The model contrasts to a "buoy model" proposed by others (see Fig 2). Finally, evidence suggests that CcmF acts as a heme reductase, reducing holoCcmE (to Fe^{+2}) through a transmembrane electron transfer conduit, which initiates a complicated series of events at the active site.

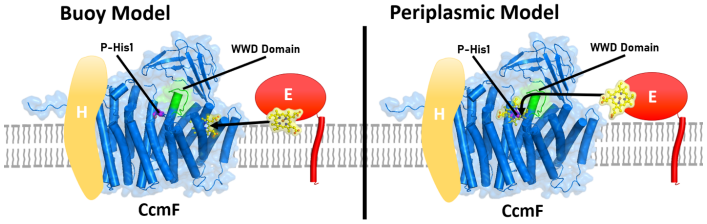

Fig 2. Mechanisms for heme access to the CcmF active site

Science priorities for the next year (2023-2024):

- We will explore further the CXXCH binding sites on CcsBA and CcmF/H using CryoEM and computational docking programs (Aim 1). Our hypothesis is that CcmF/H acts as an oxidoreductase and cyt c synthase by a reduction relay involving a TM-heme to conserved tryptophans in TMs. We have produced a video of this mechanism (JBC, in press below), whereby the reduced heme in the active site (WWD/P-His domain) is reduced by this relay, for attachment to cyt c. We will test this by mutating the tryptophans in the relay and examining cyt c synthesis (Aim2).
- We have developed a hypothesis on how the heme attached to cyt c is released from the active sites of CcsBA and CcmF/H. This hypothesis involves the distortion of heme upon thioether attachment, which weakens binding to the cyt c synthase active sites. The hypothesis is based in part on our studies of the human cyt c synthase, HCCS, where we showed this distortion occurs at the active site. We will initially analyze these distortions using our CryoEM densities of CcsBA open complex that has heme at the active site, then determine if distortion of heme alters the binding parameters (Aim 3).
- Finally, the PI (Kranz) has been invited by editors of Annual Review of Microbiology to write a comprehensive review article titled "Bacterial Heme Transport and Incorporation into Cytochrome c". This is due by end of 2024 and it will cite the DOE. It will also provide blueprints and approaches for future studies on the big gaps in the field, including controlling cyt c biosynthesis, new substrates for unique heme proteins, and novel chemicals that control cyt c synthases.

My major scientific area(s) of expertise is/are: Biochemistry, microbiology, cytochromes, heme, membrane proteins and complexes, bioenergy, biogenesis of bioenergy molecules.

To take my project to the next level, my ideal collaborator would have expertise in: I am thinking about the possibility of collaborating with an expert in substrate docking (computational), whereby our real active site structures(CryoEM) can be further evaluated for engineering unique acceptors (to CXXCH) that will fit the active site of the cyt c synthases.

Publications supported by this project (new award in 2023):

Huynh JQ, Lowder EP, Kranz RG, "Structural basis of membrane machines that traffick and attach heme to cytochromes." J Biol Chem. 2023 Oct 10:105332. doi: 10.1016/j.jbc.2023.105332. Online ahead of print. PMID: 37827288

Diversification and function of bilin chromophores in oxygenic photosynthesis

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Overall research goals:

Our overarching hypothesis is that bilin biosynthesis is ubiquitous in oxyphototrophs because bilins are required to maintain sufficient Chl biosynthesis in an oxic environment. Work under the current proposal seeks to test this hypothesis by addressing three fundamental questions about the functions of bilins in oxygenic photosynthetic organisms.

1. How do GUN4 and bilins stimulate MgCh activity and stabilize the CHLH subunit?
2. Are bilin-binding GUN4 proteins generally present in photosynthetic eukaryotes?
3. How was bilin metabolism integrated with Chl synthesis in early cyanobacteria?

Aim 1: Characterization of interactions between GUN4, bilin, and CHLH. Our earlier work has established that bilin protects CrCHLH1 against light-dependent degradation in vivo. The combination of light and oxygen is known to inactivate bacterial BchH subunits, resulting in loss of MgCh activity, so we hypothesize that CrGUN4 prevents this process. We are implementing experiments to understand how GUN4 and bilins stimulate MgCh activity and stabilize CHLH.

Aim 2: Diversity of GUN4•bilin interactions in photosynthetic eukaryotes. A critical unanswered question for the role of GUN4•bilin complexes in MgCh function is whether bilin binding is a general property of GUN4 orthologs. To address this, we need to determine whether bilin-binding GUN4 proteins are widespread in photosynthetic eukaryotes. By combining biochemical characterization of CrGUN4 variants with the availability of crystal structures of SyGUN4 with bound bilin, we were able to identify a conserved bilin-binding LxNxLR motif containing an Asn residue that is essential for phycocyanobilin (PCB) binding in CrGUN4 (see below). This motif shows systematic variation in eukaryotic algae that is roughly correlated with which FDBR enzymes are present in those algae to carry out the final step(s) of bilin biosynthesis.

Aim 3. Bilin metabolism, GUN4, and bilin-binding globins in early cyanobacteria. In the previous period, we successfully identified the origin of PCB biosynthesis in cyanobacteria: cyanobacterial PcyA evolved from pre-PcyA proteins found in heterotrophic bacteria and was introduced to cyanobacteria via HGT along with a cluster of other genes including distant phycobiliprotein homologs (see below). We are building on this success while continuing to focus on the roles of bilin and GUN4 in early oxygenic photosynthetic organisms. Work under this Aim thus examines how bilin metabolism was integrated with Chl synthesis and light harvesting in early cyanobacteria.

Significant achievements (2021-2023):

• **Evolution of GUN4•bilin interactions (publication #2, below).** Using phylogenetic analysis, site-directed mutagenesis, and spectroscopic characterization of recombinant proteins, we have demonstrated that GUN4 appeared early in cyanobacterial evolution, that a key bilin-binding motif is conserved in GUN4 orthologs from oxyphototrophs, and that this motif is not conserved in paralogs. These results implicate conserved GUN4•bilin complexes in diverse eukaryotic algae.

• **Evolution of bacterial PCB biosynthesis and bilin-binding globins (publication #3).** PCB plays a critical role in the function of the phycobilisome. In cyanobacteria, PCB is synthesized from biliverdin IXα by the FDBR PcyA. Surprisingly, we have shown that PcyA evolved from "pre-PcyA" FDBRs found in heterotrophic bacteria and was acquired by cyanobacteria via horizontal gene transfer (HGT). This HGT event also introduced distant phycobiliprotein relatives (bilin biosynthesis-associated globins, or BBAGs) and other proteins into cyanobacteria. These results elucidate the origins of PCB biosynthesis in cyanobacteria and illuminate the importance of HGT in the evolution of oxyphototrophs.

Science priorities for the next year (2023-2024):

- **Aim 1: Characterization of interactions between GUN4, bilin, and CHLH.** We have improved expression of CHLH by co-expressing with GUN4 and bilin. However, the resulting expression system gives variable results, so in the coming year we are working to improve its reliability by exploiting plasmid systems developed under Aim 3 (published in paper #3 below).
- **Aim 2: Diversity of GUN4•bilin interactions in photosynthetic eukaryotes.** We are currently screening diverse GUN4 proteins (including candidate paralogs) for bilin binding and will extend these results by examining the function of cognate FDBRs.
- **Aim 3. Bilin metabolism, GUN4, and bilin-binding globins in early cyanobacteria.** We are completing follow-up studies of BBAGs for publication. We have recently shown that the bilin-binding configuration of one BBAG is bilin-dependent and that a cyanobacterial BBAG from Synechocystis does not bind significant levels of bilin in a heterologous expression system. These results implicate a model wherein an ancestral bilin-binding globin could autocatalytically bind a range of bilins in varying conformations, behavior that was then lost in both phycobiliproteins and cyanobacterial BBAGs. We will test additional cyanobacterial BBAGs (predicted not to bind bilin) and will test whether other BBAGs from heterotrophic bacteria show similar variability in bilin-binding configuration.

Our major scientific area(s) of expertise is/are: Bilin chemistry, bilin biosynthesis, structure/function relationships in bilin-binding proteins, photobiology of bilin-binding photoreceptors, photobiology of tetrapyrroles.

To take our project to the next level, our ideal collaborator would have expertise in: Single-molecule approaches for studying GUN4•CHLH complexes; expression of eukaryotic proteins in *Bacillus* for purification of naïve CHLH and GUN4 proteins in the complete absence of protoporphyrin IX

Publications supported by this project :

1. R.D. Willows, J. C. Lagarias, D. Duanmu (2023) "Tetrapyrrole biosynthesis and signaling (chlorophyll, heme, and bilins)." in **The *Chlamydomonas* Sourcebook - Volume 2: Organellar and Metabolic Processes**; A. Grossman and F. A. Wollman, eds; Academic Press (Elsevier); Chapter 21 (pp. 691-731) [https://doi.org/10.1016/B978-0-12-821430-5.00021-3]
2. N. C. Rockwell, J. C. Lagarias, "GUN4 appeared early in cyanobacterial evolution." PNAS Nexus 2(5):pgad131 (2023) [doi: 10.1093/pnasnexus/pgad131]
3. N. C. Rockwell, S. S. Martin, J. C. Lagarias, "Elucidating the origins of phycocyanobilin biosynthesis and phycobiliproteins." Proc. Natl. Acad. Sci. USA. 120(17):e2300770120 (2023) [doi: 10.1073/pnas.2300770120]

Elucidating the Principles that Control Electron and Proton-Coupled Electron Transfer in Photosystem II and Photosystem I.

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Senior Collaborators: G. W. Brudvig (Yale), J. H. Golbeck (Penn State) and O. G. Poluektov (Argonne)

Overall Research Goals. The light reactions of photosynthesis take place in the Type II and Type I reaction centers (RC), photosystem II (PSII) and photosystem I (PSI). PSII oxidizes water to dioxygen, while PSI generates and stores the reducing equivalents required for carbon fixation. Our research is focused on elucidating the mechanism of electron and proton-coupled electron transfer (PCET) in PSII and PSI. **(i)** The water oxidation reaction in PSII is one of the most energetically demanding reactions in nature. PCET reactions, which are exquisitely tuned by smart protein matrix effects, are central to the water-splitting chemistry. The first objective of this proposal is to elucidate the role of PCET reactions in the water oxidation reaction of PSII. **(ii)** RCs use the energy of a photon to generate charge-separated hole-electron pairs against a highly unfavorable thermodynamic gradient, and stabilize this state by transferring the electron/hole through a series of cofactors to lengthen the lifetime of the donor-acceptor pair. The second objective is to obtain a deeper understanding of charge separation by studying the cofactor-protein interactions and transfer of quantum states from the primary donor to the acceptors of PSI.

Significant Achievements 2021-23. (A) ***Probing the mechanism of substrate delivery in PSII***: The light-driven four-electron water oxidation reaction occurs at the Mn_4Ca-oxo cluster in the oxygen-evolving complex (OEC) of PSII. Recent structures provide a model for the OEC, however, the mechanism of delivery and binding of substrate remain uncertain. In previous studies, we had unambiguously resolved the individual spectroscopic signatures of the amino acid and water ligands in the S_2 state of the OEC (*Energy Environ. Sci.*, 2012, 5, 7747; *Phys. Chem. Chem. Phys.*, 2014, 16, 20834). **(1)** The substrate analog, methanol, is known to access the OEC in PSII isolated from higher plants but not cyanobacteria. Recent computational studies attributed this difference to the D1-87 residue, which is Ala (D1-A87) and Asn (D1-N87) in spinach and cyanobacteria, respectively. In the present study, using 2D ^{13}C HYSCORE and DFT, for the first time, we demonstrate the binding of ^{13}C-methanol to the Mn_4Ca-oxo cluster in the S_2 state of D1-N87A PSII from *Synechocystis* sp. PCC 6803, providing insight on the delivery of substrate molecules.[1] **(2)** We use the binding of the substrate analog, ammonia, to provide valuable insights on substrate delivery in the OEC. We performed 2D HYSCORE spectroscopy and DFT that provide direct 'snapshots' of the binding of NH_3 and water molecules in the S_2 state of the Mn_4Ca-oxo cluster.[2] These results, for the first time, unambiguously assign the binding sites of ammonia and water ligands in the S_2 state. **(3)** The D1-S169 residue in the OEC is hydrogen (H)-bonded to the waters, W_1 and W_X, which likely participate in substrate delivery. We performed HYSCORE measurements that reveal a new water-derived ligand, W_X, that binds to the Mn4(IV) ion, in addition to the two existing waters, W_1 and W_2, in the S_2 state of D1-Ser169Ala PSII.[3] This suggests that the replacement of D1-Ser169 affects the energetics and stability of the S-state intermediates, allowing us to trap a new intermediate that is similar to the S_3 state. **(B)** ***Discovery of a quantum-delocalized proton in the S_2 state of PSII***: Current mechanistic models for water oxidation include the possible participation of exchangeable water-derived ligands and μ-oxo-bridge O atoms of the cluster. However, the protonation states of these ligands remain poorly defined. We had previously determined the hyperfine couplings of H atoms of the water-derived ligands in the S_2 state. We recently performed QM/MM and DFT of water-derived ligands in the presence of an extensive network of H-bonds in the S_2 state.[4] We identify a quantum-delocalized proton between the water-derived ligand, W_1, and D1-D61 residue, where delocalization of the proton leads to a lower zero-point energy which precludes its release in the $S_1 \rightarrow S_2$ transition. **(C)** ***PCET at the Y_Z and Y_D residues of PSII***. PSII contains two symmetrically placed tyrosines, Y_D and Y_Z, which are chemically identical but functionally distinct. It is important to determine the electronic structure of Y_Z and Y_D in both the neutral and radical state to elucidate the functional differences. A detailed understanding of PCET at Y_Z is complicated as it is kinetically competent in water oxidation. We recently utilized 2D ^{14}N HYSCORE and DFT to investigate the electronic structure of an artificial reaction center, benzimidazole-phenol porphyrin ($BiP–PF_{15}$), that mimics the PCET reactions at Y_Z. The results demonstrate the significance of steric effects and charge delocalization on the tuning of PCET, suggesting that these features must be considered in predicting of electronic properties of Y_Z. **(D)** ***Revealing the dimeric nature of the primary acceptor in PSI***:

Recent work on PSI has suggested that the six core chlorophyll (Chl) *a* molecules are highly coupled, allowing for efficient creation and stabilization of the charge-separated state. Involved in this coupled complex is the primary acceptor, A_0. While the properties of the intermediate and terminal acceptors are fairly well characterized, the role of A_0 and the factors that contribute to its ultrafast processes and redox properties remain unclear. We employed a combination of 2D HYSCORE spectroscopy and DFT to explore the nature the $A_0^{\cdot-}$ state. The results indicate that A_0, once thought to be a Chl *a* monomer, functions as a dimer (Chl_2/Chl_3) with an asymmetric distribution of electron density favoring Chl_3.[5] Interestingly, this dimerization occurs independently of the axial ligand.[6] The dimerization of the primary acceptor likely serves to ensure charge separation is energetically downhill, and that subsequent recombination is slowed. **(E)** ***Investigating the electronic structure of the primary donors and acceptors of RCs***: We have been interested in the factors that influence the early stages of light-driven charge separation in RCs. With the recent publication of several RC structures, we probed the geometric and electronic structure of an array of (B)Chl and (B)Pheo primary electron donors and acceptors from both Type I and Type II RCs. This has facilitated a detailed comparison of the natural design of the primary donors[7] and acceptors[8] of the Type I and Type II RCs from both an evolutionary and application based perspective. **(F)** ***Electronic structure and energetics of the heterodimeric BChl g'/Chl a' special pair in dioxygen exposed H. modesticaldum***: Heliobacteria are anoxygenic phototrophs that have a Type I homodimeric RC containing BChl *g*. Previous experimental studies have shown that in the presence of light and dioxygen, BChl *g* is converted into 8^1-OH-chlorophyll a_F (Chl a_F), with an accompanying loss of light-driven charge separation, suggesting that the RC only loses the ability to transfer electrons once both BChl *g'* molecules of the special pair are converted to Chl a_F'. Using hyperfine couplings obtained from Q-band ^{1}H ENDOR, 2D ^{14}N HYSCORE and DFT, we directly demonstrate that the partially converted BChl *g'*/Chl a_F' special pair remains functional when exposed to dioxygen.[9]

Science Objectives for 2023-24. (i) We are working on understanding the role of the amino acids that influence the delivery of water and egress of protons during the water oxidation reaction of PSII. (ii) We are investigating the effect of H-bonding and hydrophobic interactions on the tuning of the primary donor, P_{700}, and (iii) developing a system to investigate the transmission of quantum electron spin states in PSI.

My major scientific areas of expertise are EPR and solids NMR spectroscopy, DFT, electron and PCET reactions, water oxidation and the isolation, purification and light-driven reactions of photosynthetic systems. My ideal collaborator, in addition to my current collaborators, would have expertise in electrochemistry.

Partial List of References to Work Supported by this Project 2020-23 (three years).

1. Kalendra *et al*. "Binding of the Substrate Analog Methanol in the Oxygen-evolving Complex of Photosystem II in the D1-N87A Genetic Variant of Cyanobacteria" (2022) *Faraday Disc*. 234, 195.
2. Kalendra *et al*. "Mechanism of Binding of Ammonia as a Substrate Analog in the Oxygen-evolving Complex of Photosystem II" (2023) Manuscript in revision.
3. Kalendra *et al*. "Binding of Substrate Water in the S_2 to S_3 State Transition of the D1-S169A Variant of Photosystem II" (2023) Manuscript in revision.
4. Yang *et al*. "Is Deprotonation of the Oxygen-Evolving Complex of Photosystem II during the $S_1 \rightarrow S_2$ Transition Suppressed by Proton Quantum Delocalization?" (2021) *J. Amer. Chem. Soc*., 143, 8324.
5. Gorka *et al*. "A Dimeric Chlorophyll Electron Acceptor Differentiates Type I from Type II Photosynthetic Reaction Centers" (2021) *iScience* (Cell Press), 24, 102719.
6. Gorka *et al*. "Two-dimensional HYSCORE Spectroscopy Reveals Histidine as the Axial Ligand to Chl_{3A} in the $M688H_{PsaA}$ Genetic Variant of Photosystem I" (2021) *Biochim. Biophys. Acta- Bioenerg*., 1862, 148424.
7. Gorka *et al*. "Shedding Light on Primary Donors in Photosynthetic Reaction Centers" (2021) *Front. Microbiol*., 12, 735666.
8. Gorka *et al*. "Chlorophylls as Primary Electron Acceptors in Reaction Centers: A Blueprint for Highly Efficient Charge Separation in Bio-inspired Artificial Photosynthetic Systems" in Photosynthesis: From Plants to Nanomaterials (2023) (Eds. H. J. M. Hou and S. I. Allakhverdiev) Elsevier, Amsterdam, The Netherlands.
9. Kaur *et al*. "Electronic Structure and Energetics of a Heterodimeric BChl *g'*/Chl *a'* Special Pair Generated by Exposure of *Heliomicrobium modesticaldum* to Dioxygen" (2023) *Phys. Chem. Chem. Phys.*, 25, 26894.
10. Mark *et al*. "Determining the Electronic Structure Paramagnetic Intermediates in Membrane Proteins: A High-resolution 2D ^{1}H Hyperfine Sublevel Correlation Study of the Redox-active Tyrosines of Photosystem II" (2020) *Biochim. Biophys. Acta-Biomembranes*, 1862, 183422.
11. Charles *et al*. "The Role of Zinc-Bacteriochlorophyll *a'* in the Primary Photochemistry of *Chloroacidobacterium thermophilum*" (2020) *Phys. Chem. Chem. Phys.*, 22, 6457.
12. Mendez-Hernandez *et al*. "HYSCORE and DFT Studies of Proton-Coupled Electron Transfer in a Bioinspired Artificial Photosynthetic Reaction Center" (2020) *iScience* (Cell Press), 23, 101366.

Molecular Mechanism of Action of the Cyanobacterial Orange Carotenoid Protein and Phycobilisome

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Overall research goals:

The objective of the proposed research is the development and application of the quantitative cross-linking mass spectrometry platform that enables interrogations on the structural interfaces within cyanobacterial light-harvesting complex phycobilisome (PBS) and the interface of PBS with the two reaction centers that underlie the excitation energy transfer and regulation under eco-physiological conditions

We aim at using our recently developed combinatorial platform (Quantitative Cross-linking Mass Spectrometry, QXL-MS) to probe the structural allostery of PBS and its dynamic interaction with its downstream binding partner and energy acceptor, i.e., reaction centers (Photosystem II and Photosystem I). To achieve this goal, we will set up cross-linking chemistry, LC-MS/MS interrogation, structural modeling, bioinformatics analysis as well ultrafast laser spectroscopy that collect a wealth of information on the excitation energy landscape. Detailed quantitative structural MS analysis, accompanied by the functional decomposition analysis from molecular spectroscopy will provide rich structure-function information on the state transition at protein domain level and the corresponding cellular strategies under changing environmental conditions. The success of this project is ensured by the robust platform of advanced mass spectrometry as well time-resolved spectroscopy (absorption and fluorescence).

Significant achievements (2022-2023 DOE)

Using mass spectrometry in combination with protein chemistry techniques to probe the interface of between light-harvesting complex and reaction centers (RCs), we were able to resolve the close association of the intrinsically disordered regions (IDR) in linker proteins of PBS with their binding partners. We then use genetic platform to reengineer the IDR and interrogate the functions of those domains that are potentially involved in fine-tuning the excitation energy transfer within PBS and between PBS and RCs.

Science priorities for the next year (2023-2024):

- Cross-talk (excitation energy and protein structural dynamics) of the top cylinder (in tricylindrical PBS) with the two basal cylinders and
- The PBS interface facing the RCs, where terminal energy emitter is functionally coupled with the reducing side of PSII. Mutants generated.

My major scientific area(s) of expertise is/are: Mass spectrometry, biochemistry, protein chemistry.

To take my project to the next level, my ideal collaborator would have expertise in: Nuclear magnetic resonance spectroscopy (NMR) of proteins.

Publications supported by this project Click here to enter text.:

1. Niedzwiedzki, M.D., Magdaong, N.C.M., Su, X*, Adir, N., Keren, N., Liu, H. (2023) Mass spectrometry and spectroscopy characterization of a tetrameric photosystem I supercomplex from *Leptolyngbya ohadii*, a desiccation-tolerant cyanobacterium ***Biochim Biophys Acta*** (Bioenergetics) 1864 (2),148955 * Undergraduate researcher

2. Liu, H. (2023) Intramolecular allostery in cyanobacterial phycobilisome as revealed by quantitative mass spectrometry ***Biochemistry*** 62 (7), 1307-1320
3. Mummadisetti, M., Su, L*., Liu, H. (2022) An approach to nearest neighbor analysis of pigment-protein complexes using chemical cross-linking in combination with mass spectrometry ***Methods in Enzymology***. doi.org/10.1016/bs.mie.2022.08.004 * Undergraduate researcher
4. Puskar, R Truong, C.D., Swain, K., Chowdhury, S., Chan, K-Y., Li, S., Cheng, K-W., Wang, T.Y., Poh, Y-P., Mazor, Y., Liu, H., Chou, T-F., Nannenga, B.L., Chiu, P-L (2022) Molecular asymmetry of a photosynthetic supercomplex from green sulfur bacteria ***Nature Communications*** doi.org/10.1038/s41467-022-33505-4
5. Liu, H. (2022) AlphaFold and structural mass spectrometry enable interrogations on the intrinsically disordered regions in cyanobacterial light-harvesting complex phycobilisome ***J Mol Biol***. 434(21):167831. doi: 10.1016/j.jmb.2022.167831.
6. Niedzwiedzki, M.D., Magdaong, N.C.M., Su, X*, Liu, H. (2022) Biochemical and spectroscopic characterizations of the oligomeric antenna of the coral symbiotic Symbiodiniaceae Fugacium kawagutii ***Photosynthesis Res*** doi: 10.1007/s11120-022-00951-6 * Undergraduate researcher
7. Johnson, V.M., Biswas, S., Roose, J.L., Pakrasi, H.B., and Liu, H. (2022) Psb27, a photosystem II assembly protein, enables quenching of excess light energy during its participation in the PSII lifecycle. ***Photosynth Res*** Jan 5th doi: 10.1007/s11120-021-00895-3.
8. Magdaong, N.C.M., Buhrmaster, J.C., Faries, K.M., Liu, H., Tira, G.A., Lindsey, J.S., Hanson, D.K., Holten, D.J., Laible, P.D., and Kirmaier, C. (2021) In Situ, protein-mediated generation of a photochemically acitve chlorophyll analogue in a mutant bacterial photosynthetic reaction center. ***Biochemistry*** 60 (16):1260:1275
9. Liu, H., Zhang, M.M., Weisz, D.A., Cheng, M., Pakrasi, H.B., Blankenship, R.E. (2021) Structure of Cyanobacterial Phycobilisome Core Structure by Structural Modeling and Chemical Cross-linking. ***Sci Adv.*** 7 (2), eaba5743
1.

NREL Photosynthetic Energy Transduction Core Program - Modulation of PSI Oligomeric Form, Spectral Capacity and Electron Flux in *Synechocystis* sp. PCC 6803

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Overall research goals:

The overall goal of the Photosynthetic Energy Transduction core program at NREL is to understand the fundamental mechanisms that support integration of reaction center photochemistry with electron transport and reduction-oxidation reactions of enzymes. This integration serves to maintain the function of photosynthetic components as cells respond to changing energy and growth conditions. Research areas include elucidating the properties of Photosystem I (PSI) in response to variable electron/photon flux, the roles of ferredoxins in mediating electron transport and mechanisms of redox enzymes for converting electrons into reduced products. These studies are revealing how photosynthetic components successfully manage variations in electron-transport that result from variable growth conditions in converting sunlight into chemicals and fuels.

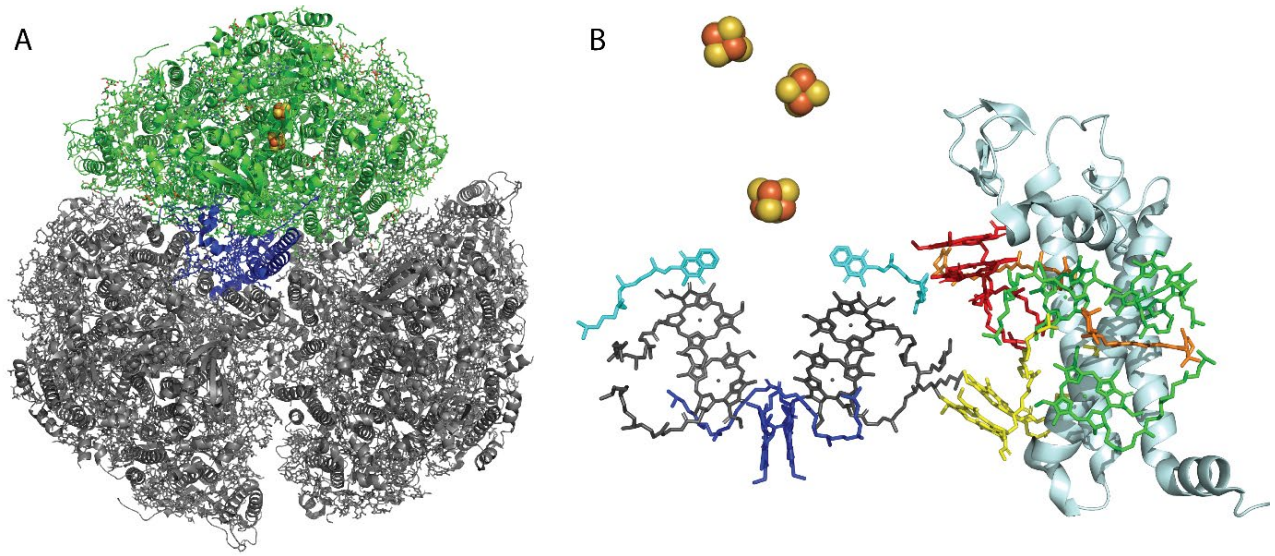

Figure. PsaL in relation to A) PSI trimeric structure as viewed from the stromal surface (PsaL in blue), B) the electron transfer chain consisting of P_{700} (blue), chlorophylls (grey), quinones (light blue), and iron-sulfur clusters (spheres), PsaL (cyan ribbon) and associated chls (green) and b-carotenes (orange), and nearby red chl pairs B37/B38 (red) and A32/B7 (yellow).

We aim to understand the properties of PSI that enable the dynamic coupling of photochemistry to enzymatic reactions under variable energy flux conditions. PSI couples photon absorption with the generation of reducing equivalents, and is susceptible to perturbations in light quality/quantity and downstream electron utilization. Under conditions where the peripheral O_2 reduction reaction (ORR) pathway is removed, we observe widespread changes to PSI, including in its oligomeric composition, P_{700} photooxidation capacity, and spectral properties which are further exasperated in fluctuating and high light. Recently, we have demonstrated that these changes result from a modulation of the protein and pigment environments around the PsaL subunit. PsaL exhibits increased accessibility, particularly in the regions associated with or near locations of chlorophyll and carotenoid binding. Changes to the abundance and compositions of carotenoids in PSI are also observed. In ORR strains, these physical

modifications of PSI in the region of PsaL perturb the spectral properties that co-occur with changes in PSI photochemistry. Because PsaL is located at the trimer interface, this may also account for the observed increase in PSI monomerization. Collectively, our findings reveal new insights on mechanisms that organisms can employ to control PSI photochemistry and finely tune energy and electron transfer processes.

Significant achievements (2021-2023)

- **Control of Photosystem I photochemistry by activity of electron utilization pathways.** We established a functional relationship between Photosystem I photochemistry and electron utilization by dissecting the properties of PSI reaction centers isolated from strains defective in the oxygen reduction reaction (ORR) catalyzed by flavodiiron proteins. Mass spectrometry and fluorescence emission spectroscopy on PSI isolated from ORR strains revealed changes in red chlorophylls, lower oxidation capacity and decreased transfer of electrons to flavodoxin. Together with increased cyclic electron flow, the modifications to PSI modulate use of excitation energy and reductant generation under changing environmental conditions (*RSC Advances*, 12, 14655, (2022)).
- **Physical modifications of PSI under conditions of decreased electron demand and increased photon availability.** We demonstrated increased accessibility of the PsaL subunit to proteolysis using mass spectrometric analysis (in collaboration with Dr. B. Bothner, Montana State University), with regions of increased accessibility associated with or near pigment binding in WT PSI. Pigment analysis revealed changes in the overall abundance of carotenoids, and increased echinenone and zeaxanthin, in PSI monomers and trimers. These findings indicate a flexibility in PSI physical properties to enable the tuning of photochemistry in response to dynamic energy fluxes (*J. Biol. Chem.*, *In review* (2023)).

Science priorities for the next year (2023-2024):

- Determine the structural properties of PSI oligomeric complexes isolated under altered cellular redox conditions (in collaboration with Dr. P. Fromme at Arizona State University).
- Probe how the oligomeric and structural changes of ORR PSI impact the interaction with redox partners to partition reducing equivalents among downstream pathways.
- Investigate alterations that occur in all PSI subunits, as well as subunit-subunit interactions that underpin the transition to monomerization under disrupted electron utilization conditions.

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

To take my project to the next level, my ideal collaborator would have expertise in: Structural determination of large pigment-protein complexes; spectroscopy on the excitation energy transfer and electronic structure of red chlorophylls.

Publications supported by this project:

1. C. E. Lubner. "Bacteria 'Read' Light to Gain a Competitive Advantage". *J. Bacteriol.,* 201:e0082-19. (2019). DOI: 10.1128/JB.00082-19.
2. Brown, K. A., Guo, Z., Tokmina-Lukaszewska, M., Scott, L. W., Lubner, C. E., Smolinski, S., Mulder, D. W., Bothner, B., King, P. W. (2019) The oxygen reduction reaction catalyzed by *Synechocystis* sp. PCC 6803 flavodiiron proteins. *Sustainable Energy Fuels*, 3, 3191.
3. S.L. Smolinski, C.E. Lubner, Z. Guo, J.H. Artz, K.A. Brown, D.W. Mulder, and P.W. King. "The influence of electron utilization pathways on photosystem I photochemistry in *Synechocystis* sp. PCC 6803", *RSC Advances*, 12, 14655, (2022). DOI:10.1039/D2RA01295B.
4. S. L. Smolinski, M. Tokmina-Lukaszewska, J. M. Holland, Z. Guo, B. Bothner, P. W. King and C. E. Lubner. "Modulation of PSI spectral capacity and electron flux in response to changes in electron flow pathways and photon flux in *Synechocystis* sp. PCC 6803", *J. Biol. Chem.*, (2023). *In review.*

Optimizing photosystem performance through a bioengineered red sites library

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[2] - Department of Chemistry, Massachusetts Institute of Technology

All photosynthetic organisms collect and concentrate sunlight using biological antennae systems. Biological antennae contain hundreds of chemically identical light harvesting pigments commonly bound by membrane proteins. The properties of each embedded pigment are modified or tuned by its specific environment in ways which contributes to the remarkable properties of biological antennae, high quantum efficiency, tunability and tolerance towards high light in the presence of oxygen. We explored two aspects biological antennae in this work, first is the energetic landscape of the photosystem I (PSI) core antennae and second, the maintenance of high excitation energy transfer efficiencies in the face of large-scale structural heterogeneities in the PSI-IsiA system from cyanobacteria. We constructed a series of chimeric PSI complexes with precisely defined mutations and spectral differences. This library allows for experimental assignments of red chlorophylls in the PSI core and is being used to establish the relationship between red site location and electronic structure to transfer efficiency and photoprotection in PSI. In the PSI-IsiA system we identified that this PSI – antennae super complex exists as a population of heterogenous structures. Shifts as large as 30 Å in the position of individual IsiA subunits across the PSI-IsiA population were detected. We found that in most of these extreme structural shifts excitation energy transfer rates from IsiA to PSI are faster and this effect depends on specific IsiA chlorophylls (Chls). We suggest that mitigating the effect of large-scale structural transitions plays an important part in determining the positions of chls between PSI and IsiA and this may extend to other large antennae – photosystem super-complexes.

Molecular Genetics of Chloroplast Fe homeostasis

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Overall research goals:

The Fe quota of a cell is determined by the stoichiometries of various Fe-cofactor-containing proteins whose abundances change in response to metabolic demand for the pathways in which they function. Because Fe is a growth-limiting micronutrient, mechanisms for reducing this quota have evolved. One such mechanism is Fe sparing, where an Fe-independent protein can cover the function of the Fe-containing one: the replacement of ferredoxin (Fd) by flavodoxin (Fld) is a widespread example of Fe sparing. Fe sparing by replacement typically operates on abundant proteins, like ferredoxins, so that the replacement can have a notable impact on the quota. In the absence of an external source of Fe, the abundant Fe proteins serve as internal reservoirs: the proteins are degraded and the Fe is re-used / re-cycled for use in metabolically prioritized pathways, like ROS management or respiration. Some essential Fe-containing proteins that cannot be replaced, like PSI in photoautotrophic cells, are reduced in abundance, concomitant with re-organization of the macromolecular complex.

The immediate questions are as follows: Which Fe proteins (besides Fd) are targeted for degradation? Which Fe proteins (besides FeSOD) are prioritized for maintenance in face of poor Fe nutrition? How does Fe-sparing and Fe-reallocation impact the chloroplast Fe quota? What is the pathway for Fe recycling? Are there modifications (composition, subunit stoichiometry) to PSI-LHCI in Fe-limited cells, and is there an impact of trophic conditionis on such modifications?

In this context, we have the following Aims.

1) We will use quantitative proteomics to a) estimate the chloroplast luxury vs. economy Fe quota in photoheterotrophic vs. photoautotrophic cells based on the inventory of plastid Fe proteins, using a compiled portfolio of Chlamydomonas Fe proteins deduced from the improved v6 genome assembly and including outputs from computational predictions and orthology to plant Fe proteins, and b) estimate the changes in the absolute abundances of PSI and LHCI polypeptides in Fe economy mode, especially in the context of trophic transitions (CO2 to acetate).

2) We will use single particle electron microscopy under cryogenic conditions to capture structural modifications to the PSI-LHCI super-complexes in Fe-limited photoautotrophic algal cells, especially with respect to the number, type, and orientation of peripheral antenna proteins, and with reference to the output from quantitative comparative proteomics.

Significant achievements (2021-2-24

Previously, we showed that Dunaliella species maintain photosynthesis and thrive in low-iron environments. They are extremophile organisms who like a saline environment (0.5 to 2M NaCl). We produced high quality genome assemblies and transcriptomes for two *Dunaliella* species (*salina* and *tertiolecta*) to identify a host of iron-uptake proteins in both species that includes a massive expansion of iron-binding transferrin proteins and a novel family of siderophore-iron uptake proteins. Complementing these multiple iron-uptake routes, ferredoxin functions as a large iron reservoir that can be released by replacement of ferredoxin with flavodoxin. Besides these iron scavenging and sparing mechanisms, we noted as well that photosystem I (with its high Fe content) was specifically down-regulated relative to PSII, as also were the associated light harvesting chlorophyll binding proteins, except for a novel chlorophyll-protein, named TIDI (for thylakoid iron-deficiency-induced). The expression of TIDI is tightly correlated with Fe status. It is barely expressed in Fe-replete conditions and

massively up-regulated in Fe-deplete cells: 1200-fold induction in *D. salina*. TIDI shares sequence similarity with Lhca3, but it is distinct in that TIDI is extended at the N-terminus and carries a larger extra-membrane loop between transmembrane helices 1 and 2. Phylogenomic analysis identified TIDI in other chlorophytes as well, speaking to its functional importance in low iron environments. Therefore, we initiated a collaboration with Dr. Masakazu Iwai (LBNL) and Dr. Grob (Nogales group, UC Berkeley) to determine a structure of *Dunaliella* PSI from Fe-deficiency (relative to Fe-replete) by single particle analysis using cryo-EM. Samples were prepared and datasets collected on the Krios G2 300 kV CryoTEM. Preliminary analysis of Dunaliella PSI isolated from Fe-deficiency reveals structural differences compared to the Fe-replete structure, especially with respect to antenna organization.

Science priorities for the next year (2023-2024):

- We expect to resolve the entire PSI-LHC complex structures from both organisms at < 3 A resolution by the end of the next project period.
- We will also complete the inventory of Chlamydomonas Fe proteins in replete vs. deficient conditions.

My major scientific area(s) of expertise is/are: trace metal biology, algal quantitative systems biology, genetics.

To take my project to the next level, my ideal collaborator would have expertise in: biochemistry and structure of photosynthetic complexes.

Publications supported by this project [Enter Publications Supported by This Grant/FWP since 11/2021:

1. Hui, C., Schmollinger, S., Strenkert, D., Holbrook, K., Montgomery, H., Chen, S., Nelson, H., Weber, P., Merchant, S.S. (2022) Simple steps to enable reproducibility: Culture conditions affecting Chlamydomonas growth and elemental composition. Plant J. 111:995-1014. doi: 10.1111/tpj.15867

2. Hui, C.H., Schmollinger, S., Glaesener, A. G. (2023) Growth Techniques in The Chlamydomonas sourcebook, 3rd edition, Volume 1, Introduction to Chlamydomonas and its Laboratory Use ed. Wollman, Grossman, Goodenough, Elsevier. Chapter 11.

3. Blaby-Haas, C.E., Merchant, S.S. (2022) Trace metal nutrition and response to deficiency in The Chlamydomonas sourcebook, 3rd edition, ed. Wollman, Grossman, Goodenough.

4. Schmollinger, S., Merchant, S.S. (2023) Quantitative elemental imaging in eukaryotic algae. Metallomics 15:mfad025 doi: 10.1093/mtomcs/mfad025

5. Glaesener, A.G., Merchant, S. S., (2023) Optimizing Fe nutrition for algal growth. Methods in Molecular Biology. Methods Mol Biol. 2665:203-215. doi: 10.1007/978-1-0716-3183-6_16.

6. Davidi, L., Gallaher, S.D., Ben-David, E., Purvine, S.O., Filmore, T.L., Nicora, C.D., Craig, R.J., Schmollinger, S., Roje, S., Blaby-Haas, C.E., Auber, R.P., Wisecaver, J., Merchant, S.S. (2023) Pumping Iron: A Multi-omics Analysis of Two Extremophilic Algae Reveals Mechanisms of Iron Economy. Proc. Natl. Acad. Sci. USA 120:e2305495120. doi: 10.1073/pnas.2305495120

Collaborative Project: Dynamics and consequences of PSI Supercomplexes

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Overall research goals:

The overall research goal of this project is to understand the diversity of PSI supercomplexes (SC), their contribution to PSI-driven cyclic electron flow (CEF), and the roles of CEF in stress acclimation over short- and long-term time scales. Our major objectives are: **(O1)** Determine the structure and localization of the *C priscuii* (formerly *Chlamydomonas* sp. UWO241) PSI SC using single particle and in situ imaging; **(O2)** Determine the contribution of PSI SC variability and CEF to supporting growth and stress response during long-term stress; **(O3)** Consider the contributions of high CEF to supporting growth and stress response during long-term stress.

Significant achievements (2022-2023):

- During the first year of our renewal project, PI Morgan-Kiss and her students (*, undergraduate student; **, graduate student) have made progress on the following major activities:

O1: Cryo-ET and Cryo-EM studies (*Lydia Marie Joubert, Wah Chiu, Jay-How Yang, Petra Fromme*). We hosted Dr. Yang for 2 weeks in Fall 2023 to work on isolation of SC isolation from fresh cultures of *C. priscuii*. We are supplying Dr. Joubert with fresh cultures and thylakoids for Cryo-ET studies.

O2: Impact of long-term salinity stress on CEF and state transitions in *C. reinhardtii*, *C. priscuii* and *Chlamydomonas* sp. ICE-MDV (*I. Kalra*** et al. 2023). We compared photobiology and state transitions across three *Chlamydomonas* spp. with variable tolerance to high salt. Long-term acclimation to high salt resulted in attenuation of state transition capacity across all strains: state transition capacity was also negatively corelated with salt tolerance. All strains exhibited higher CEF and produced a PSI SC under high salt acclimation. Maximum rates of CEF were correlated with high tolerance to salt stress. We have generated a working model for the role of CEF and formation of various kinds of PSI SCs in *Chlamydomonas* strains exhibiting a gradient of stress tolerance (Fig. 1).

O2: A role for CEF in *C. priscuii* exposed to short- vs. long-term high light stress (*D. Popson***, S. *D'Silva**, ms in prep). Despite long-term isolation in extreme shade, *C. priscuii* possesses remarkable ability to grow in low temperature/high light conditions. Overall, *C. priscuii* exhibits constitutive ability to maintain very low levels of ROS (H_2O_2) as well as reduced photodamage to PSI, compared with *C. reinhardtii*. Acclimation to either long-term high light or low temperature stress is associated with high CEF and confers additional resistance to photoinhibition.

O2: The impact of mixotrophic growth on long-term acclimation to high salt and high light in *C. reinhardtii* (*K. Wheeless***). We are investigating how the function of CEF is altered in *C. reinhardtii* when it is grown under autotrophic vs. mixotrophic conditions. Under mixotrophic conditions, CEF is upregulated under both control and stress conditions, while under autotrophic conditions, CEF is only activated under long-term stress. Furthermore, high CEF is associated with activation of NPQ in autotrophic but not mixotrophic-grown cultures, suggesting that the function of CEF is dependent upon trophic status.

O3: The role of high CEF in energy homeostasis (*K. Wheeless***). We have preliminary data on energy charge status (ATP/[ADP+ATP]) of *C. reinhardtii* and *C. priscuii* grown under variable conditions.

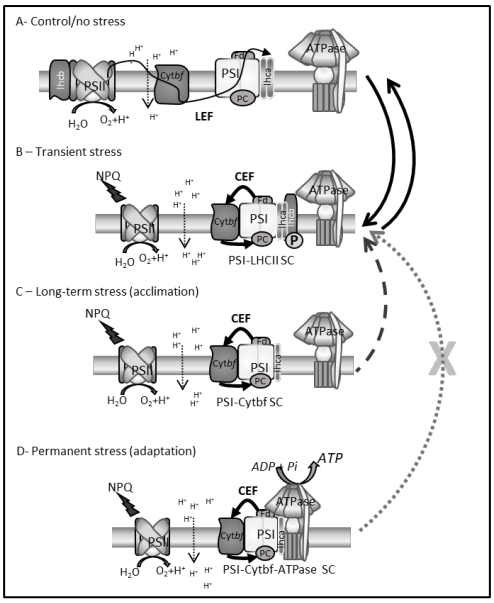

Figure 1. Models for formation of CEF associated PSI SCs during short- and long-term stress in *Chlamydomonas* spp. A. Structure of the photosynthetic electron transport chain (PETC) during optimal growth condition. **B.** PETC under conditions of state 2. **C.** PETC configuration after long-term acclimation to salinity stress. **D.** PETC configuration after adaptation to permanent salinity stress, as in case for halotolerant psychrophile *C. priscuii* (Kalra et al. 2023).

- We also made advances in other related activities: [1] *C. priscuii* workshop. In summer 2023, PI Morgan-Kiss hosted an international workshop for researchers working with *C. priscuii*. [2] New polar *Chlamydomonas* strains. We have started collaborations with one current (Marina Cvetkovska, U Ottawa) and one new researcher (C. Ribeiro, Universidad Mayor, Chile) to study CEF in four new polar *Chlamydomonas* spp. isolated from the Arctic and marine Antarctic regions. [3] Acquisition of new instrumentation. PI Morgan-Kiss received two internal grants to fund the purchase of a JTS150 spectrometer and three FMT150 photobioreactors. We have a working protocol for the JTS: the ECS signal appears to be distinct in *C. priscuii* compared with *C. reinhardtii*. Pilot experiments have been conducted in the photobioreactors for *C. reinhardtii* and *C. priscuii*.

Science priorities for the next year (2023-2024):

While we have made progress on Yr 1 on several activities, our research was impacted by a 6-month pause in growing the psychrophiles due to issues with contamination and reproducible growth. These problems were recently resolved, and we are eager to move forward with a renewed focus on the SC structural studies of Objective 1. In addition, we will be continuing to perfect protocols for the JTS150 and photobioreactors to support experiments focused on the temporal trends of CEF activation, functional consequences of CEF, and isolation of PSI SCs from different conditions to validate our working models shown in Figure 1(Objective 2). In parallel, we will be measuring energy charge in cultures from various conditions and growth stages (Objective 3). We are also preparing several manuscripts from results of Objective 2 activities.

My major scientific area(s) of expertise is/are: algal photobiology, cultivation of extremophilic algae, diversity of native algal and microbial eukaryote communities, Antarctic field biology, molecular microbial ecology.

To take my project to the next level, my ideal collaborator would have expertise in: JTS150 instrument, particularly interpreting ECS data; BN-PAGE; Metabolomics

Publications supported by this project :(*, undergraduate student; **, graduate student; **bold**, PI or Co-PI on this project).

1. **Morgan-Kiss R.M.** ¥, Popson D.**, Pereira R.**, Dolhi-Binder J.**, Teufel A.**, Li W.**, Kalra I.**, Sherwell S.**, Reynebeau E., and Takacs-Vesbach C. Sentinel protist taxa of the McMurdo Dry Valley lakes, Antarctica. *Frontiers in Ecology and Evolution. In Review MS# 1343472*
2. Kalra I.**, **Wang X., Zhang R.** and **Morgan-Kiss R.M.** (2023) High salt-induced PSI-supercomplex is associated with high CEF and attenuation of state transitions. *Photosynthesis Research*, 157: 65-84. https://doi.org/10.1007/s11120-023-01032-y
3. Poirier M., Osmers P., Wilkins K. and **Morgan-Kiss R.M**. and Cvetkovska M. (2023) Aberrant light sensing and motility in the green alga *Chlamydomonas priscuii* from the ice-covered Antarctic Lake Bonney. *Plant Signaling & Behavior*, *18*; 2184588. https://doi.org/10.1080/15592324.2023.2184588
4. Hüner, N.P., Smith, D.R., Cvetkovska, M., Zhang, X., Ivanov, A.G., Szyszka-Mroz, B., Kalra, I.** and **Morgan-Kiss, R. M.** (2022) Photosynthetic adaptation to polar life: Energy balance, photoprotection and genetic redundancy. *Journal of Plant Physiology (Invited Review)*, 268: 153557
5. Stahl-Rommel S.**, Kalra I.**, D'Silva S.*, Hahn M.M.*, Popson D.**, Cvetkovska M. and **Morgan-Kiss R.M.** ¥ (2021) Cyclic electron flow (CEF) and ascorbate pathway activity provide constitutive photoprotection for the photopsychrophile, *Chlamydomonas* sp. UWO241 (renamed *Chlamydomonas priscuii*). *Photosynthesis Research* 151: 235-250. https://doi.org/10.1007/s11120-021-00877-5
6. Zhang X., Cvetkovska M., **Morgan-Kiss R.M**., Hüner N.P.A., and Smith D.R. (2021) Draft genome sequence of the Antarctic green alga *Chlamydomonas* sp. UWO241. *iScience* 102084-102084. https://doi.org/10.1016/j.isci.2021.102084
7. Kalra I.**, **Wang X.,** Cvetkovska M., Jeong J., McHargue W., **Zhang R.**, Hüner N.P.A., Cvetkovska M. **Morgan-Kiss, R.M.** (2020) *Chlamydomonas* sp. UWO 241 exhibits high cyclic electron flow and rewired metabolism under high salinity. *Plant Physiology* 183: 588-601. https://doi.org/10.1104/pp.19.01280
8. Raymond J.A., **Morgan-Kiss R.M.,** and Stahl-Rommel S.** (2020) Glycerol is an osmoprotectant in two Antarctic *Chlamydomonas* species from an ice-covered saline lake and is synthesized by an unusual bidomain enzyme. *Frontiers in Plant Science* 11: 1259-1259. https://doi.org/10.3389/fpls.2020.01259

NREL Photosynthetic Energy Transduction Core Program – Tuning of Electron-transfer and Catalysis in Photosynthetic Energy Conversion

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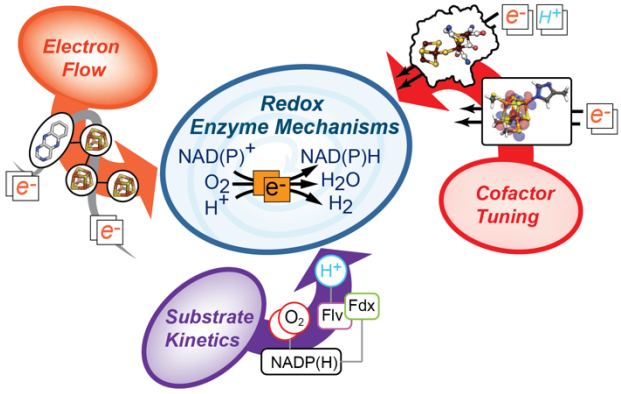
Overall research goals:

The overall goal of the Photosynthetic Energy Transduction core program at NREL is to understand the fundamental mechanisms that support integration of reaction center photochemistry with electron transport and reduction-oxidation reactions of enzymes. This integration serves to maintain the function status of photosynthetic components as cells respond to changing energy and growth conditions. Research areas include elucidating the properties of Photosystem I in response to variable electron/photon flux, the roles of ferredoxins in mediating electron transport, and mechanisms of redox enzymes for converting electron flux into reduced products. These studies are revealing how photosynthetic components successfully manage variations in electron-transport that result from variable growth conditions in converting sunlight into chemicals and fuels.



To address how photosynthetic flux is coupled to the catalytic activation of small molecules, we have been probing electron-transfer (ET) and catalytic mechanisms in model proteins that display a wide range of metal sites and cofactors, ET properties, and catalytic reactivity. Using a combined biochemical and spectroscopic approach, we are determining how cofactor tuning mechanisms relate to fundamental properties that control electron-transport in ferredoxin-mediated reactions, directional ET in the HoxEFU sub-complex mediating electron-exchange reactions between ferredoxin and pyridine nucleotide pools, and the vastly different catalytic bias observed for [FeFe]-hydrogenases catalyzing the activation of H_2 from protons and electrons. For the latter case, we have been investigating enzymes that favor either H_2 uptake or H_2 production to understand the relationship between electron/proton transfer and catalytic reactivity. We hypothesize that differences in reactivity are due to differences in the electronic structure of the active-site H-cluster, and profound changes in stabilization of reaction intermediates. Our results are revealing a common set of principles for how structural and electronic properties of cofactors are tuned through protein interactions to impart exquisite control over ET and catalysis.

Significant achievements (2021-2023)

- **Understanding iron-sulfur cluster function in electron transport**. Electron transport chains of the Cyanobacterial [NiFe]-hydrogenase, HOX, and some [FeFe]-hydrogenases contain a [4Fe-4S] cluster with site-differentiation. Our combined computational models and experimental studies show that His-coordination spatially organizes unoccupied molecular orbitals and tunes reduction potentials to control electron transport (Lubner et al. *Chem. Sci.* 2022). Studies of electron relay iron-sulfur clusters in [FeFe]-hydrogenase CpII is revealing the role of spin coupling in the control of electron transport for H_2 oxidation catalysis.
- **Understanding how proteins tune cofactor properties for catalytic H_2 activation.** [FeFe]-hydrogenases catalyze reversible H_2 activation in photosynthetic algae and anaerobic microbes. We

have shown that distinct enzyme classes can have significant variation in reactivity from differential tuning of iron-sulfur cofactors. Our studies of these enzymes are revealing how distinct examples of [FeFe]-hydrogenases have evolved to favor either H_2 production or H_2 uptake reactions (Kisgeropoulos et al. *Front. Microbiol.* 2022; in review at *J. Am. Chem. Soc*. 2023).

- **Redox landscape of HoxEFU and role of HoxE.** HoxEFU catalyzes the diaphorase reaction coupling NAD(P)H reduction-oxidation to the ferredoxin pool in *Synechocystis* (Artz et al. *J. Biol. Chem*. 2020). HoxE is required for reactivity with ferredoxin, and we have used EPR and potentiometric studies on purified HoxE that show it coordinates a [2Fe-2S] cluster with an E_m that changes from -395 mV to -520 if alone or in a HoxEFU complex, suggesting the role of HoxE in mediating ET may be complex.

Science priorities for the next year (2023-2024):

- Understand the properties of ferredoxins that mediate electron transport in *Synechocystis* PCC 6803.
- Develop understanding of the properties of redox cofactors in mediating electron transport in model redox enzymes, and in the control of catalytic directionality in the activation of H_2 and O_2.
- Determine how structural and electronic protein-cofactor interactions modulate directional electron-transfer mechanism across large distances for the model HoxEFU diaphorase.

My major scientific area(s) of expertise is/are: Application of EPR spectroscopy and structural methods to the study of hydrogenases, redox enzymes, and protein-bound iron-sulfur clusters.

To take my project to the next level, my ideal collaborator would have expertise in: Synchrotron based techniques for probing electronic spin structure of metallocofactors.

Publications supported by this project:

1. K.A. Brown, Z. Guo, M. Tokmina-Lukaszewska, L.W. Scott, C.E. Lubner, S. Smolinski, D.W. Mulder, B. Bothner, P.W. King. "The oxygen reduction reaction catalyzed by *Synechocystis* sp. PCC 6803 Flavodiiron proteins." *Sustain. Energy Fuels*. **3**:3191-3200. (2019).
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Modular Synthesis of Biohybrid Assemblies for Studying Photosynthetic Mechanisms and Solar Fuels Catalysis

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Overall research goals:

Research of natural photosynthesis accomplished by reaction center (RC) proteins has provided incredible insight into the molecular and electronic structure of the cofactors that manage light-harvesting, highly efficient electron transfer, and redox catalysis. These basic studies have given us the foundational knowledge for how to utilize nature's optimized photochemistry to drive non-native chemical reactions. Toward uncovering new photosynthetic pathways and products, biohybrid work in this program is focused on augmenting the protein structure with abiotic cofactors and new inter-protein connections to provide 1) well-defined but synthetically untenable light-harvesting and electron transfer pathways between molecular photosensitizers and catalysts, and 2) unique microenvironments for abiotic cofactors, potentially enabling activity or mechanisms not possible in homogeneous solution. Recent work has focused on utilizing the extraordinarily strong non-covalent binding between streptavidin and biotin to ensure stoichiometric and stable interaction between light-harvesting or electron transfer proteins and the abiotic molecular photosensitizers or catalysts. The streptavidin-biotin linking chemistry is enabling us to access biohybrid assemblies that effectively capture low-energy red light to drive H_2 evolution, and also to understand the molecular design features that will allow us to utilize earth-abundant first row transition metal based molecular photosensitizers for biohybrid photocatalysis. We anticipate that our group's combined efforts in protein manipulations, design and synthesis of abiotic cofactors, and spectroscopic analyses will provide a deep mechanistic understanding of electron and energy transfer pathways, and catalytic mechanisms for solar fuels generation.

Significant achievements (2022-2023):

Mini-RC biohybrids augmented with red-light absorbing antenna proteins. The mini-RC biohybrids our team has worked with up to this point consist of Ferredoxin (Fd) or Flavodoxin (Fld) labeled with a Ru(II)tris(2,2'-bipyridine) analog that can selectively label free cysteine residues (RuPS, Figure 1b). A limitation of using RuPS is that its MLCT absorption is relatively narrow and limited to the high-energy blue region of the solar spectrum. Recent work has shown that a red-light absorbing antenna protein R-phycoerythrin (RPE) can transfer energy to Ru(bpy)$_3$ complexes that then go on to initiate photoredox catalysis. To build on this concept and introduce potentially new energy and electron transfer pathways into our mini-RC biohybrids, we have developed a stepwise approach to assembling RPE with Ru-Fd-Co using streptavidin-biotin interactions (Figure 1a). We observe that the RPE-

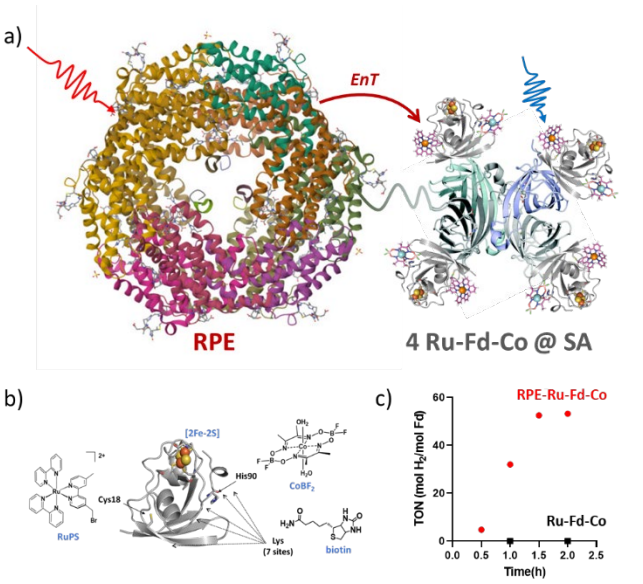

Figure 1. a) Proposed scheme for multi-wavelength excitation of RPE-Ru-Fd-Co biohybrid and energy and electron transfer pathways. b) Structure of Ru-Fd-Co biohybrid assembly labeled with biotin to bind to streptavidin. c) Comparison of red-light initiated H_2 photocatalysis by Ru-Fd-Co with and without RPE antenna.

Ru-Fd-Co biohybrid is capable of generating H_2 from water using both red and blue illumination, where the biohybrid without RPE is not active with exclusively red light (Figure 1c). We hypothesize that targeting specific illumination wavelengths will initiate either energy or electron transfer mechanisms within the RPE-Ru-Fd-Co biohybrid, and that simulated solar light will activate both mechanisms, providing insight into the efficiency of both pathways and synergistic interactions.

Protein confinement to manipulate excited state kinetics of earth-abundant molecular photosensitizers. We have recently initiated an effort to investigate Cu(I)bis(phenanthroline) complexes in protein environments as a replacement for the prototype $Ru(bpy)_3$ photosensitizers typically used in biohybrid assemblies. A hurdle to using copper-based photosensitizers to drive multi-electron redox catalysis is their rather short excited state lifetimes, which is an outcome of a dramatic structural distortion in their MLCT state. We hypothesized that the microenvironment exhibited by natural proteins may impact this distortion and have a substantial effect on the excited state kinetics. To test this hypothesis, we have synthesized three Cu(I)bis(phen) complexes with different spacer lengths between the Cu(I) photosensitizer core and a biotin group. Incubation of these complexes with streptavidin yields stoichiometric binding, and the MLCT band of the Cu(I) center is preserved in the Cu-SA biohybrids. Preliminary ultrafast and nanosecond transient absorption spectroscopy measurements show that the Cu(I)bis(phen) excited state lifetime is measurably longer when embedded in the streptavidin pocket, and also extremely sensitive to the spacer length. We anticipate that the results of these studies will provide design principles to realize biohybrid solar fuels catalysts using all earth-abundant elements.

Science priorities for the next year (2023-2024):

- Compare H_2 evolution activity from RPE-Ru-Fd-Co biohybrids under simulated solar light to that with exclusively blue or red illumination, isolate energy and electron transfer pathways.
- Analysis of Cu(II) environment, electronic structure, and Cu-Cu distance in Cu-SA biohybrids using cw and pulsed EPR spectroscopy.
- Computational analysis of Cu-SA biohybrids to visualize copper microenvironment and help interpret experimental results (collaboration with Prof. Kristy Mardis, Chicago State University).

My major scientific area(s) of expertise is/are: Design and synthesis of molecular photosensitizers and catalysts; analysis of photoinduced electron transfer kinetics; evaluation of electro- and photocatalytic mechanisms.

To take my project to the next level, my ideal collaborator would have expertise in: site-selective mutagenesis; transient absorption spectroscopy of PSI and light-harvesting proteins.

Publications supported by this project:

1. L. M. Utschig, C. L. Duckworth, J. Niklas, O. G. Poluektov, "Intrinsic photosystem I-ferredoxin interactions related to linear electron flow in spinach and cyanobacterial thylakoid membranes" *Photosyn. Res.* **2023**, submitted.
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Regulation of Photosynthesis

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Overall research goals:

Photosynthetic light-harvesting systems are regulated, protected against photo-oxidative damage, self-assembled into supercomplexes, and rely on a subtle balance of electronic interactions to produce a highly robust system. In this project, we apply a multidisciplinary approach that combines genetics, biochemistry, and structural biology with ultrafast spectroscopy and modeling to understand the mechanisms by which oxygenic photosynthetic organisms regulate light harvesting. In response to fluctuations in light intensity, non-photochemical quenching (NPQ) mechanisms are induced and relaxed on different timescales. In algae and plants, the qE type of NPQ is turned on and off rapidly by changes in thylakoid lumen pH, qZ operates on an intermediate timescale of several minutes and depends on zeaxanthin, whereas the slower qH and qI types downregulate PSII during long-term light stress. Our specific aims are (1) to elucidate the mechanisms and locations of qE and qZ in algae and plants, (2) to investigate how NPQ affects the generation and temporal dynamics of ^{3}Chl* and $^1O_2$*, (3) to construct a quantitative model for NPQ responses, (4) to reconstitute NPQ in a simplified membrane system, (5) to understand how specific amino acid residues and domains of LHCs affect their structure and function, and (6) to visualize the dynamics of essential NPQ components.

Significant achievements (2021-2023):

- We performed fluorescence lifetime snapshot experiments with *Nannochloropsis* under regular and irregular illumination sequences. In collaboration with Limmer, we developed models based on the xanthophyll cycle and protonation of LHCX1, which are able to quantitatively describe the dynamics of NPQ.
- We successfully isolated LHCX1-containing supercomplexes from *Nannochloropsis* and performed negative stain electron microscopy (EM) to demonstrate their suitability for single-particle cryo-EM analysis.
- We isolated LHCSR- and PsbS-containing super- and mega-complexes from various NPQ-related mutants and collected single-particle cryo-EM datasets in collaboration with Nogales.
- By monitoring NPQ in intact cells of *Chlamydomonas* throughout high light/dark cycles of various illumination periods, we showed that LHCSRs play a major role during the light phases and revealed an activation of NPQ during the dark phases that arises from state transition.
- We generated homozygous CRISPR knock-outs of *PSBS*, *ZEP*, *VDE*, and *LUT2* in *Nicotiana benthamiana*.
- We performed annihilation-free transient absorption (TA) and snapshot TA spectroscopy on thylakoids from our *N. benthamiana* mutants and showed that (1) excitation energy transfer (EET) to the S1 state of zeaxanthin is temporally correlated with qE and (2) qE decreases the exciton diffusion length (L_D).
- We transiently expressed 63 PsbS orthologs across the Viridiplantae in the *N. benthamiana psbs* mutant and showed that land plant-like PsbS activity likely arose first in streptophyte green algae.
- We successfully obtained crystals of LHCII from spinach, performed XFEL experiments, and obtained structures at 1.9 Å resolution in collaboration with Kern, Yachandra, and Yano.
- To generate background strains for mutagenesis of LHCII in vivo, we have made *Arabidopsis* CRISPR knock-out lines that contain only specific LHCII isoforms.

Science priorities for the next year (2023-2024):

- Determine the locations of LHCX1, LHCSR, and PsbS in photosystem II.
- Measure timescales of charge transfer (CT) quenching in thylakoids from *N. benthamiana* mutants.

Our major scientific area(s) of expertise is/are: plant and algal genetics, membrane protein biochemistry, ultrafast spectroscopy.

To take our project to the next level, our ideal collaborator would have expertise in: developing new cryo-EM data analysis algorithms.

Publications supported by this project (2021-2023):

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4. Short AH, Fay TP, Crisanto T, Hall J, Steen CJ, Niyogi KK, Limmer DT, Fleming GR (2022). Xanthophyll-cycle based model of the rapid photoprotection of *Nannochloropsis* in response to regular and irregular light/dark sequences. *J Chem Phys* 156: 205102. DOI: 10.1063/5.0089335
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10. Perin G, Bellan A, Michelberger T, Lyska D, Wakao S, Niyogi KK, Morosinotto T (2023). Modulation of xanthophyll cycle impacts biomass productivity in the marine microalga *Nannochloropsis*. *Proc Natl Acad Sci USA* 120: e2214119120. DOI: 10.1073/pnas.2214119120
11. Teh JT, Leitz V, Holzer VJC, Neusius D, Marino G, Meitzel T, García-Cerdán JG, Dent RM, Niyogi KK, Geigenberger P, Nickelsen J (2023). NTRC regulates CP12 to activate Calvin-Benson cycle during cold acclimation. *Proc Natl Acad Sci USA* 120: e2306338120. DOI: 0.1073/pnas.2306338120
12. Fleming GR, Minagawa J, Renger T, Schlau-Cohen GS (2023). Photosynthetic light harvesting and energy conversion. *J Chem Phys* 159: 100401. doi: 10.1063/5.0170807
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Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosynthetic Reaction Centers

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Overall goals of the project:
In oxygenic photosynthesis, the initial charge separation events occur with near unit quantum efficiency in the photosystem II reaction center (PSII RC). PSII is the only known natural enzyme that uses solar energy to split water. The primary motivation of the proposed work is to *elucidate the design principles critical for our fundamental understanding of photosynthesis and for our ability to mimic PSII's remarkable properties*. This goal requires developing new approaches to connect structure to function; a particularly challenging task in PSII where the overlapping electronic transitions of the many pigments obscure their individual roles in the system's electronic excited states and charge separation process. With the overarching goal of improving our understanding of the structure-function relationship in the PSII RC, we propose parallel studies of spectroscopically simpler systems including PSII grown under far red light, model dyads and other reaction centers including the purple bacterial reaction center (BRC) and the heliobacterial reaction center (HbRC). We aim to leverage and extend the multidimensional spectroscopic tools we have developed in previous funding periods to advance structure-based excitonic and charge separation models in these systems. We propose a synergistic series of experiments on wild-type and mutant RCs and simpler dyad systems aimed at addressing the following questions:

1) How can we test and refine structure-based exciton models of photosynthetic reaction centers?
2) How can we relate structure to charge separation mechanisms in photosynthetic reaction centers?
3) Do electronic-vibrational resonances enhance photosynthetic energy transfer and charge separation?

Significant achievements (2021-2023):
Charge Separation in the Photosystem II Reaction Center Resolved by Multispectral Two-Dimensional Electronic Spectroscopy (*1*) While the PSII-RC has been studied extensively, the similar timescales of energy transfer and charge separation, and the severely overlapping pigment transitions in the Q_y region have led to multiple models of its charge separation mechanism and excitonic structure. We combined two-dimensional electronic spectroscopy (2DES) with a continuum probe and two-dimensional electronic vibrational spectroscopy (2DEV) to study the cyt *b*559-D1D2 PSII-RC at 77K. This multispectral combination correlates the overlapping Q_y excitons with distinct anion and pigment-specific Q_x and mid-IR transitions to resolve the charge separation mechanism and excitonic structure. Through extensive simultaneous analysis of the multispectral 2D data we find that charge separation proceeds on multiple timescales from a delocalized excited state via a single pathway in which $Pheo_{D1}$ is the primary electron acceptor, while Chl_{D1} and P_{D1} act in concert as the primary electron donor.

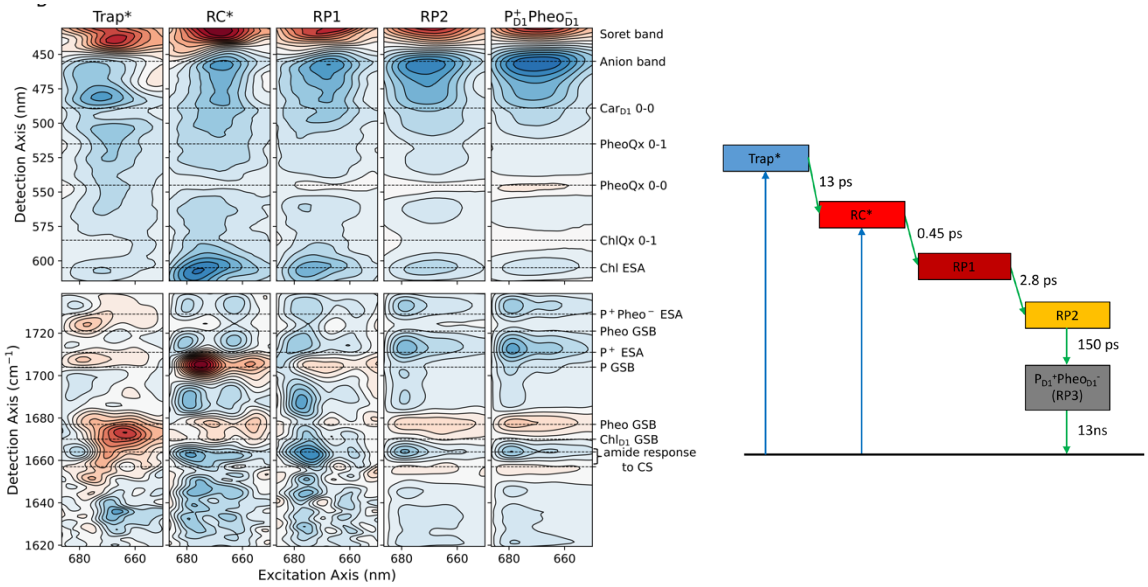

Figure 1: Global target model from simultaneous fitting of the multispectral 2D data (*1*) (Right) Five-compartment model with one trap state and three RP states. (**B**) 2D Species Associated Spectra (SAS) of each compartment, with both visible (top) and mid-IR (bottom) components.

Objectives for the coming year

- Continued tests of excitonic models of the PSII RC and BRC using multispectral 2DES
- Multispectral 2DES measurements of BRC mutants and model dyads to probe the importance of electronic-vibrational resonance on energy transfer and charge separation

My major scientific area(s) of expertise is/are: Multidimensional coherent spectroscopy and microscopy.

To take my project to the next level, my ideal collaborator would have expertise in: Theoretical modeling of energy transfer and charge separation, simulations of multidimensional spectra, synthetic chemist/biochemist creating biomimetic structures and reaction center mutants.

Publications supported by this award(*1-5*)

1. H. H. Nguyen, Y. Song, E. L. Maret, Y. Silori, R. Willow, C. F. Yocum, J. P. Ogilvie, Charge separation in the photosystem II reaction center resolved by multispectral two-dimensional electronic spectroscopy. *Science Advances* **9**, eade7190 (2023).
2. K. Zakutauskaitė, M. Mačernis, H. H. Nguyen, J. P. Ogilvie, D. Abramavičius, Extracting the excitonic Hamiltonian of a chlorophyll dimer from broadband two-dimensional electronic spectroscopy. *The Journal of Chemical Physics* **158**, 015103 (2023).
3. Y. Silori, R. Willow, H. H. Nguyen, G. Shen, Y. Song, C. J. Gisriel, G. W. Brudvig, D. A. Bryant, J. P. Ogilvie, Two-Dimensional Electronic Spectroscopy of the Far-Red-Light Photosystem II Reaction Center. *The Journal of Physical Chemistry Letters*, 10300-10308 (2023).
4. V. R. Policht, A. Niedringhaus, R. Willow, P. D. Laible, D. F. Bocian, C. Kirmaier, D. Holten, T. Mančal, J. P. Ogilvie, Hidden vibronic and excitonic structure and vibronic coherence transfer in the bacterial reaction center. *Science Advances* **8**, eabk0953 (2022).
5. Y. Song, R. Sechrist, H. H. Nguyen, W. Johnson, D. Abramavicius, K. E. Redding, J. P. Ogilvie, Excitonic structure and charge separation in the heliobacterial reaction center probed by multispectral multidimensional spectroscopy. *Nature Communications* **12**, 2801 (2021).

Assembly and Repair of Photosystem II, a membrane protein complex

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Overall research goals:

The long-term objective of this project is to unravel the mechanisms that regulate protection and repair of the photosynthetic apparatus in oxygenic organisms. Specifically, we are aiming to understand the molecular details of the life cycle of PSII. Our experimental organism is the cyanobacterium *Synechocystis* 6803. Photosystem II naturally undergoes *de novo* assembly and repair processes, making it one of the pigment-protein complexes with the highest turnover rate. We are analyzing the intermediate complexes that accumulate either during PSII assembly or repair. In addition, our efforts have also focused on studying the excitation-energy transfer characteristics of one of these intermediate complexes, the CP43-preassembly (pCP43) complex. We are also examining biosynthesis and energy transfer mechanisms in the iron-stress induced (Isi) A protein, a close relative of CP43.

Significant achievements (2021-2023):

- We identified and analyzed a previously unknown PSII intermediate complex, no-reaction center (NRC) complex that is formed during the PSII repair cycle.
- We developed a reversible and inducible CRISPR (CRISPRi) system to target key proteins involved in PSII and PSI assembly. Such a system provides the advantage of segregating the assembly and repair processes that naturally overlap, making it easier to study the two processes individually.
- We have identified a novel protein HltA that confers high-light tolerance in cyanobacteria. HltA has a GAF domain attached to a phosphatase domain, unique to these oxygenic phototrophs .
- We studied the role of a conserved cysteine residue of IsiA protein in quenching excess light energy in PSI-free IsiA complexes. On replacing the valine residue of CP43 that aligns with the cysteine residue of IsiA, we were able to engineer high-light tolerance in *Synechocystis* 6803.
- Our study of PSII function in *Cyanothece* 51142 challenged a long-standing notion that PsbA4, a member of the D1 protein family, is involved in PSII disassembly in the dark to facilitate nitrogen fixation, opening new avenues to explore the role of the PsbA4 protein.
- We assessed the energy-dissipation efficiency of the pCP43 complex to determine that β-carotene mediated chlorophyll *a* quenching is inefficient. The pCP43 antenna complex is not very well suited to cope with excessive light if it is separated from the rest of PSII.
- Our efforts at reevaluating the evolution of antenna proteins in cyanobacteria have prompted us to propose that the PcbC protein is the probable ancestor of IsiA and not CP43.

Science priorities for the next year (2023-2024):

- Our research priority for the next year is to further our understanding of the pCP43 complex. This will include ultrafast spectroscopic characterization of different subspecies of the pCP43 complex as well as its protein subunits.
- We are also studying the functional roles of Psb30, PsbK, and PsbZ, components of the pCP43 complex, on the spectroscopic properties of pCP43.
- Both subaims belong to the overarching aim of understanding the photoprotective mechanisms in chlorophyll-binding protein complexes (assembly intermediates) that do not have reaction centers.

My major scientific areas of expertise are: Biochemistry and Genetics of cyanobacteria.

To take my project to the next level, my ideal collaborator would have expertise in: Structural Biology, using cryo-electron microscopy and/or x-ray crystallography.

Publications supported by this project:

1. S. Biswas, D.M. Niedzwiedzki, M. Liberton, H.B. Pakrasi, "Phylogenetic and spectroscopic insights on the evolution of core antenna proteins in cyanobacteria." Photosynthesis Research, Sep 22 (2023). doi:10.1007/s11120-023-01046-6.
2. S. Biswas, D.M. Niedzwiedzki, H.B. Pakrasi, "Energy dissipation efficiency in the CP43 assembly intermediate complex of photosystem II. Biochimica et Biophysica Acta-Bioenergetics, 1864(3), 148982 (2023). doi:10.1016/j.bbabio.2023.148982.
3. M. Liberton, S. Biswas, H.B. Pakrasi, " Photosynthetic modulation during the diurnal cycle in a unicellular diazotrophic cyanobacterium grown under nitrogen-replete and nitrogen-fixing conditions." Scientific Reports, 12, 18939 (2022). doi:10.1038/s41598-022-21829-6.
4. S. Biswas, D.M. Niedzwiedzki, H.B. Pakrasi, "Introduction of cysteine-mediated quenching in the CP43 protein of photosystem II builds resilience to high-light stress in a cyanobacterium." Biochimica et Biophysica Acta-Bioenergetics, 1863(7), 148580 (2022). doi:10.1016/j.bbabio.2022.148580.
5. V.M. Johnson, H.B. Pakrasi, "Advances in the understanding of the lifecycle of photosystem II." Microorganisms, 10(5), 836 (2022). doi:10.3390/microorganisms10050836.
6. V.M. Johnson, S. Biswas, J.L. Roose, H.B. Pakrasi, H. Liu, "Psb27, a photosystem II assembly protein, enables quenching of excess light energy during its participation in the PSII lifecycle." Photosynthesis Research, 152(3), 297-304 (2022). doi: 10.1007/s11120-021-00895-3.
7. P.L. Walker, H.B. Pakrasi, "A ubiquitously conserved cyanobacterial protein phosphatase essential for high light tolerance in a fast-growing cyanobacterium." Microbiology Spectrum, 10(4), e01008-22 (2022). doi: 10.1128/spectrum.01008-22.
8. H-Y.S. Chen, D.M. Niedzwiedzki, A. Bandyopadhyay, S. Biswas, H.B. Pakrasi, "A novel mode of photoprotection mediated by a cysteine residue in the chlorophyll protein IsiA." mBio 12(1), 1-14 (2021). doi:10.1128/mBio.03663-20.
9. H. Liu, M.M. Zhang, D.A. Weisz, M. Cheng, H.B. Pakrasi, R.E. Blankenship, "Structure of cyanobacterial phycobilisome core revealed by structural modeling and chemical cross-linking." Science Advances, 7(2), eaba5743 (2021). doi:10.1126/sciadv.aba5743.
10. D. Liu, V.M. Johnson, H.B. Pakrasi, "A reversibly induced CRISPRi system targeting photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803." ACS Synthetic Biology, 9(6), 1441-1449 (2020). doi: 10.1021/acssynbio.0c00106.
11. C.J. Knoot, S. Biswas, H.B. Pakrasi, "Tunable repression of key photosynthetic processes using Cas12a CRISPR interference in the fast-growing cyanobacterium *Synechococcus* sp. UTEX 2973." ACS Synthetic Biology, 9 (1), 132-143 (2020). doi:10.1021/acssynbio.9b00417.
12. D.A. Weisz, V.M. Johnson, D.M. Niedzwiedzki, M.K. Shinn, H. Liu, C.F. Klitzke, M.L. Gross, R.E. Blankenship, T.M. Lohman, H.B. Pakrasi, "A novel chlorophyll protein complex in the repair cycle of photosystem II. Proceedings of the National Academy of Sciences of the United States of America 116(43), 21907-21913 (2019). doi:10.1073/pnas.1909644116.
13. A. Nagarajan, M. Zhow, A.Y. Nguyen, M. Liberton, K. Kedia, T. Shi, P. Piehowski, A. Shukla, T.L. Fillmore, C. Nicora, R.D. Smith, D.W. Koppenaal, J.M. Jacobs, H.B. Pakrasi, "Proteomic insights into phycobilisome degradation, a selective and tightly controlled process in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973." Biomolecules, 9(8), 374 (2019). doi:10.3390/biom9080374.
14. H. Liu, D. Weisz, M. Zhang, H. Zhang, M. Cheng, G. Gerstenecker, H.B. Pakrasi, M. Gross, R.E. Blankenship, "Phycobilisomes harbor FNRL in cyanobacteria." mBio, 10, e00669-19 (2019). doi: 10.1128/mBio.00669-19.
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Revealing Nature's Optimized Quantum Spin Coherences in Photosynthetic Proteins

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Overall research goals:

The Quantum Information Science, QIS, program is focused on discovering mechanisms that control and manipulate quantum phenomena following light-induced charge transfer events that will enable the design of novel quantum information systems. To reach this goal, we will advance the mechanistic understanding of light-triggered quantum phenomena in natural photosynthetic systems wherein long-lived electron spin-based entanglement in the form of spin-correlated radical pairs, SCRPs, is readily detected and achieved. Photosynthetic proteins are well-defined, experimentally tunable molecular systems, and as such, are ideal model systems for studying the creation and control of coherent phenomena. This research program features a combined use of experimental and theoretical approaches that include biochemical and genetic manipulation of photosynthetic proteins, application of a suite of advanced EPR techniques, as well as theoretical description of decoherence using semiclassical methods for atomistic simulations.

This program has a strong synergy with the "Fundamental Mechanisms for Solar Energy Conversion in Photosynthesis" program as both use specialized isotopically-labeled photosynthetic proteins and advanced EPR techniques to discern the roles heterogeneous local protein-cofactor environments play in controlling and optimizing solar energy conversion mechanisms.

Significant achievements (2022-2023):

Even though SCRPs have been extensively used in quantum sensing to unravel structure-function relationships in photosynthetic proteins (for some recent work see: Poluektov, Utschig, *J. Phys. Chem B*, 2021; Poluektov, Niklas, Utschig, *J. Phys. Chem. B*, 2019), the spin coherence properties of SCRPs have not been addressed before. We have carried out the first systematic study of light-induced electron spin pair decoherence in photosynthetic proteins. Photosystem I (PSI) from three biological species: *Synechococcus lividus, Synechococcus leopoliensis,* and *Thermosynechococcus vestitus* were studied. Decoherences were measured using two-pulse electron spin echo decay as a function of interpulse time, typically referred to as phase memory time, T_M. These measurements were performed over a large range of temperatures (10 – 220 K). Coherence/phase memory times T_M were recorded for transient SCRP states as well as for stable unpaired electron spin states generated after laser illumination at low temperatures. This study reveals that T_M exhibits minimal dependence on the biological species, biochemical treatment, and deuterium isotope enrichment of PSI. Analysis indicates that nuclear spin diffusion (NSD: flip-flops of nuclear spins coupled to electron spin) as well as instantaneous diffusion (ID: electron spin-spin interactions due to high local electron spin concentration) cannot explain the observed coherence times.

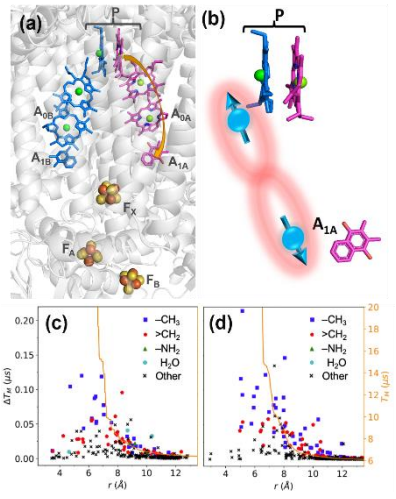

Fig. a) ET pathway in PSI and **b)** schematic representation of SCRP $P_{700}^{+}A_{1A}^{-}$. **c)** and **d)** Calculated contribution of different side groups to decoherence ΔT_M for A_{1A}^{-} and P_{700}^{+} respectively.

Theoretical modeling was used to clarify these experimental findings. Knowledge of the PSI crystal structure allowed large scale spin dynamics calculations to be performed. The effects of NSD due to protein microenvironment on coherence times T_M were studied. Closely positioned pairs (doubles) and triples of protons located in the 5 to 8 Å range from the unpaired electron spin were shown to largely control T_M. Various spin dynamics calculations were performed where specific cofactors and amino acid residues were either removed or replaced which allowed the identification of specific proton pairs and triples that are highly effective in inducing decoherence. Furthermore, we identified features of the microenvironment that control the anisotropy of spin decoherence. By comparison with experimental results, we show that NSD alone cannot explain the 3-times shorter experimentally observed coherence times. We suggest that low-temperature dynamics of methyl and potentially amino groups in the protein surrounding the unpaired electron spin centers is the main factor governing the loss of spin coherence in PSI.

Science priorities for the next year (2023-2024):

- Compare electron spin coherences of SCRPs in bacterial photosynthetic RCs and PSI.
- Develop a theoretical/computational approach for explicit inclusion of methyl group tunneling/rotation effect on coherence time.
- Correlate electron spin coherences with ET kinetics of photosynthetic proteins from different biological species as well as biochemically modified proteins.
- Investigate the lack of isotope (deuteration) effect on the electron spin coherence in photosynthetic proteins.

My major scientific area(s) of expertise is/are: advanced EPR spectroscopy and its application to photochemistry, catalysis, natural photosynthesis, electron transfer related spin dynamics.

To take my project to the next level, my ideal collaborator would have expertise in: transient optical measurements of ET kinetics in natural photosynthetic proteins in nanoseconds to seconds region.

Publications supported by this project (2021-2023):

Y. Jeong, J. Bindra, J. Niklas, L.M. Utschig, O.G. Poluektov, and A.W. Jasper, "Theoretical Prediction of Light-Induced Spin Coherences in Photosystem I" *Appl. Phys. Lett.*, **2023**, under review.

J.K. Bindra, J. Niklas, Y. Jeong, A.W. Jasper, J. Kern, L.M. Utschig, O. G. Poluektov, "Coherences of Photoinduced Electron Spin Qubit Pair States in Photosystem I" *J. Phys. Chem. B*, **2023**, DOI:10.1021/acs.jpcb.3c06658

U. Zvi, D. R. Candido, A. Weiss, A. R. Jones, L. Chen, I. Golovina, X. Yu, S. Wang, D. V. Talapin, M. E. Flatté, A. P. Esser-Kahn, P. C. Maurer, "Engineering spin coherence in core-shell diamond nanocrystals", *Nat. Mater.*, under review; arXiv:2305.03075 (**2023**). https://doi.org/10.48550/arXiv.2305.03075

N.C. Magdaong, K. Faries, J. Buhrmaster, G. Tira, R. Wyllie, C. Kohout, D. Hanson, P. Laible, D. Holten, C. Kirmaier, "High Yield of B-side Electron Transfer at 77 K in the Photosynthetic Reaction Center Protein from *Rhodobacter sphaeroides.*" *J. Phys. Chem. B,* **2022**. (special issue in honor of Steve Boxer) 126(44): 8940-8956. DOI: 10.1021/acs.jpcb. 2c05905

V.R. Policht, A. Niedringhaus, C. Spitzfaden, P.D. Laible, D. Bocian, C. Kirmaier, D. Holten, T. Mančal and J. P. Ogilvie. "Hidden Vibronic and Excitonic Structure and Vibronic Coherence Transfer in the Bacterial Reaction Center." *Sci. Adv.* **2022**, 8 (1). DOI: 10.1126/sciadv.abk0953

Trafficking of Metabolites and Reductant between the Chloroplast and Other Subcellular Compartments

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Overall research goals:

Our overarching research is investigating the functions of triose-phosphate transporters, oxaloacetate/malate transporters, NAD kinase and carbon assimilation enzymes involved in cellular redox trafficking in *Picochlorum celeri* and probing their potential roles in photoautotrophic growth rates. The experiments will be done using mutants in the wildtype background and in transformant cell lines that have significant pigment reduction. The proposed experiments will dovetail with the Chlamydomonas experiments in the Grossman/Burlacot laboratories to establish whether conserved mechanisms for metabolite channeling and redox control are found in distinct algae or whether unique features are used by these different algae. In *Picochlorum celeri*, we will construct and express fluorescent fusion proteins of interest and use fluorescent protein expression and confocal microscopy imaging to localize targeted proteins within the cells. Cas9 gene editing is well established in *P. celeri* and we will use this technique to make functional knockouts of targeted proteins hypothesized to be critical for redox balance. Photoautotrophic growth rates in transformants will be characterized in custom bioreactors that mimic outdoor diel growth regimes. These experiments will biochemically establish the metabolites trafficked by the putative TPT/OMP homologs in *Picochlorum celeri* and begin to establish a network of photosynthetic metabolite movement between organelles in this alga. Photosynthetic O_2 evolution will be quantified at light levels ranging from 10 to 2,500 (P-I curve) and used to quantify P_{max} and I_k changes in the transformants relative to control cell lines. These experiments will establish how metabolite transport influences photosynthetic activity. Lastly, we will use MIMS to determine whether H_2O-H_2O cycles are activated in various cell lines.

Significant achievements (2023):

Picochlorum celeri was isolated from alga enrichments under the selective pressures of high salt, high temperature, high light and rapid dilutions. Under idealized conditions, *P. celeri* is capable of 2-hour photoautotrophic doublings. For the last five years, *P. celeri* was grown outdoors at the Arizona State University algal testbed and is the current DOE state of technology summer strain capable of attaining 40 $g/m^2/d$ of biomass on ideal days outdoors. To understand the basic underpinnings of its outlying photosynthetic properties, we are systematically using CRISPR gene editing tools to knockout and study reductant transporters, carbonic anhydrase enzymes and carbon partitioning enzymes. We have initiated our studies by targeting two NAD kinases for CRISPR disruption. These studies will focus on transforming the wildtype background and mutants already in hand with dramatically (>50%) reductions in photosynthetic pigment levels that have alterations in electron transport properties. The low-pigment mutants will be used to probe energy management in these cells relative to the wildtype. We have generated a robust cassette for localizing proteins with GFP and have initiated studies to localize targeted proteins within the cell. We have also systematically knocked out most of the carbonic anhydrase enzymes in *P. celeri* and have determined that the majority of these enzymes are not involved in photosynthetic carbon assimilation. Despite rapid growth in high CO_2, *P. celeri* grows slowly in air. Two of the carbonic anhydrase enzymes appear to have novel roles in controlling final cells densities and are important for maintaining viability in stationary phase. Using our recently developed protein visualization tools we have initial evidence that one of these carbon anhydrases is outside of the chloroplast/mitochondrion/cytosol, which is consistent with our phenotype characterizations to date (Figure 1). Lastly, we have generated a large suite of heavily depigmented mutants by disrupting photosystem assembly/import proteins, pigment

biosynthesis genes and/or light-harvesting complex proteins. We are in the process of down-selecting to a single line for comparative metabolite shuttling relative to the wildtype.

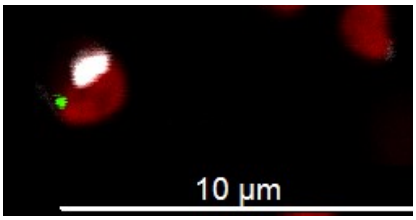

Figure 1. GFP carbonic anhydrase fusion indicating localization outside of the chloroplast (red autofluorescence) and mitochondrion (white rhodamine fluorescence).

Science priorities for the next year (2023-2024):

- Attain functional characterization of carbonic anhydrase 7 and build data sets to define physiological functions.
- Attain CRISPR knockouts of NAD kinase and initiate physiological characterizations
- Attain CRISPR knockouts of TPT transporters

My major scientific area(s) of expertise is/are: Photosynthetic oxygen evolution, algal metabolism, strain engineering, gene editing.

To take my project to the next level, my ideal collaborator would have expertise in: Characterization of electron transport and photoprotection mechanisms in mutants with altered electron flow.

Publications supported by this project: 2023:

Huang, W., Krishnan, A., Plett, A., Meagher, M., Linka, N., Wang, Y., Ren, B., Findinier, J., Redekop, P., Fakhimi, N., Kim, R. G., Karns, D. A., Boyle, N., Posewitz, M.C. and Grossman, A.R. (2023) Chlamydomonas mutants lacking chloroplast TRIOSE PHOSPHATE TRANSPORTER3 are metabolically compromised and light-sensitive, *The Plant Cell*, **35**, 2592-2614.

Saroussi, S., Redekop, P., Karns, D.A.J., Thomas, D. C., Wittkopp, T. M., Posewitz, M. C., and Grossman A. R. (2023) Restricting electron flow at cytochrome b_6f when downstream electron acceptors are severely limited, *Plant Physiology*, **192**, 789-804.

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Hemschemeier, A., Posewitz, M. C., Happe T., (2023) Hydrogenases and hydrogen production, in in *The Chlamydomonas Sourcebook*, F.A. Wollman, et al., Editors. Elsevier, Academic Press: Oxford.

Protein phosphorylation and oxidative protein modification in photosystem II disassembly and repair

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Overall research goals:

Our overall objective is to understand the molecular mechanisms that aid in the repair of plant photosystem II (PSII). The light-driven water-oxidation reaction of PSII exposes its key reaction center proteins to irreversible photooxidative damage. A rapid repair cycle replaces the photodamaged core subunits, but how the large antenna-core supercomplex structures of plant PSII disassemble for repair is not currently understood. We examine the role of post-translational modifications in PSII disassembly and repair. We are especially interested in elucidating the role of protein phosphorylation and protein oxidative modifications in PSII disassembly. The two specific aims of the current project are:

1. Examine the role of protein phosphorylation in PSII disassembly.

In this aim, we explore the role of core protein phosphorylation in PSII disassembly. Our hypothesis is that phosphorylation of strategic antenna attachments sites and dimerization motifs promote dissociation of the peripheral antenna from the core and core monomerization. We will test this hypothesis with plants that contain altered core phosphorylation sites or hypophosphorylated or hyperphosphorylated cores.

2. Examine the role of oxidative protein modification in PSII disassembly.

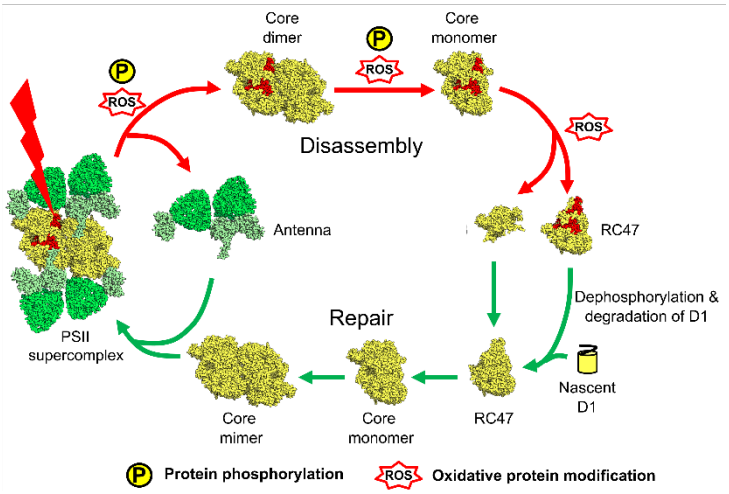
Here we examine the role of protein oxidative modification in PSII disassembly. Our hypothesis is that oxidative protein modification is an active mediator of PSII disassembly, especially in the conversion of damaged monomeric cores into two smaller subcomplexes. We will test this hypothesis by treating thylakoid membranes and isolated PSII to reactive oxygen species under in vitro and in vivo conditions.

Significant achievements (2000-2023):

1. PSII core phosphorylation promotes peripheral antenna dissociation and core monomerization. Using *Arabidopsis thaliana* mutants with hypophosphorylated and hyperphosphorylated cores, we demonstrate a specific involvement of phosphorylation in removing the peripheral antenna from the core and in monomerization of the dimeric cores. We further showed that phosphorylation protects plant PSII from photooxidative damage in the short-term by decreasing excitation pressure through removal of the peripheral antenna. Using an in vitro assay to phosphorylate thylakoid membranes, we have demonstrated that phosphorylation is sufficient to induce PSII disassembly, and that core and antenna proteins within antenna-core supercomplexes are phosphorylated. Core proteins show differing degrees of phosphorylation, raising the possibility that a certain stoichiometry of phosphorylation must be reached for antenna dissociation and core monomerization. We further discover PsbL, PsbF, PsbQ-2, and PsbR as new core phosphoproteins of plant PSII using an MS-based phosphoproteomic approach.

2. Oxidative protein modification is an active mediator of PSII disassembly. Analysis of *Arabidopsis* mutants that altogether lack core protein phosphorylation demonstrated that the disassembly of the monomeric cores into a CP43-free RC47 complex and a CP43-precomplex proceeds independently of phosphorylation. This points to other unknown mechanisms of core disassembly. Using H_2O_2 as an exogenous reaction oxygen species, we show that oxidative protein modification fulfills this disassembly role. The damage-mediated disassembly of PSII reaction center cores could be repeated with isolated cores

from PSII kinase mutants. Our results also reveal novel disassembly intermediates in the kinase mutant in the form of a monomeric core with an RC47 complex (C-RC47) and a dimer of RC47 complexes (RC47-RC47). We further demonstrate that the damage-mediated disassembly occurs under high light. Using mass spectrometry, we additionally map sites of oxidatively modified amino acid residues under innate and exogenous peroxide-driven oxidative stress. The oxidative protein modifications are more numerous in highly disassembled species of PSII as the disassembly correlates with the degree of oxidative damage. The oxidatively modified amino acid residues map at the interface of D1 and CP43 subunits, providing a potential dissociative pathway for the monomeric cores along these subunits. Taken together, oxidative damage-mediated disassembly may represent an economical process in which only damaged cores disassemble while undamaged cores reassemble and continue with photochemical activity.



A scheme for plant PSII repair cycle. The damaged D1 protein is depicted in red. Phosphorylation and oxidative protein modification are suggested to act synergistically for the sequential and economical disassembly of photodamaged PSII.

Science priorities for the next year (2023-2024):

For the next year, we will build on our observations of phosphorylation-mediated antenna disassociation by analyzing disassembly in site-directed tobacco mutants of PSII core phosphosites. The oxidative modifications that are critical for core monomer disassembly will be identified. We will also examine PSII disassembly in in vivo systems that overproduce H_2O_2 and in plants that are prone to photodamage. Using a structural biology approach, we will also characterize the features of the damaged RC47 complex.

My major scientific area(s) of expertise is/are: Our areas of expertise are in biochemical, biophysical, and physiological analyses of plants, thylakoid membranes, and isolated PSII. We have successfully used mass spectrometry to determine the composition, post-translational modification, and relative abundance of PSII.

To take my project to the next level, my ideal collaborator would have expertise in: Structural analysis of PSII by cryo-electron microscopy (Cryo-EM) and molecular modeling of protein-protein interaction.

Publications supported by this project:

1. Puthiyaveetil S and McKenzie SD (2023) Plant photosystem II assembly: TROL2 to the rescue. *Mol Plant*, 16, 1719-1721. doi: 10.1016/j.molp.2023.09.014.
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3. Svoboda V, Oung HMO, Koochak H, Yarbrough R, Mckenzie SD, Puthiyaveetil S, and Kirchhoff H (2023) Quantification of energy-converting protein complexes in plant thylakoid membranes. *BBA-Bioenergetics*, 1864, 148945. doi: 10.1016/j.bbabio.2022.148945.
4. Ibrahim IM, McKenzie SD, Chung J, Aryal UK, Leon-Salas WD, and Puthiyaveetil S (2022) Photosystem stoichiometry adjustment is a photoreceptor-mediated process in Arabidopsis. *Sci Rep*., 12, 10982. doi: 10.1038/s41598-022-14967-4.
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The Type I homodimeric reaction center in *Heliobacterium modesticaldum*

Kevin Redding, Principal Investigator
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Overall research goals:

- Understand electron transfer within the heliobacterial reaction center (HbRC), using a combination of biochemistry, biophysics, site-directed mutagenesis, computational chemistry and molecular modeling.
- Understand the cyclic electron transport pathways driven by the HbRC in heliobacterial cells using a combination of computational and experimental approaches.

Significant achievements (2022-2023):

- **Created set of site-directed mutations in the HbRC:** A set of 37 site-directed variants in the *pshA* gene have been made, including all mentioned in our proposal. All mutant genes are in shuttle vectors for conjugation and expression. The majority (24) have been introduced into the Δ*pshA* mutant and the transformants have been characterized spectroscopically to see if they make the mutant HbRC and how much. A more limited subset is now being purified for ultra-fast pump-probe spectroscopic work.
- **Completed a comprehensive analysis of heliobacterial ferredoxins:** The 7 small (<100 residues) dicluster ferredoxins predicted from the heliobacterial genome were expressed recombinantly and purified as holoproteins with iron-sulfur clusters inserted. All ferredoxins were characterized spectroscopically (optical and EPR), redox titrated, and tested as redox partners for the HbRC and the pyruvate:ferredoxin oxidoreductase (PFOR), a key enzyme in carbon flow. We found that only 3 of the ferredoxins (PshB1, PshB2, and Fdx3) could be reduced by the HbRC, and the same 3 were the only redox partners for PFOR as well. Proteomic studies were performed, and it was found that these 3 ferredoxins were the 3 most highly expressed ferredoxins (with PshB1 > PshB2 > Fdx3), in agreement with our former transcriptomic data. Each of the genes for these three ferredoxins was deleted individually, and a double mutant in which *pshB1* and *pshB2* were both deleted was also constructed. Each single mutant could grow in a variety of conditions: chemotrophic in the dark using pyruvate (fermentable) as carbon source, mixotrophic in the light with pyruvate, phototrophic in the light with acetate (nonfermentable), and diazotrophic (N_2 fixing) with pyruvate in the light. Thus, available evidence suggests that these 3 ferredoxins are largely redundant and form a pool that interacts with both the HbRC and key metabolic enzymes.
- **Heliobacterial NfnAB NADH-dependent ferredoxin-NADP$^+$ oxidoreductase (FNR):** After exhaustively searching for a gene product that could potentially serve as an FNR in heliobacteria, we realized that the most likely protein serving that role would actually be an electron-bifurcating enzyme encoded by the *nfnAB* genes. It typically catalyzes this reaction: 2 NADPH + 2 Fd^{ox} + $NAD^+ \rightarrow$ 2 $NADP^+$ + 2 Fd^{red} + NADH. The gene products were expressed recombinantly and purified, characterized spectroscopically (optical and EPR), and assayed enzymatically. We found that it has [2Fe-2S] and [4Fe-4S] clusters, binds flavin, and possesses NADH-dependent FNR activity.
- Constructed an all-atom model of the HbRC (with surrounding lipid bilayer and water) for classical MD simulations. We are now running MD simulations (3 replicas) for 1 μs each and calculating the activation barriers for two reaction channels: A_0 chlorophyll to F_X Fe-S cluster or to menaquinone.
- Performed time-dependent density functional theory computations for computing electron-transfer states between the key donor and acceptor sites. Performed quantum calculations of molecular polarizabilities and electronic couplings between the HbRC cofactors.
- Developed computational algorithms integrating quantum calculations with classical MD to arrive at rates of electronic hopping transitions. The model has the potential for predicting pathways of electronic transport in bacterial photosynthesis.
- Constructed two macromolecular structural models of the heliobacterial membrane (300 nm x 300 nm in size), corresponding to low light and high light adapted compositions, comprising over a hundred HbRC each and accompanying proteins, to be used as the basis of overall energy conversion calculations.

- Constructed a structural rate kinetics framework for energy conversion based on the aforementioned macromolecular models, which determines the ATP production rate as a function of illumination. By computing the surplus ATP production beyond base metabolism, the energy-return-on-investment by the heliobacterial membrane is estimated, allowing an estimate of the cell doubling time.

Science priorities for the next year (2023-2024):

- Complete analysis of all site-directed mutants in the HbRC.
- Create a system for study of MQ reduction in the HbRC by pump-probe spectroscopy.
- Delete the *nfnAB* genes using a system to complement the mutation by expression of a repressible version.
- Repeat MD simulations with isoprenyl-phosphate inserted into the menaquinone site.
- Run simulations of the supramolecular structural models using coarse-grained MD methods. The rate limitation by the diffusion of charge carriers (cytochrome *c*, ferredoxin, and MQ) will be determined at various light intensities. The diffusion behavior will be analyzed using a Fokker-Planck based continuum model, which accounts for membrane heterogeneity based on the ensemble of MD trajectories.
- The structural rate kinetics framework will be refined to account for cycle switching at the HbRC, specifically integrating long cycle vs short cycle energy conversion modalities, which have different energy-return-on-investment as well as different channel capacities.

My major scientific area(s) of expertise is/are: Photosynthesis, light-driven electron transfer, phototrophic metabolism.

To take my project to the next level, my ideal collaborator would have expertise in: Cryo-electron tomography, Proteomics (especially of membrane proteins).

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

1. Layton, A. and K.E. Redding (2022) Examination of Genetic Control Elements in the Phototrophic Firmicute *Heliomicrobium modesticaldum*. *Microorganisms* **10**:876-887. 10.3390/microorganisms10050876

2. Orf GS, Gisriel C, Baker P, and Redding KE (2022) The PshX subunit of the photochemical reaction center from *Heliobacterium modesticaldum* acts as a low-energy antenna subunit. *Photosynth Res.* **151**:11-30. doi: 10.1007/s11120-021-00871-x

3. Leung SW, Baker PL, and Redding KE (2021) Removal of the cytochrome *bc* complex from *Heliobacterium* modesticaldum results in viable cells incapable of phototrophy. *Photosynth Res.*, **148**:137-152.

4. Song Y, Sechrist R, Nguyen HH, Johnson W, Abramavicius D, Redding KE, and Ogilvie JP. (2021) Excitonic structure and charge separation in the Heliobacterial Reaction Center probed by multispectral multidimensional spectroscopy. *Nature Comm.* **12**:2801-2808.

5. Orf GS, Redding KE (2021) Perturbation of the primary acceptor chlorophyll site in the heliobacterial reaction center by coordinating amino acid substitution. *Biochim Biophys Acta Bioenerg* **1862**(1):148324. doi:10.1016/j.bbabio.2020.148324

6. Johnson WA & Redding KE (2020) Reconstitution of the heliobacterial photochemical reaction center and cytochrome c_{553} into a proteoliposome system. *Photosynth Res*. **143**(3):241-250 doi:10.1007/s11120-019-00695-w

7. Orf GS & Redding KE (2019) Expression and purification of affinity-tagged variants of the photochemical reaction center from *Heliobacterium modesticaldum*. *Photosynth Res* **142**(3):335-348. doi:10.1007/s11120-019-00672-3

8. Baker PL, Orf GS, Kevershan K, Pyne ME, Bicer T, & Redding KE (2019) Using the Endogenous CRISPR-Cas System of *Heliobacterium modesticaldum* To Delete the Photochemical Reaction Center Core Subunit Gene. *Appl Environ Microbiol* **85**(23). doi:10.1128/AEM.01644-19

9. Baker PL, Orf GS, Khan Z, Espinoza L, Leung S, Kevershan K, & Redding KE (2019) A Molecular Biology Tool Kit for the Phototrophic Firmicute *Heliobacterium modesticaldum*. *Appl Environ Microbiol* **85**(19). doi:10.1128/AEM.01287-19

Structural Tuning of Photosynthetic Light Harvesting

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Overall research goals:

This project aims to develop a transferable toolkit for identifying and controlling structure-based optical tuning in photosynthetic pigment-protein complexes (PPCs). The three core components of this toolkit – (a) robust site energy prediction methods, (b) structure-dependent vibronic mixing models, and (c) experimentally calibrated quantum dynamics simulations – will be parameterized directly against an experimental library of mutagenesis data on model PPCs, establishing a quantitative connection between theory and experiment. Finally, these methods will be applied to (d) resolve the long-standing question of the structural origins and functional significance of the low-energy trap states of cyanobacterial photosystem II through a combination of structure-based spectroscopic simulations and toolkit-guided mutagenesis. A key goal is to produce a strain of the cyanobacterium Synechocystis PCC6803 that lacks low-energy trap states in photosystem II (PSII), allowing the functional role of these states to be directly assessed.

Significant achievements (2022 - 2023):

- Scaled up mutagenesis/reconstitution experiments with the water-soluble Chlorophyll protein (WSCP) of green plants, allowing us to screen structure-based tuning in several dozen new point mutants.
- Produced WSCP complexes with absorption peaks ranging from 639 nm to 670 nm by combining point mutations with metal-substitution in Chl *a* and Chl *b*.
- Demonstrated that electronic coupling strongly enhances vibrational sideband intensities in WSCP fluorescence via selective suppression of the purely electronic transition.
- Found that 695 nm fluorescence in cyanobacterial Photosystestem II can be both red- and blue-shifted by single point mutants near Chl B16 in the CP47 protein.

Science priorities for the next year (2023-2024):

- Optimize theoretical frequency-shift models against new WSCP data.
- Explore how elimination/alteration of the 695 nm fluorescent state alters PSII function.
- Use 5 K spectroscopy to get high-resolution vibrational tuning parameters.
- Implement higher-level theory and simulation methods into our PigmentHunter simulation tool.

My major scientific area(s) of expertise is/are: Spectroscopy, Energy Transfer, Molecular Dynamics, Quantum Dynamics, Mutagenesis.

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

Publications supported by this project

1. Reppert, Mike; "Bioexcitons by Design: How Do We Get There?". *The Journal of Physical Chemistry B*, 127, 1872-1879 (2023).

Photosynthetic membrane lipid transport through chloroplast membrane contact site homologs

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Overall research goals:

The ***overall goal*** is to learn more about lipid transport within the chloroplast contributing to the biogenesis and repair of the thylakoid membrane. We ***hypothesize*** that proteins at internal chloroplast membrane contact sites (MCS) enable efficient lipid transport to the thylakoids.

1) ***Test the sub-organellar location and membrane perturbing roles of our candidate proteins.*** Our 7 candidate chloroplast MCS proteins are homologous to lipid-transferring members of MCS in other systems. We have shown that 4 are exclusively located in the chloroplast when expressed transiently in *N. benthamiana*. We begin by identifying their sub-organellar location by chloroplast fractionation and then immunogold transmission electron microscopy. Then we test the candidates' membrane perturbing capacity.
2) ***Compare lipid transport to thylakoids mediated by MCSs and stromal vesicles.*** Our preliminary data suggests one of our MCS candidates affects MCS formation, and another affects stromal vesicles. We will use chloroplasts isolated from Arabidopsis loss-of-function mutants of priority MCS candidates to quantitatively measure the efficiency of lipid transport. We will measure photosynthetic phenotypes in the same mutants. Objective 2 directly tests our hypothesis that MCS proteins enable efficient lipid transport.
3) ***Expand our understanding of protein components at chloroplast MCSs.*** We use Split-TurboID to identify proteins at thylakoid and inner envelope MCS in an unbiased fashion, while immunoprecipitating protein partners of current candidate proteins.

Significant achievements (2023-2026):

Since funding began in October,

- we increased the number of top-priority candidates for which loss-of-function mutants affect thylakoid and inner envelope connectivity to 3.
- we have begun making Flag-tagged proteins for these top three priority candidates.
- we have crossed the top priority candidates with *cpsfl1*, a vesicle protein contributing to thylakoid formation.
- we have used co-expression in *N. benthamiana* to test the second subcellular location for candidates that were not exclusively in the chloroplast and have begun collaborating with another group to test if these also impact thylakoid biogenesis.
- we have received radioisotope approval to start measuring lipid transport.
- we have confirmed that our split-TurboID system biotinylates more proteins in *N. benthamiana* with the addition of both biotin and rapamycin (no current publications use split-Turbo inside chloroplasts).

Science priorities for the next year (2023-2024):

- A top priority is completing the TurboID unbiased proteomic identification of additional contact site proteins.
- We will measure lipid transport phenotypes in loss-of-function plants of our three priority candidates.

- We will measure photosynthetic phenotypes in loss-of-function plants of our three priority candidates.
- We will express our top three priority proteins at high concentrations in heterologous systems.

My major scientific area(s) of expertise is/are: Lipid metabolism, chloroplast biology, protein biochemistry.

To take my project to the next level, my ideal collaborator would have expertise in: Cryo-ET, FIB-SEM, or similar visualization systems that we could use with our mutant membrane contact site proteins. Other high performance microscopy systems could also be of interest.

Publications supported by this project [N/A]

Intelligent mutagenesis of photosynthetic protein: Programming the function

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Overall research goals:

The objective of the proposed research is the development of novel synergistic approaches that enable control of energy transfer in photosynthetic and artificial pigment-protein complexes via the introduction of point mutations that fine-tune properties of protein-bound chromophores in a quantitatively controlled way.

Significant achievements (2020-2023):

The main goal of the project for the previous period of funding was to develop a new experimental technique, time-resolved circular dichroism (TRCD) spectroscopy, that is sufficiently sensitive to study photosynthetic protein complexes. The second goal of the project was to develop a QM/MM computational protocol capable of obtaining electronic properties of pigment-protein complexes from first principles solely from x-ray structure and assist in the interpretation of experimental data on excitation dynamics in photosynthetic complexes. As the result:

- A TRCD spectrometer is developed with sensitivity in $\Delta A_{CD} < 10^{-6}$ and $< 10^{-7}$ in nanosecond and femtosecond modes, respectively. This is at least 50 times more sensitive than typical designs proposed earlier by other groups. For the first time, the measurements of the transient CD signals from photosynthetic complexes became possible.
- QM/MM computational protocol is now available to predict *quantitatively* electronic properties of pigments embedded in a protein matrix. It is shown that to model such complex systems correctly, QM calculations on a large number of structural snapshots need to be averaged to capture the effect of the thermal motion of atoms in the system on the electronic properties of chromophores. It was also demonstrated, that utilizing a polarizable description of the protein (via the effective fragment potential (EFP) polarizable force field) is also critical for achieving quantitative agreement with experimental data.
- TRCD technique and computational protocols were applied to study the Fenna Mathews Olson (FMO) photosynthetic complex, which has also served as a testbed for refining new experimental techniques and computational protocols. The figure below demonstrates the agreement between experimental measurements of ΔA_{CD} and in ΔA and the corresponding first-principle predictions.

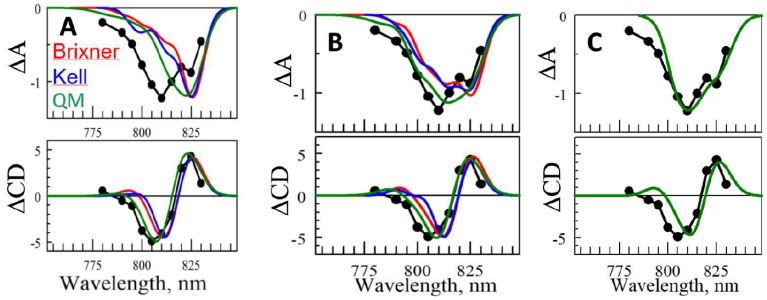
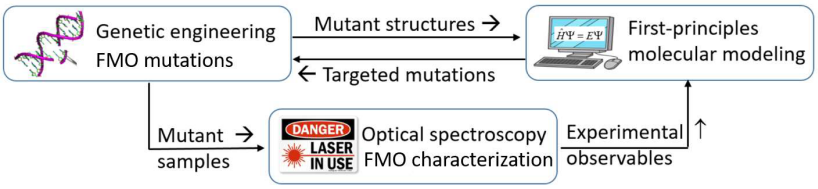


Fig. 1. The decay-associated spectrum of the longest decay component (55 μs) is measured (black dots) and reproduced by QM/MM/EFP computations. Panes A, B, C represent different refinement steps of the computational protocol. A: Fit in the simplest model assumes decay of the triplet state localized exclusively on pigment 3. B: Fit that assumes decay of a Boltzmann-equilibrated triplet state. C: In addition to B, fit accounts for the electrochromic shift of diagonal terms in Hamiltonian due to the electric field of BChl in the triplet state. Green curves (QM) represent fits using Hamiltonians obtained from first principles, and red and blue use empirical Hamiltonians proposed earlier (Brixner, Kell).

- Using the *same* computational protocol with the starting point being the wild-type complex x-ray structure, it was also possible to reproduce the measured effects of point mutations on the electronic properties of the FMO complex.

Science priorities for the next funding period 2023-2026:

Today, targeting properties of specific chromophores by mutations is largely empirical. While there are some general motifs that govern a choice of specific amino acids for substitution, the effect of a particular mutation is not known *a priori.* As a result, the mutational work is often based on a trial-and-error approach, where a number of empirically feasible mutations are first generated, and then their effect on the targeted properties is quantified experimentally. The remarkable agreement between the first-principle computational predictions and experimental measurements achieved during the past funding period opens an avenue to transform this arduous try-and-test method in mutagenesis into the predict-and-design approach that eliminates the necessity of generating and testing a large number of mutations and focusing only on the mutations with the targeted outcomes quantified *a priori*. In this grant period, we aim at developing and validating a computational protocol that will enable us to quantitatively predict changes in the electronic properties of target chromophores due to mutations, with a sole starting point being the x-ray structure of the original wild type (wt) pigment-protein complex. The success of the project is ensured by a synergistic collaboration of three PI's: Dr. Savikhin (optical spectroscopy), Dr. Slipchenko (multiscale molecular modeling) and Dr. Liu (mutagenesis), as outlined in the figure below:



The proposed research consists of two major stages: (i) the refinement of molecular modeling strategies based on the existing pool of mutants of the FMO complex, (ii) application of the validated computational protocol to alter the function of FMO in a controlled way. In the second stage, a new set of mutants targeting the electronic properties of specific pigments will be determined computationally, generated by mutagenesis, and the predicted properties and functional effects will be verified in optical experiments. The two stages will overlap in the second year of the project, when the perspective mutations will be proposed by computation, and new mutagenesis work will be initiated.

In 2023-2024, the work will concentrate on Stage I, where the first-principle computational protocols will be further refined using several existing mutants of FMO (Y16F, Y345F, S37A, Y184F), which will be characterized experimentally, and their properties compared with computational predictions.

My major scientific area(s) of expertise is/are: PI Savikhin: optical (time-resolved) spectroscopy; co-PI Slipchenko: multiscale molecular modeling; co_PI Liu: mutagenesis .

Publications supported by this project (2020-2023) :

1. Y Kim, Z Mitchell, J Lawrence, D Morozov, S Savikhin, LV Slipchenko (2023). Predicting Mutation-Induced Changes in the Electronic Properties of Photosynthetic Proteins from First Principles: The Fenna–Matthews–Olson Complex Example The Journal of Physical Chemistry Letters 14 (31), 7038-7044
2. Y Watanabe, BM Washer, M Zeller, S Savikhin, LV Slipchenko, A Wei (2022). Copper (I)–Pyrazolate Complexes as Solid-State Phosphors: Deep-Blue Emission through a Remote Steric Effect Journal of the American Chemical Society 144 (23), 10186-10192
3. Zakharov, S Savikhin, Y Misumi, G Kurisu, WA Cramer (2022). Isothermal titration calorimetry of membrane protein interactions: FNR and the cytochrome b6f complex. SD Biophysical Journal 121 (2), 300-308
4. I. V Kurashov, G Milanovsky, L Luo, A Martin, AY Semenov, S Savikhin…(2021). Conserved residue PsaB-Trp673 is essential for high-efficiency electron transfer between the phylloquinones and the iron-sulfur clusters in Photosystem, Photosynthesis research 148 (3), 161-180
5. Y Kim, D Morozov, V Stadnytskyi, S Savikhin, LV Slipchenko (2020). Predictive first-principles modeling of a photosynthetic antenna protein: The Fenna–Matthews–Olson complex. The Journal of Physical Chemistry Letters 11 (5), 1636-1643

Spectroscopic studies of protein-protein association in model membranes

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Overall research goals:

The goal of this project is to apply spectroscopy to synthetic membrane-protein systems, known as nanodiscs, to reveal how photosynthetic light-harvesting proteins and their interactions produce efficient and regulated energy transport in the membrane environment. We build networks of light-harvesting proteins in the nanodiscs to systematically alter the association of the proteins and probe the ultrafast dynamics with time-resolved fluorescence, ultrabroadband 2D, and transient absorption spectroscopy.

Significant achievements (2021-2023):

Energy transfer in purple bacteria. In purple bacteria, the light energy is absorbed by light-harvesting complex 2 (LH2) and transferred across a network of multiple such antenna complexes before it reaches a dedicated protein, LH1-RC, for charge separation. Previous studies of the photophysics were carried out on native membrane fragments, which have a heterogeneous and congested organization that obscures individual inter-complex energy transfer steps, or on detergent-solubilized proteins, which introduce non-native perturbations. Thus, a better understanding of the energy transfer dynamics within the photosynthetic membrane is needed.

(i) Distance-dependent energy transfer between LH2 variants. We embedded two variants of LH2 from *Ph. molischianum* together into a nanodisc and integrated ultrafast transient absorption spectroscopy, quantum chemical calculation, and cryogenic electron microscopy to determine inter-protein energy transfer timescales (Figure). By varying the diameter of the nanodiscs, we characterized inter-protein energy transfer across the physiological range of distances. The closest distance possible between neighboring LH2, which is the most common in native membranes, is 25 Å and resulted in a timescale of 5.7 ps. Larger distances of 28-31 Å resulted in timescales of 10-14 ps. Overall, our results introduce a framework for well-controlled studies of inter-protein energy transfer dynamics and suggest that protein pairs serve as the primary pathway for the efficient transport of solar energy [1].

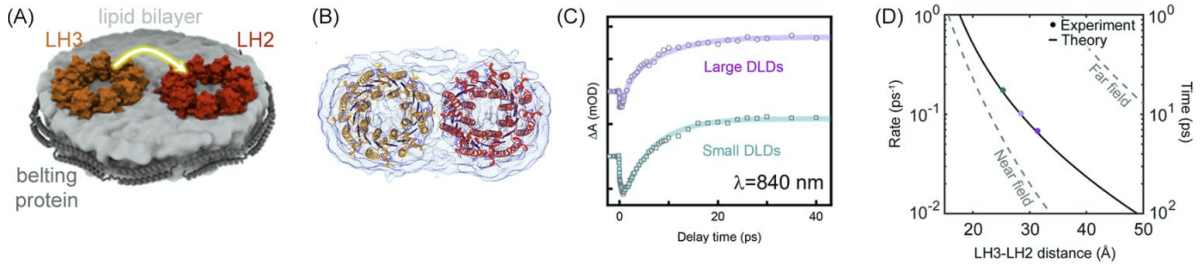

Figure. LH3-to-LH2 energy transfer in nanodisc. (A) Schematic of LH2 and LH3 embedded in a nanodisc. (B) Cryo-EM analysis of small doubly-loaded discs (DLDs) showing density maps with docked crystal structures. (C) Absorption transients of small (teal) and large (purple) LH2-LH3 DLDs at 840 nm. Experimental values are shown as open symbols, and the fitted kinetics are shown as solid lines. (D) The theoretical generalized FRET rate for inter-protein energy transfer across the biologically relevant range. The experimentally measured rates for the small and large DLDs are indicated in teal and two shades of purple dots, respectively.

Stress response in cyanobacteria. The aqueous habitats of cyanobacteria are commonly iron limited owing to its low solubility in water. Under low iron or high light conditions, an additional light harvesting antenna protein is expressed, the iron-stress induced protein A (IsiA) antenna. IsiA adopts various organizations within the membrane and, correspondingly, various functional roles have been proposed. Thus, knowledge of the photophysics of both the building block and the complex assemblies is needed.

(ii) Structural and functional heterogeneity of the IsiA-PSI supercomplex. We performed Cryo-EM and single-molecule measurements on the IsiA-PSI supercomplex, which revealed large-scale structural heterogeneity and signatures of transiently decoupled IsiA, respectively. Structure based calculations showed that rapid IsiA-to-PSI energy transfer is always maintained within the coupled complex, and even increases by three-fold in rare conformations via IsiA-specific chlorophylls. We postulate that antennae design mitigates structural fluctuations, providing a mechanism for robust energy transfer in the flexible membrane [2].

Science priorities for the next year (2023-2024):

- We incorporated LH1-RC into nanodisc with varying lipid compositions. A comparison of LH1-to-RC energy transfer revealed a lipid dependence, suggesting the lipids play a role in regulating the redox state of the RC. Complementary molecular dynamics simulations showed preferential association of certain lipids to the complex, which may be the underlying biophysical mechanism behind the functional importance of membrane composition.
- We prepared spectral and structural variants of LH2 from different purple bacteria species. Structural characterization with cryo-EM has shown different chromophore-protein interactions and surface charge distributions. Transient absorption studies will provide insights into the variants' spectral tuning mechanism and intra-protein energy transfer dynamics.
- We also prepared new doubly-loaded nanodiscs using the LH2 variants. Structural characterization will show how the electrostatic interactions mediate the inter-protein association. A comparison of inter-complex energy transfer dynamics for nanodiscs loaded with different LH2 variants will also show how the spectral shifts affect the inter-protein energy transfer.

My major scientific area(s) of expertise is/are:
photosynthetic light harvesting, energy transfer, ultrafast spectroscopy, and model membranes.

To take my project to the next level, my ideal collaborator would have expertise in:
light harvesting in purple bacteria or cyanobacteria, energy transfer theory, and cryogenic electron microscopy of photosynthetic complexes.

Publications supported by this project 2021-2023:

1. D. Wang, O. C. Fiebig, D. Harris, H. Toporik, Y. Ji, C. Chuang, M. Nairat, A. L. Tong, J. I. Ogren, S. M. Hart, J. Cao, J. N. Sturgis, Y. Mazor, **G. S. Schlau-Cohen**, "Elucidating interprotein energy transfer dynamics within the antenna network from purple bacteria", *Proc Natl Acad Sci*, **120**, e2220477120 (2023). [10.1073/pnas.2220477120]
2. D. Harris, H. Toporik, **G. S. Schlau-Cohen**[#], Y. Mazor[#], "Energetic robustness to large scale structural fluctuations in a photosynthetic supercomplex." *Nat Commun*, **14**, 4650 (2023). [10.1038/s41467-023-40146-8]
3. Q, Li, K. Orcutt, R. L. Cook, J. Sabines-Chesterking, A. L. Tong, **G. S. Schlau-Cohen**, X. Zhang, G. R. Fleming, K. B. Whaley, "Single-photon absorption and emission from a natural photosynthetic complex." *Nature*, **619**, 300-304 (2023). [10.1038/s41586-023-06121-5]
4. O. C. Fiebig, D. Harris, D. Wang, M. P. Hoffmann, **G. S. Schlau-Cohen**, "Ultrafast dynamics of photosynthetic light harvesting: Strategies for acclimation across organisms." *Annu Rev Phys Chem*, **74**, 493–520 (2023). [10.1146/annurev-physchem-083122-111318]
5. M. Son, A. Pinnola, R. Bassi, **G. S. Schlau-Cohen**, "Protein-protein interaction enhances photoprotective dissipation in arrays of the plant light-harvesting complex, LHCII," *J Am Chem Soc*, **143**, 17577 (2021). [10.1021/jacs.1c07385]
6. M. Son, S. M. Hart, **G. S. Schlau-Cohen**, "Investigating carotenoid photophysics in photosynthesis with two-dimensional electronic spectroscopy", *Trends Chem*, **3**, 733 (2021). [10.1016/j.trechm.2021.05.008]
7. A. M. Hancock, M. Son, M. Nairat, **G. S. Schlau-Cohen**[#], P. G. Adams[#], "Ultrafast energy transfer between lipid-linked chromophores and plant light-harvesting complex II," *Phys Chem Chem Phys*, **23**, 19511 (2021). [10.1039/D1CP01628H]
8. P. Manna, T. Davies, M. Hoffmann, M. P. Johnson, **G. S. Schlau-Cohen**, "Membrane-dependent heterogeneity of LHCII characterized using single-molecule spectroscopy", *Biophys J*, **15**, 3091 (2021). [10.1016/j.bpj.2021.06.010]

Understanding phycoerythrin biogenesis: Structural and biochemical studies of bilin lyase-isomerase MpeV

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Overall research goals:

Cyanobacteria synthesize, degrade, and modify their light harvesting proteins, called phycobiliproteins, in response to such parameters as light intensity, light wavelength, and nutrient availability. The phycobiliproteins (phycocyanin, phycoerythrin, and allophycocyanin) absorb light in the visible region between 470-670 nm due to one or more linear tetrapyrroles (bilins) that are attached at cysteine residues. Cyanobacteria are extremely important contributors to primary productivity in the oceans, and some marine *Synechococcus* can harvest the blue light that dominates these environments due to the presence of their chromophore called phycourobilin.

Small unicellular cyanobacteria such as *Synechococcus* RS9916 are estimated to contribute 25-30% of the total marine productivity. *Synechococcus* is likely to be one of the most ubiquitous phytoplanktonic organisms in the world's oceans and numerically the second most abundant marine phototroph in this ecosystem. This dominance is in large part due to its remarkable phenotypic plasticity in its use of a diverse set of photosynthetic pigments and a complex set of genes and regulatory systems that control the expression of these pigments. Much of this diversity occurs within the protein Phycoerythrin (PE), which has 5 phycoerythrobilin (PEB) or phycourobilin (PUB) chromophores attached at 6 cysteine residues. Phycobiliprotein biogenesis involves the attachment of chemically distinct bilin pigments to these proteins via enzymatic reactions catalyzed by bilin lyases. Notably, some members in this enzyme group, called bilin lyase-isomerases, are able to attach a green-absorbing bilin (PEB) and isomerize it to a blue-absorbing bilin (PUB). These lyases and lyase-isomerases play a key role in a widespread phenomenon, called Type IV Chromatic Acclimation (CA4), present in about half of all *Synechococcus* cells. CA4 causes these cells to change color as a result of the massive restructuring of their phycobilisome antennae, after switching between blue and green light. The ability to perform CA4 is conferred by a small genomic island composed of 4-5 genes, at least one of which is a bilin lyase. However, the mechanism by which *Synechococcus* utilizes different bilin lyases and lyase-isomerases to achieve their pigment diversity remains elusive at the molecular level. My collaborators and I are working to elucidate the structures, functions and mechanisms of bilin lyases responsible for CA4 and to dissect how these important enzymes confer their substrate specificity in pigment type and site during CA4.

These studies will also biochemically characterize enzymes which will have very important potential biotechnological and cell biology applications for the use of phycoerythrins as fluorescent tags.

Significant achievements (DOE funding 2023-2026):

Recently, we have characterized a group of novel bilin lyases that have the unique capability of attaching a bilin chromophore via double ligation at Cys50 and Cys61 on the β-subunits of phycoerythrin. Our initial sequence analysis and structural modeling of three closely related proteins MpeV, MpeU, and CpeF (denoted VUF) suggest a novel molecular mechanism for attachment with the active site geometry and protein architecture distinct from all known bilin lyases. We have shown that CpeF is a PEB lyase while MpeV and MpeU are lyase-isomerases that convert PEB to PUB. In this proposal, we will focus on the lyase-isomerase MpeV to address two important questions central to our

mechanistic understanding of this VUF family bilin lyases. *First*, how do VUF enzymes accomplish the bilin isomerization upon ligation? *Second*, how do they catalyze the double ligation reactions to attach bilin at both Cys50 and Cys61? We will employ an integrated approach of biochemistry, mutagenesis, spectroscopy, bioinformatics, and structural biology to pursue the following aims:

Aim 1. To identify key residues underlying the lyase-isomerase function of MpeV, we will perform comparative bioinformatics analysis on a collection of annotated protein sequences of lyases (CpeF) and lyase-isomerases (MpeU/V). Using activity assays we have established, we will then perform site-directed mutagenesis and examine the roles of candidate residues in conferring the isomerase activity.

Aim 2. To identify key residues underlying the double ligation reaction of MpeV, we will explore possible modes of enzyme-substrate interactions based on homology models, sequence conservation and site-directed mutagenesis. We aim to pinpoint the active site(s) for bilin attachment, and determine the sequence and interplay of the double ligation events at Cys50 and Cys61. In addition, we will examine the role of CpeZ, a chaperone-like protein essential for the action of MpeV.

Aim 3. To determine the structural basis of the double ligation and bilin isomerization of MpeV, we will make enzymatically active fusion proteins to promote formation of stable enzyme-substrate complexes for structural studies. Our supporting data demonstrate that the MpeV and CpeZ proteins purified from *E. coli,* are soluble and adequate for structural studies. To obtain stable complexes, we will a) tether MpeV and CpeZ via fusion or linkers guided by modeling (Aim 2), and b) arrest the pre-ligation structure by removing one or both cysteine anchors in the CpeB substrate. The crystal structure of the complex will elucidate the active site geometry and key interactions underlying the double bilin attachment reaction.

The proposed studies will provide mechanistic insights into two intriguing aspects of MpeV, a bilin lyase that catalyzes the isomerization and double ligation reactions of the bilin chromophore. This research will advance our fundamental understanding of the important VUF enzyme family critical to light harvesting and pigment diversity allowing efficient photosynthesis of cyanobacteria in different light niches. The mechanistic understanding to be gained from this research will lay a groundwork for designing artificial photosynthetic light harvesting systems and biohybrid photocatalysts, and set the stage for developing tool enzymes for fluorescent protein production.

Since funding arrived this Fall, we are focused on making the fusion protein constructs for MpeV-CpeZ and are generating some site directed mutants to test the importance of various amino acid residues in the putative active site for MpeV. We hope to have preliminary data to report soon.

Science priorities for the next year (2023-2024):

- We are focusing our initial efforts on making some site-directed mutants in MpeV and CpeZ and on obtaining a fusion protein of MpeV-CpeZ that is enzymatically active.

My major scientific area(s) of expertise is/are: Photosynthesis, post-translational modifications of proteins, phycobiliprotein biosynthesis.

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

Publications supported by this project: N/A (new award)

A molecular thermostat for post-translational regulation of light harvesting complex biogenesis

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Overall research goals:

The long term goal of this project is to decipher the molecular basis of light harvesting complex (LHC) biogenesis in molecular and energetic detail, by integrating biochemistry with high resolution biophysical tools including nuclear magnetic resonance (NMR) and fluorescence spectroscopy. In addition to a better understanding of the bioassembly of this fundamental complex, this project will elucidate general principles of membrane protein biogenesis and provide valuable knowledge and tools for the engineering of analogous processes in the future.

Specific objectives:

Objective 1. Understand how the Alb3 translocase remodels cpSRP43 to induce LHCP release.
Objective 2. Understand how cpSRP43 interacts with and protects LHCPs.
Objective 3. Understand how cpSRP43 provides thermoprotection of TBS enzymes.

Significant achievements (2022-2023):

1. **A molecular thermostat for post-translational regulation of light harvesting complex biogenesis**

The biogenesis of light harvesting complex (LHC) of photosynthetic systems is a rate-limiting step in biomass production from solar energy and requires a conserved chaperone, the chloroplast signal recognition particle 43 kDa subunit (cpSRP43). cpSRP43 mediates the membrane transport of chlorophyll a,b-binding proteins (LHCPs) and protects multiple enzymes in the chlorphyll biosynthesis pathway, which supplies chlorophylls critical to LHCP folding. How the two activities of cpSRP43 are coordinated to maintain LHC homeostasis is unclear. Here we show that cpSRP43 does so via a temperature-sensitive conformational change between a well-folded 'closed' state and a partially disordered 'open' state. Using rational mutations that modulate the open-to-closed rearrangement, we found that closed cpSRP43 specifically protects LHCP during its *de novo* biogenesis, whereas open cpSRP43 is responsible for protecting chlorophyll biosynthesis enzymes from heat-induced misfolding. High temperature shifts cpSRP43 to the open state, thereby unleashing its thermoprotection activity. Our results provide the molecular basis of a post-translational mechanism to coordinate the two branches of LHC biogenesis. They also demonstrate how an ATP-independent chaperone uses conformational dynamics to alter its activity and client selectivity, thereby adapting to cellular proteostatic demands under shifting environmental conditions.

2. Progress towards deciphering the interaction of cpSRP43 with LHCPs.

The isolation of cpSRP43 mutants locked in the closed conformation, that stably binds the LHCPs (Aim 1), provides new opportunities to study the interaction of this chaperone with LHCP using NMR spectroscopy, an effort that is currently in progress. As complementary approaches, we incorporated a photocrosslinker, Bpa, into specific sites in LHCP and identified their interaction sites on cpSRP43 using mass spectrometry (MS). Preliminary results show that different structural elements on LHCP

crosslink to cpSRP43 at distinct sites and suggest that LHCP is anchored on cpSRP43 via sequence-specific recognition of an L18 motif in a loop sequence, whereas the transmembrane domains (TMDs) of LHCP interact with cpSRP43 in a dynamic but topology-dependent manner.

3. Progress towards deciphering the interaction of cpSRP43 with TBS enzyme.

We proposed to use GUN4 as a model substrate to probe the client interaction of cpSRP43 with TBS enzymes. Circular dichroism (CD) measurements show that cpSRP43 induces partial unfolding of GUN4 at room temperature and maintains GUN4 in this conformational state under heat stress conditions. This suggests that cpSRP43 recognizes GUN4 in a near native state and that this interaction can be probed by NMR at room temperature.

Science priorities for the next year (2023-2024):

- Characterize the effects of conformation-specific cpSRP43 mutants in Arabidopsis to understand the *in vivo* role of the different conformational states of cpSRP43.
- Test the mode of cpSRP43-LHCP interaction with additional crosslinking-MS experiments. Use time-resolved fluorescence-resonance energy transfer (FRET) measurements to further probe the dynamics of the chaperone-TMD interaction.
- In preliminary experiments, the NMR spectrum of GUN4 is dominated by N-terminal disordered sequences. We will engineer new constructs to improve the NMR behavior of GUN4 and enable the use of NMR spectroscopy to probe the interaction of cpSRP43 with this TBS client.

My major scientific area(s) of expertise is/are: protein biochemistry and biophysics; protein folding and quality control; molecular chaperones.

To take my project to the next level, my ideal collaborator would have expertise in: environmental regulation of light harvesting complex biogenesis: response to draught, salinity, day/light cycles.

Fundamental Research Aimed at Diverting Excess Reducing Power in Photosynthesis to Orthogonal Metabolic Pathways

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Email: aus40@psu.edu; Website: https://sites.psu.edu/silakovlab/

Overall research goals:

In this project, we aim to establish basic science principles that will allow the efficient coupling of enzymes to cyanobacterial Photosystem I and the diversion of excess energy available under high-light conditions to produce chemical bonds instead of heat. Our goal is to re-engineer cyanobacterial Photosystem I (PS I) to donate electrons to an orthogonal H_2 production pathway by fusing its stromal subunits with [FeFe] hydrogenase (H_2ase). We hypothesize that it is possible to take advantage of the three iron-sulfur clusters (F_X, F_A, and F_B) on the stromal side of PSI to efficiently deliver electrons to an [FeFe] H_2ase and drive light-dependent hydrogen production. In the first specific aim of the current project, we investigate the structure-function determinants associated with the coupling of PSI and [FeFe] H_2ase-PsaE fusion proteins. This research is instrumental in evaluating the efficiency and sustainability of the H_2-producing nanoconstruct in cyanobacteria. The second part of the project addresses the plausibility of activating the engineered [FeFe]H_2ase:PSI complex *in vivo*. For this purpose, we study the strategy for transforming a marine cyanobacterium *Synechococcus sp.* PCC 7002 (*S.*7002) with the three strictly required maturation enzymes that do not exist in the wild-type organism.

Significant achievements (2022-2023):

- Understanding factors influencing O_2-tolerance in [FeFe] hydrogenases is essential for delineating potential pitfalls of the PSI-[FeFe] H_2ase coupling strategies. To this end, we achieved a milestone by demonstrating the unusual flexibility of the protein environment around the active center of O_2-tolerant [FeFe] H_2ase from *Clostridium beijerinkii* (*Cb*HydA1) using EPR spectroscopy.[4] Our data accumulated to date suggest that the ability to access the aberrant protein conformation around the active center is related to the O_2-tolerance of this enzyme.[2-4]
- We also successfully constructed and investigated a *Cb*HydA1-PsaE fusion. We have found that the addition of PsaE does not affect the structure of the H-cluster but does lower its activity compared to WT-*Cb*HydA1. We attributed the lower activity to an increased propensity of the *Cb*HydA1-PsaE fusion to transition to the inactive, O_2-protected state. The addition of PsaE to *Cb*HydA1 made the construct more O_2-tolerant![3]
- Encouraged by these results, we investigated the effect of the *Cb*HydA1-PsaE binding on charge transfer kinetics of the photoexcited PS I from *S.* 7002. First, we have realized that the ΔPsaE-variant of PS I exhibits prolonged charge recombination compared to wild-type, an effect that is yet to be understood and needs to be investigated. Upon addition of *Cb*HydA1-PsaE fusion, ΔPsaE-PS I exhibits the recombination kinetics at the wild-type PS I levels, indicating an almost quantitative binding of the nanoconstruct. Removal of hydrogen from the atmosphere showed an appearance of a ~30% contribution of a >1s charge recombination phase, indicating a loss of electron to the *Cb*HydA1 domain. The rates of anaerobic light-induced H_2 production are on par or better than those reported for other systems under similar conditions. We are also happy to report that the designed nanoconstruct exhibited a

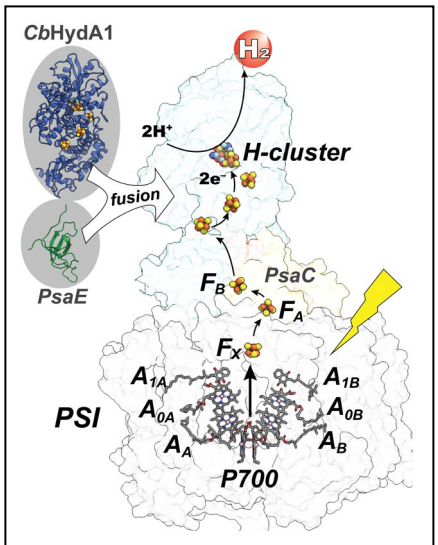

Figure 1. Schematic depiction of [FeFe]-H_2ase-PsaE fusion strategy for photo-H_2 production.

sustained H_2 production on air, albeit at a lower rate. Overall, the research is highly encouraging, showing the viability of the PsaE-based fusion strategy.[3]

- We have successfully obtained CryoEM image sets of WT PS I from S.7002 and the ΔPsaE PS I variant and extracted electron densities of both trimeric complexes at 2.8Å resolution. Modeling of the densities is currently underway. We have also performed a preliminary EPR study of the PSI complexes to show that the ratio of reduced F_A and F_B in cryogenic photoreduction experiments changes from WT to the ΔPsaE variant. This effect suggests PsaE's role in tunning the electronic properties of the FeS clusters in PsaC.[1]
- Pertinent to the program's success, we have successfully identified other O_2-tolerant [FeFe] hydrogenases with the aid of a bioinformatics strategy developed by us, expanding the library of alternative candidates for *in vivo* work in cyanobacteria.[2]

Science priorities for the next year (2023-2024):

- Continue the comparative investigation of ΔPsaE PS I and WT PS I complexes using EPR with a focus on the structural and redox properties of the F_A/F_B clusters.
- Resolving structures of PS I complexes is another priority area. We will continue using CryoEM to resolve structures of WT PS I and ΔPsaE PS I. We will also perform CryoEM experiments on ΔPsaE PS I:*Cb*HYdA1-PsaE complex, the analysis of which will be instrumental in delineating the disposition of the hydrogenase domain. This information will be invaluable for resolving structural perturbations caused by the fusion construct and creating the next generation of nanoconstructs.
- Installation of active [FeFe] hydrogenases in cyanobacteria is thwarted by the absence of host-native maturation factors necessary to synthesize the active cofactor. Therefore, we will next evaluate the cyanobacterial environment's ability to support the maturation of [FeFe] hydrogenases.

My major scientific area(s) of expertise is/are: EPR, FTIR and optical spectroscopies, electrochemistry, aerobic and anaerobic enzymology, [FeFe] hydrogenases, cyanobacterial systems.

To take my project to the next level, my ideal collaborator would have expertise in: structural biology, quantum chemical computations, algal systems.

Publications supported by this project:

1. Gorka, M., Rumbaugh T., Silakov, A., Investigation of the ΔPsaE deletion variant of Photosystem I from *Synechococcus sp.* PCC 7002. *In submission 2024*
2. Khundoker, R., Gorka, M., Silakov, A., A bioinformatic method for identification of O_2-tolerant [FeFe] hydrogenases. *In submission 2023*
3. Rumbaugh, T, Gorka, M, Baker, C.S., Golbeck, J.H., Silakov, A. PsaE-based hydrogenase fusion proteins coupled to Photosystem I for light induced H_2 generation. *In submission 2023*
4. Corrigan P.S., Majer S.H., Silakov A. Evidence of atypical structural flexibility of the active site surrounding of [FeFe] hydrogenase from *Clostridium beijerinkii*. Journal of the American Chemical Society (2023), 145, 20, 11033
5. Brimberry M., Corrigan P., Silakov A., Lanzilotta W.N., Evidence for Porphyrin Mediated Electron Transfer in the Radical SAM Enzyme HutW (2023) 62, 6, 1191-1196. doi: 10.1021/acs.biochem.2c00474
6. Gorka M., Baldansuren A., Malnati A., Gruszecki E., Golbeck J.H., Lakshmi K.V. Shedding Light on Primary Donors in Photosynthetic Reaction Centers. Front Microbiol (2021) 12, 735666. doi: 10.3389/fmicb.2021.735666
7. Gorka, M., Gruszecki, E., Charles, P., Kalendra, V, Lakshmi, K.V., and Golbeck, J.H. Two-dimensional HYSCORE Spectroscopy Reveals a Histidine Imidazole as the Axial Ligand to Chl3A in the M688HPsaA Genetic Variant of Photosystem I. Biochim Biophys Acta Bioenerg. (2021) 1862, 7, 148424. doi: 10.1016/j.bbabio.2021.148424
8. Gorka, M., Charles, P., Kalendra, V., Lakshmi, K.V., and Golbeck, J.H., A. Dimeric Chlorophyll Electron Acceptor Differentiates Type I from Type II Photosynthetic Reaction Centers. iScience (2021) 24, 7, 102719, doi: 10.1016/j.isci.2021.102719
9. Corrigan, P., Tirsch, J. Silakov, A. Investigation of the unusual ability of the [FeFe] Hydrogenase from *Clostridium beijerinkii* to access an O_2-protected state. Journal of the American Chemical Society (2020) 142, 28, 12409, doi: 10.1021/jacs.0c04964
10. Gorka, M., Cherepanov, D.A., Semenov, A.Y., and Golbeck, J.H. Control of electron transfer by protein dynamics in photosynthetic reaction centers. Critical Reviews in Biochemistry and Molecular Biology. 2020, 55, 5, 425
11. Gorka, M., Golbeck, J.H. Generating dihydrogen by tethering an [FeFe]hydrogenase via a molecular wire to the A_{1A}/A_{1B} sites of Photosystem I. Photosynthesis Research 2020, (143) 155, doi: 10.1007/s11120-019-00685-y
12. Walters, K.A., Golbeck, J.H. Designing a modified clostridial 2[4Fe–4S] ferredoxin as a redox coupler to directly link photosystem I with a Pt nanoparticle. Photosynthesis Research 2020, (143) 165, doi: 10.1007/s11120-019-00679-w

NREL Photosynthetic Energy Transduction Core Program - Photosystem I Responses to Differential Electron Flux in *Synechocystis* sp. PCC 6803

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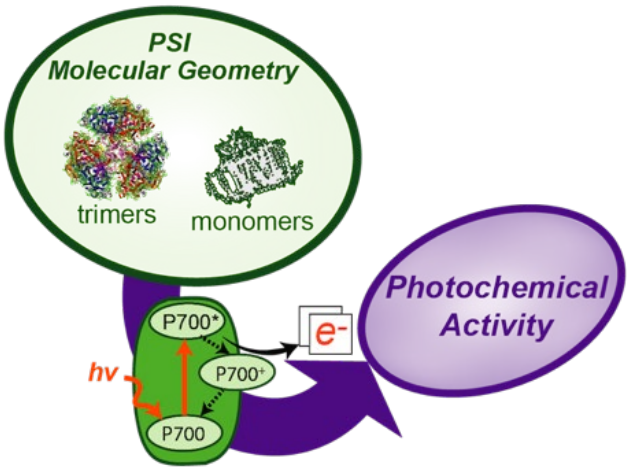
Overall research goals:

The overall goal of the Photosynthetic Energy Transduction core program at NREL is to understand the fundamental mechanisms that support integration of reaction center photochemistry with electron transport and reduction-oxidation reactions of enzymes. This integration serves to maintain the functional status of photosynthetic components as cells respond to changing energy and growth conditions. Research areas include elucidating the properties of Photosystem I (PSI) that modulate photochemistry, the roles of ferredoxins in mediating electron transport and mechanisms of redox enzymes for converting electron flux into reduced products. These studies are revealing how photosynthetic components successfully manage variations in electron-transport that result from variable growth conditions in converting sunlight into chemicals and fuels.



We are using *Synechocystis* sp. PCC 6803 as a model system to isolate PSI reaction centers from strains engineered with an altered peripheral electron flux and cultured under differential photon fluxes. The studies are revealing that the absence of a major O_2 reduction reaction (ORR) pathway causes changes in the photochemical, spectral, oligomeric, and physical properties of PSI. We hypothesize these changes serve as an adpatative mechaism to control photoexcitation and electron transport under differential flux conditions. In addition to photochemical studies, we are collaborating with Dr. P. Fromme (ASU) with the aim of solving the structures of PSI trimers and monomers prepared from cells under differential flux to identify how changes in subunit interactions and cofactor properties contribute to the observed changes in PSI photochemistry. We have also observed that the absence of ORR affects the specificities and interactions of PSI with the major phycobilisome (PBS) complexes, CpcL and CpcG. We are investigating the basis for these changes and the function in controlling excitation energy allocation to PSI. We expect that in addition to modifying PBS interactions, photoexcitation, and photochemistry that the changes in PSI also alter the binding interactions and electron transfer kinetics with ferredoxins, which is being addressed in photochemical assays of PSI preparations with isolated ferredoxins. Combined, our findings aim to provide key insights into the mechanisms that tune PSI photochemistry to enable cells to adapt to natural variations in light and electron flux conditions.

Significant achievements (2021-2023)

- Modulation of PSI photochemical and physical properties. We demonstrated that oligomeric, spectral, photochemical, and physical modifications to PSI occur in response to changes in electron demand and photon availability. Together these modifications serve as mechanisms to modulate the distribution of energy under changing cellular and environmental conditions. (*RSC Advances*, 12, 14655, 2022; *J. Biol. Chem.*, *In review).*

- Attenuated electron flow extensively remodels reaction center interactions with PBS. Strains attenuated in electron utilization by the removal of flavodiiron proteins demonstrate fluorescence changes suggesting preferences in how different forms of PBS (CpcL-PBS, CpcG-PBS) interact with PSI and PSII. We demonstrate a shift in binding preference that results from molecular changes in both the PBS and PSI.
- Emerging studies on interactions between PSI and ferredoxins. We are investigating how modifications to PSI influence interactions with ferredoxins and electron transfer kinetics.

Science priorities for the next year (2023-2024):
- Determine the structural properties of PSI oligomeric complexes isolated under altered cellular redox conditions.
- Determine how the CpcL and CpcG forms of PBS interact with PSI monomers and trimers with different oligomeric, photochemical, and physical properties.
- In continued collaboration with Dr. B. Bothner at Montana State University, determine protein-protein interactions using mass spectronomic analysis.
- In collaboration with Dr. P. Fromme at Arizona State University, determine the three-dimensional structures of PSI monomeric complexes isolated from strains with disrupted electron utilization pathways.
- Determine the photoreduction kinetics of PSI isoforms prepared from ORR strains with ferredoxins 1, 8, and 11.

My major scientific area(s) of expertise is/are: Photosynthetic electron transport processes, microbiology, molecular biology, protein expression and purification, biochemistry, spectroscopy.

To take my project to the next level, my ideal collaborator would have expertise in: Determination of the structure, cofactor composition, and protein interactions of protein complexes using mass spectrometry, cryo-EM, and protein crystallography.

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

1. C. E. Lubner. “Bacteria ‘Read’ Light to Gain a Competitive Advantage”. *J. Bacteriol.,* 201:e0082-19. (2019). DOI: 10.1128/JB.00082-19.
2. S.L. Smolinski, C.E. Lubner, Z. Guo, J.H. Artz, K.A. Brown, D.W. Mulder, and P.W. King. “The influence of electron utilization pathways on photosystem I photochemistry in *Synechocystis* sp. PCC 6803", *RSC Advances*, 12, 14655, (2022). DOI:10.1039/D2RA01295B.
3. S. L. Smolinski, M. Tokmina-Lukaszewska, J. M. Holland, Z. Guo, B. Bothner, P. W. King and C. E. Lubner. “Modulation of PSI spectral capacity and electron flux in response to changes in electron flow pathways and photon flux in *Synechocystis* sp. PCC 6803”, *J. Biol. Chem.*, (2023). *In review.*

Probing Limitations to C_4 Carbon Capture

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Overall research goals:

We seek to understand aspects of Rubisco regulation, particularly how the two forms of Rubisco activase (RCA) are functionally differentiated, as well as how activase expression itself is regulated in C_4 plants. The activation state of Rubisco is dynamically regulated by RCA, with the active state being catalytic and the inactive state being inhibited and possibly conferring increased protein stability. Plants therefore modulate the proportion of total assembled Rubisco that is activated in response to environment, substrate availability, and light.

In C_4 plants RCA is encoded by two genes, *RcaA* specifying the α form; and *RcaB* the β form. The α form bears a C-terminal extension with redox-sensitive Cys residues and is specifically induced under heat stress. The β form lacks redox regulation and is the predominant form under all growth conditions. Because the AAA+ ATPase domain of activase is heat-sensitive, we have hypothesized that the α form's induction under heat plays a role in maintaining Rubisco activation under such conditions.

Our approach has been to develop the C_4 model *Setaria viridis*, where we have created *rcaA* and *rcaB* mutants using CRISPR-Cas9, as well as transgenics overexpressing different forms of RCA including a chimeric Rca–β with a heat-tolerant AAA+ domain from *Agave tequilana*. We are seeking to address three main questions:

1. What are the functional differences between Rca-α and β, as elucidated through analysis of the gene-edited mutants?
2. What are the endogenous mechanisms limiting RCA accumulation and Rubisco activation? This is being explored through ectopic expression of foreign or disabled forms of RCA, i.e. forms unable to catalyze ATP hydrolysis, or unable to bind Rubisco.
3. The information and resources developed in the second question can be used to explore the consequences of a forced higher Rubisco activation state. For example, does it lead to Rubisco instability? We will also combine plants engineered to accumulate higher levels of Rubisco with those overexpressing RCA, through genetic crosses. This will address whether C_4 plants seek to achieve a balance between Rubisco and RCA abundance.

Significant achievements (2023-2026):

We have created mutants for both Rca-α and Rca-β in *Setaria.* Mutants lacking Rca-α have normal growth and photosynthesis under control conditions. We are current examining their performance in detail under 44°C heat stress, where α is induced in WT plants. Mutants lacking Rca-β require high CO_2 under otherwise normal growth conditions, as expected, and also have low carbon assimilation rates even under high CO_2. Under heat stress conditions, Rca-α is induced in Rca-β mutants and allows *rcaB* mutants to maintain higher levels of photosynthesis under atmospheric CO_2 levels compared to plants grown at ambient temperature with or without increased CO_2. This suggests that Rca-α is effective at activating Rubisco at high temperatures.

We have begun creating mutant forms of RCA related to Question 2. One form we have introduced into plants is the chimeric Rca-β form, denoted AtSv, that should work efficiently at high temperature, perhaps more so than SvRca-α. Plants expressing AtSv have been crossed to the *rcaB* mutant, generating plants where the native β form has been replaced with a heat-tolerant form.

We have obtained transgenic plants overexpressing three genes: Rubisco LS, Rubisco SS and the Rubisco assembly factor Raf1. In maize this combination led to 30-40% higher Rubisco levels and 8-10% higher CO_2 assimilation, the differential being due to decreased Rubisco activation state. These plants are being characterized for their assimilation rates and Rubisco accumulation and activity, and will be crossed to plants expressing AtSv to further increase their Rubisco activation state.

Science priorities for the next year (2023-2024):

- Our main emphasis for next year, apart from completing experiments in progress, will be creating several mutants of Rca-β and assaying them *in vitro* and *in vivo*. This will include two mutants that should inactivate the ATPase activity, and 3-4 mutants that should decrease or eliminate the ability of RCA to bind Rubisco LS, based on structural information. These mutant proteins will be produced in bacteria and assayed for ATPase activity, and their ability to activate Rubisco. If ATPase activity is present and Rubisco is not activated or is poorly activated, this will help define residues important for LS interaction in C_4 systems. When expressed *in vivo*, we will observe whether plants still limit their accumulation, addressing whether either ATPase activity or Rubisco binding is a sensor for RCA regulation.

My major scientific area(s) of expertise is/are: Plant genetics and transgenics, *in vivo* assimilation measurements, gene expression, plant phenotyping.

To take my project to the next level, my ideal collaborator would have expertise in: Metabolite flux, enzyme kinetics in fluctuating light.

Publications supported by this project Click here to enter text.:

1. Salesse C, Sharwood R, Busch FA, Kromdijk J, Bardal V, Stern D (2018) Overexpression of Rubisco subunits with RAF1 increases Rubisco content in maize. *Nature Plants* 4:802-810.
2. Salesse-Smith C, Sharwood RE, Busch FA, Stern DB. (2019) Increased Rubisco content in maize mitigates chilling stress and speeds recovery. *Plant Biotech. J.* 18:1409-1420.
3. Feiz, L., Strickler, S.R., van Eck, J., Mao, L., Movahed, N., Taylor, C., Gourabathini, P., Fei, Z. and Stern, D.B., 2020. *Setaria viridis* chlorotic and seedling-lethal mutants define critical functions for chloroplast gene expression. *Plant J.* 104:917-931.
4. Hotto AM, Salesse-Smith C, Lin M, Busch FA, Simpson I and Stern DB (2021) Rubisco production in maize mesophyll cells through ectopic expression of subunits and chaperones. *J. Exp. Bot.* 72:4930-4937.

Mechanism of Protein Transport across the Chloroplast Thylakoid Membrane

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Overall research goals:

Protein targeting represents a major cellular activity through which approximately half the proteins synthesized in eukaryotic cells and 30% of the proteins made in prokaryotic cells are transported across one or more membranes en route to their final destinations. Not surprisingly, the various protein transporters governing this protein traffic are essential to the cells' survival. In plants, more than 95% of the proteins residing in chloroplasts are encoded in the nucleus and are post-translationally imported from the cytoplasm. This necessarily includes many proteins involved in photosynthesis, in both the dark and light reactions. *A long-term goal of my laboratory is the discovery of the mechanisms and energetics of protein transport into and within chloroplasts.*

Numerous proteins located in the both the thylakoid lumen and thylakoid membrane reach these compartments through the action of the Twin Arginine Translocation (Tat) pathway, a pathway to whose discovery my laboratory contributed. These include proteins critical to photosynthetic oxygen evolution. While this pathway has been under intense investigation for many years, the mechanism through which Tat substrates cross their target membranes is still unknown. We seek to correct this situation with the experiments we will perform with the current grant.

This funding period started in September 2023, and so the specific aims are largely yet to be realized. They are:

Aim #1. Develop a real-time spectroscopic assay for Tat protein transport into the thylakoid lumen.

Aim #2. Test the hypothesis that the number of Tha4 molecules associated with the Hcf106/cpTatC complex is dependent on the size of the substrate.

Aim #3. Probe Tat protein transport across artificial membranes by electrophysiology using artificial membranes suspended over an aperture separating two aqueous chambers.

Aim #4. Use single-particle cryo-EM to determine the structure of the digitonin-solubilized cpTat translocation machinery in which Tha4 is constitutively assembled into the complex.

Significant achievements: 2019 - 2023

In the previous funding period, ending in September 2023, we probed many aspects of the Tat pathway in both chloroplasts and bacteria. Significant discoveries included:

- Discernment of the molecular logic behind the short, 15 residue transmembrane helices found in TatA and TatB.
- Discovery of different specific motifs in the hydrophobic TMH of chloroplasts and of bacteria.
- Examination of the leak of ions across thylakoid membranes during Tat and Sec-dependent protein translocation events
- Development of a real-time assay for Tat protein transport in bacterial spheroplasts
- Unification of the energetic requirements for Tat protein transport in chloroplasts and bacteria

Science priorities for the next year (2023-2024):

- We will continue our efforts to collect evidence for or against the three major models for the mechanism of Tat protein transport.
- We will push hard to produce a structure for the active Tat transport machinery. To this end, we have hired a postdoc with cryoEM experience in solving structures of thylakoid protein complexes.

My major scientific area(s) of expertise is/are: Protein trafficking in chloroplasts and in bacteria. Membrane biophysics. Bioenergetics.

To take my project to the next level, my ideal collaborator would have expertise in: Protein complex purification, and structure determination by CryoEM. These are the areas of expertise of my co-PIs Terry Bricker and Maria Maldonado, respectively.

Publications supported by this project over the past 5 years:

1. Ganesan I, and Theg S.M. (2019) Structural considerations of folded protein import through the chloroplast TOC/TIC translocons. FEBS Lett. 2019 Mar;593(6):565-572. doi: 10.1002/1873-3468.13342. Epub 2019 Mar 5.
2. McKinnon LJ, and Theg S.M. (2019) Determinants of the Specificity of Protein Targeting to Chloroplasts or Mitochondria. Mol Plant. 2019 Jul 1;12(7):893-895. doi: 10.1016/j.molp.2019.05.004. Epub 2019 May 22.
3. Day PM, Inoue K, and Theg S.M. (2019) Chloroplast Outer Membrane β-Barrel Proteins Use Components of the General Import Apparatus. Plant Cell. 2019 Aug;31(8):1845-1855. doi: 10.1105/tpc.19.00001. Epub 2019 Jun 19.
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5. Li, H.M., Schnell, D., and Theg, SM. (2020) Protein Import Motors in Chloroplasts: On the Role of Chaperones. Plant Cell. 2020 Mar;32(3):536-542. doi: 10.1105/tpc.19.00300. Epub 2020 Jan 13.
6. McKinnon, L., Fukushima J., Endow, J.K., Inoue K., and Theg, S.M. (2020) Membrane chaperoning of a thylakoid protease whose structural stability is modified by the protonmotive force. The Plant Cell. 2020 May, 32:1589-1609. doi: 10.1105/tpc.19.00797. Epub 2020 Mar 13. Also, bioRxiv 788471; doi: https://doi.org/10.1101/788471
7. Klasek L, Inoue K, and Theg, S.M. (2020) Chloroplast Chaperonin-Mediated Targeting of a Thylakoid Membrane Protein. The Plant Cell 2020 Dec;32(12):3884-3901. doi: 10.1105/tpc.20.00309. Epub 2020 Oct 22.
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10. Hao, B., Zhou, W., and Theg, S.M. (2021) Hydrophobic mismatch effect is a key factor in protein transport on the Tat pathway. doi: https://doi.org/10.1016/j.jbc.2022.101991.
11. Hao, B., Zhou, W., and Theg, S.M. (2023) The polar amino acid in the TatA transmembrane helix is not strictly necessary for protein function
doi: https://doi.org/10.1016/j.jbc.2023.102998
12. Zhou, W., Hao, B., Bricker, T.M., Theg, S.M. (2023) A Real-Time Analysis of Protein Transport via the Twin Arginine Translocation Pathway in Response to Different Components of the Protonmotive Force *in vivo* bioRxiv 2023.01.12.523868; doi: https://doi.org/10.1101/2023.01.12.523868

Dynamic molecular recognition underlying protein electron transfer studied by residue-specific vibrational spectroscopy

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Overall research goals:

We aim to advance our understanding of the electron transfer reactions of photosynthesis, specifically through the development of site-selective vibrational techniques that will provide insight into the nature of the protein ensemble that supports electron transfer (ET) between plastocyanin (Pc) and cytochrome *f* (cyt *f*). A longer-term goal is to elucidate the involvement of local rearrangements of protein and water at the protein-protein interface. Toward addressing the need for tools that can probe proteins with high spatial and temporal resolution, we have been developing infrared spectroscopy applied to frequency-resolved vibrational probes introduced selectively into proteins. Aim 1 is directed at improving and expanding the methodology, while Aim 2 applies the approaches to advance understanding of ET between Pc and cyt *f*.

Aim 1. Development of site-selective vibrational spectroscopy for analysis of protein electrostatics and dynamics. (1.A.) Implement and optimize difference spectroscopy to increase sensitivity through triggering plastocyanin oxidation via attachment of a Ru phototrigger. This aim also contributes to establishing ns-ms kinetic experiments. (1.B.) Increased sensitivity will be achieved through use of a more intense quantum cascade laser (QCL) source than enables thicker pathlengths. Longer term goals are to use pulsed QCL to implement ns-ms kinetics of ET between Pc and cyt *f*. (1.C.) Development of site-specific 2D IR spectroscopy. While linear spectroscopy is informative, 2D IR spectroscopy provides richer information. We aim to generate and apply new frequency-resolved 2D IR probes of proteins and extend the approach by implementing polarization-dependence.

Aim 2: Investigate the nature of the activated step of ET in the Pc/cyt *f* complex. The complexes formed by ET partners are low affinity and highly dynamic, as required for high flux of ET within a cell. For *Nostoc* Pc and cyt *f*, the stable complex appears to consist of a majority ensemble of loosely associated encounter states. The proposed experiments apply spectroscopy with protein labeling to investigate how the nature of the complexes and perturbation to the Cu site of Pc vary under conditions reported in literature to perturb or enhance ET.

Significant achievements (2021-2023):

(1) Altered coordination in a blue copper protein upon association with redox partner revealed by carbon–deuterium vibrational probes. Although extensive investigation has illuminated how the surrounding environment impacts redox centers in individual proteins, an additional consideration for inter-protein ET is that the reaction occurs after proteins associate into a complex where the redox center potentially experiences a different environment. Comparatively little is known about how complexation might alter a redox center and its properties toward ET. We assessed impact to the Cu site of the Pc from the association with cyt *f* by introducing C-D bonds to serve as a vibrational reporter at the key cysteine ligand Cys89. Massive increase in the C-D frequencies upon association of Pc with cyt *f* indicates substantial strengthening of the interaction between the cysteine ligand and the Cu ion. The effect is observed in either redox state but not for the Zn-substituted Pc, implicating the native Cu-Cys89 bonding. The changes to the Cu site are predicted to promote rapid ET in the complex with the partner cyt *f*.

(2) The structure of plastocyanin tunes the midpoint potential by restricting axial ligation of the reduced copper ion. Plastocyanin is a blue copper protein that has served as a model for illustrating how proteins tune metal properties. Nevertheless, the mechanisms by which the protein controls the metal site remain to be fully elucidated. A hindrance is that the closed shell Cu(I) site is inaccessible to most spectroscopic analyses. Carbon deuterium (C-D) bonds used as vibrational probes afford nonperturbative, selective characterization of the key cysteine and methionine copper ligands in both redox states. The structural integrity of Nostoc plastocyanin was perturbed by disrupting potential hydrogen bonds between loops of the cupredoxin fold via mutagenesis (S9A, N33A, N34A), variably

raising the midpoint potential. The C-D vibrations show little change to suggest substantial alteration to the Cu(II) coordination in the oxidized state or in the Cu(I) interaction with the cysteine ligand. They rather indicate, along with visible and NMR spectroscopy, that the methionine ligand distinctly interacts more strongly with the Cu(I) ion, in line with the increases in midpoint potential. We show that the protein structure determines the redox properties by restricting the interaction between the methionine ligand and Cu(I) in the reduced state.

(3) Synthesis and assessment of cyanoselenocysteine as new 2D IR probe of proteins that extends experimental timescale. The vibrational lifetime of probe limits the timescale over which 2D IR can be applied to measure equilibrium dynamics. The lifetime of an established 2D IR probe or proteins, cyanocysteine, is long for a nitrile due to heavy atom decoupling of the cyano group from vibrational relaxation pathways. Based on an analogous synthetic route, we have successfully generated FMOC-cyanoselenocysteine. Characterization by 2D IR spectroscopy is underway. As anticipated the lifetime of the CN vibration is substantially lengthened.

Science priorities for the next year (2023-2024):

- Understand change in Cu center of Pc induced by binding to cyt *f*. Prior experiments were performed at low ionic strength to promote complexation. We will determine whether the change in the Cu site is similarly induced at physiological ionic strength where electrostatic interactions are more shielded.
- A major thrust will be to push forward our goal to establish QCL spectrometer to measure kinetics of protein response to phototriggered ET. Ru-labeled Pc has been generated. The next sub-goal is to collect steady-state photo-induced difference spectra. In parallel we are working on LabView software to implement time-resolved experiments with the QCL.
- 2D IR spectroscopy of Pc/cyt *f* complex. We aim to resume challenging 2D IR experiments with proteins focusing on extending prior studies using cyanophenylalanine probes of Pc to characterize how the nature of the complex depends on ionic strength.

My major scientific area(s) of expertise is/are: infrared spectroscopy, multidimensional time-resolved spectroscopy, protein engineering, biological electron transfer, metalloproteins, enzyme catalysis.

To take my project to the next level, my ideal collaborator would have expertise in: intra/inter-protein electron transfer theory and modeling, vibrational spectroscopy theory and modeling, x-ray absorption spectroscopy.

Publications supported by this project :

1. C. Mammoser, B. LeMasters, S. Edwards, E. McRae, Mullins, Y. Wang, N. Garcia, K. Edmonds, D. Giedroc, M. Thielges, "The structure of plastocyanin tunes the midpoint potential by restricting axial ligation of the reduced copper ion." Comm. Chem. 6, 175 (2023). [10.1038/s42004-023-00977-4]
2. C. Mammoser, R. Agh, N. Garcia, Y. Wang, M. Thielges, "Altered coordination in a blue copper protein upon association with redox partner revealed by carbon–deuterium vibrational probes." Phys. Chem. Chem. Phys., 24, 21588, (2022). [10.1039/D2CP03314C]
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4. M. Thielges, "Transparent Window 2D IR of Proteins." J. Chem. Phys. 1553, 040903, (2021). [10.1063/5.0052628]
5. G. Tumbic, Y. Hossan, M. Thielges, "Protein dynamics by 2D IR spectroscopy." Annu. Rev. Anal. Chem. 14, 299, (2021). [10.1146/annurev-anchem-091520-091009]
6. S. Ramos, M. Thielges, "Site-specific 1D and 2D IR spectroscopy to characterize the conformations and dynamics of protein molecular recognition." J. Phys. Chem. B 123, 3551, (2019). [10.1021/acs.jpcb.9b00969]
7. S. Ramos, A. Le Sueur, R. Horness, J. Specker, J. Collins, K. Thibodeau, M. Thielges, "Heterogeneous and highly dynamic interface in plastocyanin-cytochrome *f* complex revealed by site-specific 2D IR spectroscopy." J. Phys. Chem. B 213, 2114, (2019). [10.1021/acs.jpcb.8b12157]

Fundamental Mechanisms for Solar Energy Conversion in Photosynthesis

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Karen Mulfort, Jens Niklas, Oleg Poluektov, Co-PI(s)
Tirupathi Malavath, Postdoctoral Research Associate
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Overall research goals:

Natural photosynthetic energy research is aimed at resolving fundamental mechanisms of photochemical energy conversion in photosynthetic proteins. A key challenge for photosynthesis research is that the mechanisms of regulation and control for solar energy conversion by light-induced electron transfer are matrixed across multiple scales of hierarchical photosynthetic structure and function. This program is directed at designing and conducting experiments to resolve fundamental mechanisms controlling solar energy conversion in photosynthesis by investigating linked photosynthetic function across multiple length scales, ranging from the protein-cofactor interactions at specific sites in reaction center (RC) proteins to long-range photosynthetic function in intact thylakoid membranes. Our hypothesis is that key energy and electron transfer reactions in natural photosynthesis are controlled by dynamic, specifically-tailored protein matrices that embed the donor/acceptor cofactors and regulate protein-protein interactions. Fundamental mechanisms revealed by this research program will inform design strategies for sustainable photosynthetic-inspired systems with efficient solar energy conversion and solar fuel synthesis capabilities.

Significant achievements (2021-2023):

Biohybrid Photosynthetic Charge Accumulation. A new biohybrid-based strategy was used to truncate the native three-protein electron transfer cascade PSI → ferredoxin (Fd) → ferredoxin-$NADP^+$ reductase (FNR) to a two-protein cascade by replacing PSI with a Ru(II)poly(pyridyl) photosensitizer molecule covalently bound to Fd and flavodoxin (Fld) to form biohybrid complexes that successfully mimic PSI in light-driven NADPH formation. Selective deuteration allows resolution of the intermediate semiquinone (SQ) state of FNR by EPR. Experiments reveal nature's mechanisms that couple single electron transfer chemistry to multi-electron transfer events, providing insight into inter-flavoprotein electron transfer with implications for efficient photocatalytic solar fuel strategies.

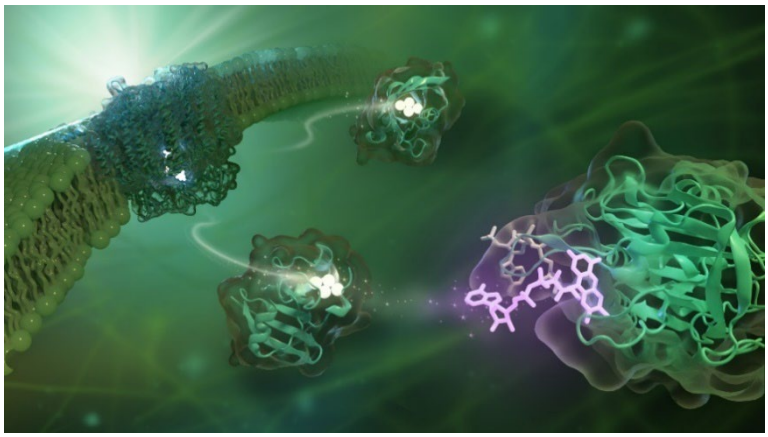

Fig 1. Photosynthetic charge accumulation via the flavin semiquinone of ferredoxin-$NADP^+$ reductase.

Intrinsic Photosystem I-ferredoxin Interactions in Thylakoids. Photoreduction of $NADP^+$ was found to occur without addition of extraneous Fd for thylakoids isolated from spinach and cyanobacteria. These findings indicate that isolated thylakoids either (i) retain a "pool" of Fd or (ii) that a fraction of PSI in thylakoid membranes is associated with Fd, possibly in a tertiary PSI-Fd-FNR complex capable of PSI → Fd →FNR electron transfer. Temperature-dependent EPR studies confirm the presence of Fd in thylakoid preparations capable of $NADP^+$ photoreduction. Quantification of EPR signals of P_{700}^+ and Fd reveal that Fd is present at a ratio of ~1 Fd per PSI monomer in both spinach and cyanobacterial thylakoids. The existence of intrinsic PSI-Fd functional associations provides evidence of nature's

mechanisms utilizing the confined thylakoid microenvironments to facilitate directional photosynthetic electron flow.

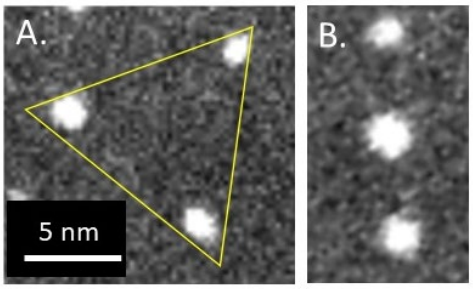

Fig 2. HAADF STEM images of cyanobacterial thylakoid_PtNP hybrids. A. PtNP trimer stromal planar view. B. PtNP linear arrangement of side view.

Imaging Catalytic Sites in Thylakoid Water-splitting Hybrids. Photosystem I-Pt nanoparticle (PtNP) hybrids are efficient for light-driven H_2 generation from water, and this work precisely demonstrates for the first time how the hybrid components interact with one another and suggests pathways to increase overall system efficiency. Argonne's Picoprobe, an advanced next generation analytical Electron Microscope was used to visualize how PtNPs interact with the protein. Hyperspectral elemental imaging confirms PtNP sites co-located to photosystem I sites in thylakoids, and the trigonal configuration of PtNPs confirm that the abiotic catalyst mimics the natural photosystem I acceptor protein.

Science priorities for the next year (2023-2024):

- Explore PSI-driven reduction of FNR in solution and in thylakoids with EPR spectroscopy.
- CryoEM structural determination of trimeric PSI-PtNP biohybrids.
- Examine electron transfer regulation between PSII and intrinsic Fd and PtNP at PSI, particularly the cytb6f-P700 step in spinach, algal, and cyanobacterial thylakoid systems.
- Examine PSI charge separation in thylakoid membranes related to intrinsic Fd and Fld binding associated conformational changes connected to low temperature interprotein electron transfer.

My major scientific area(s) of expertise is/are: photosynthetic reaction centers, bioinorganic chemistry.

To take my project to the next level, my ideal collaborator would have expertise in: transient optical measurements of PSI, kinetics of ET reactions in thylakoid membranes, green algal PSII/PSI preparations.

Publications supported by this project (2021-2023):

1. L. M. Utschig, C. L. Duckworth, J. Niklas, O. G. Poluektov, "Intrinsic photosystem I-ferredoxin interactions related to linear electron flow in spinach and cyanobacterial thylakoid membranes" *Photosyn. Res.* **2023**, submitted.

2. L. M. Utschig, N. J. Zaluzec, T. Malavath, N. S. Ponomarenko, D. M. Tiede, "Solar water splitting Pt-nanoparticle photosystem I thylakoid systems: Catalyst identification, location and oligomeric structure" *Biochim. Biophys*. *Acta Bioenerg.* **2023**, 1864, 148974. DOI: 10.1016/j.bbabio.2023.148974

3. L. M. Utschig, U. Brahamachari, K. L. Mulfort, J. Niklas, O. G. Poluektov, "Biohybrid photosynthetic charge accumulation detected by flavin semiquinone formation in ferredoxin-$NADP^+$ reductase" *Chem. Sci.* **2022**, 13, 6502-6511. DOI: 10.1039/d2sc01546c

4. L. M. Utschig "Light-harvesting biohybrids for enhanced solar-to-chemical conversion technologies" *Chem* **2022**, 8, 1-2. DOI: 10.1016/j.chempr.2021.12.011

5. J. Niklas, U. Brahmachari, L. M. Utschig, O. G. Poluektov "D-band EPR and ENDOR spectroscopy of 15N-labeled Photosystem I" *Appl. Magn. Reson.* **2022,** 53, 1175-1193. DOI: 10.1007/s00723-021-01438-8

6. O. G. Poluektov, L. M. Utschig "Quantum sensing of electron transfer pathways in natural photosynthesis using time-resolved high-field electron paramagnetic resonance/electron-nuclear double resonance spectroscopy" *J. Phys. Chem.* **2021**, 125, 4025-4030. DOI:10.1021/acs.jpcb.1c00946

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

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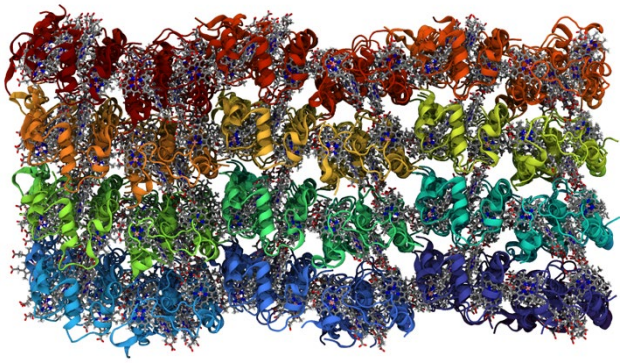
Overall research goals:

The Plant Research Laboratory brings together multiple labs as part of a larger collaborative effort to quantify and develop actionable models around light capture, downstream energy conversion, and storage in photosynthetic organisms. For organizational efficiency, the research is grouped into three themes. The first theme studies 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 mesooscale studies of carbon concentrating mechanisms or antenna complexes, or through genetic engineering of new components into chloroplasts. The third theme is to integrate photosynthetic processes into a cellular context, such as managing photorespiration and carbon fluxes throughout metabolism.

Since this is a larger collaboration, the focus her is on the contribution by Josh Vermaas. The Vermaas lab uses molecular simulations to investigates the nanoscale interactions that drive biological processes, including BES mission science around photosynthesis, bacterial microcompartments to enhance catalysis, and electron transfer. Non-BES work in the laboratory includes studying membrane-protein interaction and natural product biosynthesis through a computational lens.

Significant achievements (2023-2026):

- Measured lipid bilayer response to temperature, both with and without isoprene present. The findings highlight that isoprene likely does not directly impact membrane properties, but may instead act as a signaling molecule to regulate metabolism.
- Using Brownian dynamics simulations and an approximate packing density, we determined that about 3000 carbon dioxides are fixed for by rubisco inside a carboxysome for every one that escapes the carboxysome shell.
- Free energy perturbation calculations quantify the relative binding affinities of chlorophyll a and b to LHCII, highlighting that there is not a strong thermodynamic driving force preferring a vs. b in many binding sites.
- Developed a detailed model for how thermal motion within nanocrystals influences electron transfer through the crystal. This has recently been published in *Small*, with the following abstract:



The ability to redirect electron transport to new reactions in living systems opens possibilities to store energy, generate new products, or probe physiological processes. Recent work by Huang et al. showed that 3D crystals of small tetraheme cytochromes (STC) can transport electrons over nanoscopic to mesoscopic distances by an electron hopping mechanism, making them promising materials for nanowires. However, fluctuations at room temperature may distort the nanostructure, hindering efficient electron transport. Classical molecular dynamics simulations of these fluctuations at the nano- and mesoscopic scales allowed us to develop a graph network representation to estimate maximum electron flow that can be driven through STC wires. In longer nanowires, transient structural fluctuations at protein-protein interfaces tended to obstruct efficient electron transfer, but

these blockages are ameliorated in thicker crystals where alternative electron transfer pathways become more efficient. The model implies that more flexible protein-protein interfaces limit the required minimum diameter to carry currents commensurate with conventional electronics.

Science priorities for the next year (2023-2024):

- Small molecule permeation across carboxysome shell proteins
- Nanoscale quantification of phycobilisome interactions with PSII
- Develop the architecture for inducing phycobilisome conformation change *in silico*
- Quantify binding affinity for alternative ferredoxin isoforms to PSI

My major scientific area(s) of expertise is/are: molecular simulation and enhanced sampling techniques for free energy determination.

To take my project to the next level, my ideal collaborator would have expertise in: structure determination, in particular for the complexes involved in photosynthesis and energy transfer.

Publications supported by this project (2023-):

1. Ranepura, G.; Mao, J.; Vermaas, J.; Wang, J.; Gisriel, C. J.; Wei, R.; Ortiz-Soto, J.; Uddin, M. R.; Amin, M.; Brudvig, G. W.; Gunner, M. R. *Computing the Relative Affinity of Chlorophylls a and b to Light-Harvesting Complex II*; Preprint; Chemistry, 2023, DOI: 10.26434/chemrxiv- 2023-hsc1f.
2. Shivaiah, K.-K.; Boren, D. M.; Tequia-Herrera, A.; Vermaas, J.; Lundquist, P. K. *An Amphipathic Helix Drives Interaction of Fibrillins with Plastoglobule Lipid Droplets*; Preprint; Cell Biology, 2023, DOI: 10.1101/2023.09.28.559984.
3. Kulke, M.; Olson, D. M.; Huang, J.; Kramer, D. M.; Vermaas, J. V. Long-Range Electron Transport Rates Depend on Wire Dimensions in Cytochrome Nanowires. *Small* 2023, 2304013, DOI: 10.1002/smll.202304013.
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5. Weraduwage, S. M.; Sahu, A.; Kulke, M.; Vermaas, J. V.; Sharkey, T. D. Characterization of Promoter Elements of Isoprene-responsive Genes and the Ability of Isoprene to Bind START Domain Transcription Factors. *Plant Direct* 2023, *7*, e483, DOI: 10.1002/pld3.483.
6. Sarkar, D.; Kulke, M.; Vermaas, J. V. LongBondEliminator: A Molecular Simulation Tool to Remove Ring Penetrations in Biomolecular Simulation Systems. *Biomolecules* 2023, *13*, 107, DOI: 10.3390/biom13010107.

Assembly and Repair of the Photosystem II Reaction Center

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Overall research goals:

- We are studying the dependence of soluble proteins, pH, chloride, and specific amino acid residues on photo-assembly of the Photosystem II (PSII) Mn_4CaO_5 oxygen-evolving complex (OEC).
- We aim to identify and characterize protein-protein interactions that facilitate the assembly of PSII in biogenesis centers in cyanobacteria.
- We aim to identify and characterize proteases and translation factors that facilitate PSII turnover in *Chlamydomonas*.

Significant achievements (2019-2024):

Assembly of the PSII OEC is controlled by proton release events.
The OEC is assembled from free Mn^{2+}, Ca^{2+}, and H_2O in situ and does not require chaperones or scaffolds. Building this Mn_4CaO_5 active site requires multiple water deprotonation events to form μ-(hydr)oxo ligands. Earlier, in collaboration with Prof. Marilyn Gunner's group, we showed that the rate-limiting step of OEC assembly involves deprotonation. Chloride facilitates this process by tuning the pK_a's of side chains and waters (Vinyard, Badshah, Riggio, Kaur, Fanguy, & Gunner, *Proc. Natl. Acad. Sci* 2019).

Using electron paramagnetic resonance (EPR) spectroscopy, we have shown that formation of the first Mn^{3+} intermediate in OEC assembly involves a deprotonation event facilitated by the presence of chloride. Ca^{2+} must bind before or simultaneously with Mn^{2+} for Mn^{3+} to be accumulated (Russell and Vinyard, *Photosyn. Res.* 2022).

The extrinsic PSII subunit PsbO is well known to stabilize the OEC. D1 residue R334 interacts with PsbO through a single hydrogen bond. By studying D1-R334G and Δ*psbO* mutants in *Synechocystis* sp. PCC 6803, we have characterized the "broad" channel that connects the OEC to the lumen in two distinct conformations. The conformation present when PsbO is bound supports efficient proton release to the lumen. The conformation present with PsbO is absent supports OEC assembly (Russell and Vinyard, *Biochim, Biophys. Acta – Bioenerg.* 2024).

Chaperones facilitate PSII assembly in cyanobacterial biogenesis centers.
In *Synechocystis* 6803, early steps of PSII biogenesis occur in PratA-Defined Membranes (PDMs) at the intersection of the plasma and thylakoid membrane systems. Results from in vitro pull down study suggested that PratA interacts with chaperones including DnaK2. In Δ*pratA* mutants, PSII activity is highly attenuated, some PSII subunits are present at low levels, and less DnaK2 is membrane associated compared to wild type. Preliminary results show that fluorescently tagged DnaK2 is observed in puncta at the periphery of cells. Together, these results support a model in which PratA recruits chaperones like DnaK2 to biogenesis centers to facilitate efficient PSII assembly (Mehra, Russell, and Vinyard, in preparation).

A psbA translation factor was identified and characterized in Chlamdyomonas.
We screened a home-made mutant pool of *Chlamydomonas reinhardtii* for strains deficient in PSII activity. The causative gene in the mutant *ami6* was mapped to *CrHCF244* and confirmed with CRISPR-Cas9 knock-out mutants and complements. In plants, HCF244 is known to function in a complex with chlorophyll-binding proteins OHP1/OHP2 and is involved in PSII assembly.

Arabidopsis HCF244 partially complements *ami6*. While this study experimentally verified the functional conservation of this protein in algae and plants, we also observed differences. Unlike the case in plants, the inability to accumulate D1 in *ami6* is not accompanied with a decrease in chlorophyll, supporting the uncoupling of D1 translation and chlorophyll association in *Chlamydomonas* (Wang, Cheramie, Wang, Dassanayake, Moroney, and Vinyard, in preparation).

Extending our expertise in EPR spectroscopy to other systems.
Finally, we have collaborated with local colleagues on EPR spectroscopy measurements and analyses. Work with Prof. George Stanley (LSU Department of Chemistry) led to two significant publications on cobalt hydroformylation catalysts.

Science priorities for the next year (2023-2024):

- We have preliminary evidence that some point mutants in *Synechocystis* 6803 have differences not only in proton release but also in the Mn^{2+}, Ca^{2+}, and Cl^{-} concentration requirements for efficient OEC assembly. Our goal is to exploit these differences to trap and characterize metal cluster intermediates not previously observed.
- The potential interaction between PratA and DnaK2 will be confirmed both in vivo and in vitro. We will extend our tagging and microscopy studies to other cyanobacterial chaperones and co-chaperones.
- Our efforts to understand the D1 turnover cycle in *Chlamydomonas* have yet to uncover the specific protease(s) that makes the first cuts in D1 loops during its degradation. We are currently localizing all 12 Deg proteases in *Chlamydomonas* using fluorescent fusion proteins. Those localized to the chloroplast will be inactivated using CRISPR-Cas9 methods to determine their potential role in the PSII repair cycle. We anticipate that Deg proteases may have redundant functions and will use genetic approaches to create strains with combinations of Deg deletions.

My major scientific area(s) of expertise is/are: EPR spectroscopy, molecular biology, protein purification and analysis.

To take my project to the next level, my ideal collaborator would have expertise in: High field and/or pulsed EPR experiments, cryo-EM, high-resolution fluorescence microscopy.

Publications supported by this project over the past four years:

1. B. P. Russell, D. J. Vinyard, "Conformational changes in a Photosystem II hydrogen-bond network stabilize the oxygen-evolving complex." Biochimica et Biophysica Acta, 1865, 149020 (2024). [https://doi.org/10.1016/j.bbabio.2023.149020.]
2. D. J. Vinyard, "A low-cost and realistic noisy light system for studying photosynthesis." Photosynthesis Research, 157, 37 (2023). [https://doi.org/10.1007/s11120-023-01012-2]
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4. D. J. Vinyard, C. F. Yocum, T. M. Bricker, "Preface: Special issues on Photosystem II." Photosynthesis Research, 152, 87 (2022). [https://doi.org/10.1007/s11120-022-00930-x]
5. B. P. Russell and D. J. Vinyard, "Chloride facilitates Mn(III) formation during photo-assembly of the Photosystem II oxygen-evolving complex." 152, 283 (2022). [https://doi.org/10.1007/s11120-021-00886-4]
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The green alga *Auxenochlorella protothecoides* as a chassis to study molecular players of non-photochemical quenching

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Plants and algae must constantly balance the light energy harvested for photosynthesis and the energy dissipated to avoid photooxidative damage. Non-photochemical quenching (NPQ) is a mechanism by which excess energy is dissipated as heat. It has been found to involve several components that function at different timescales for its induction and relaxation. Excitation energy-dependent quenching (qE) is the component of NPQ occurring with the fastest kinetics, and it involves two distinct protein families, LHCSR/LHCX and PSBS, as major contributors. Green algae like *Chlamydomonas reinhardtii* possess genes encoding both LHCSR and PSBS, but qE functions mostly through LHCSR. In contrast, most plants have lost LHCSRs and rely solely on PSBS to carry out qE. In contrast, the moss *Physcomitrium patens* uses both proteins for qE, leading to the general hypothesis that the function of PSBS as a major player in qE arose during the evolution of terrestrial plants from algae. *Auxenochlorella protothecoides* is a highly oleaginous Trebouxiophyceaen green alga that can grow photoautotrophically and heterotrophically. Strikingly, its genome contains a gene that is PSBS-like but lacks LHCSR, which raised the question whether *A. protothecoides* is an exceptional green alga that uses PSBS for qE as in plants. To test this, we generated knock-out mutants of PSBS and found that it has only a modest, if any, role in NPQ. The NPQ observed in wild-type cells is low in magnitude and displays slow kinetics resembling a zeaxanthin-dependent quenching (qZ). We are now leveraging homologous recombination and synthetic biology in *A. protothecoides* to test hypotheses to understand the molecular mechanisms of different NPQ components, such as (1) is the introduction of LHCSR sufficient to confer qE?, (2) is the introduction of PSBS from plants sufficient to confer qE?, (3) which of the two types of violaxanthin de-epoxidase (plant-type and CVDE) is involved in qZ?, and (4) what other molecular players might be involved?

Quantum Light Studies of Photosynthetic Light Harvesting at the Single-Photon Level

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Overall research goals:

Our work in the DOE Solar Photosynthesis program focuses on study of the microscopic mechanism for capture and transduction of light energy by natural photosynthetic light harvesting systems at the single- photon level relevant to the weak sources of light in natural conditions. Our focus on study at the single- photon level differentiates our approach from that of the majority of studies of photosynthetic light harvesting, which primarily study excitonic dynamics of large ensembles that are initiated by excitation with laser pulses containing many photons (typically ~10^{10}). Our long-term goals are to gain a full microscopic understanding of the nature of photosynthesis under the optimal in vivo conditions of weak illumination by sunlight where unusually high values of quantum efficiency (larger than 95%) are encountered.

To enable studies of photosynthesis induced by individual single photons, we have developed new experimental probes and new theoretical methods for analysis of experiments conditioned on interaction with and emission of single photons. We employ the experimental and theoretical methods in a synergistic manner to probe the response of photosynthetic systems to incident single photons and to learn how the energy of individual photons is converted to the electronic energy that initiates the subsequent 'dark' chemical reactions of photosynthesis.

Significant achievements (2021-2023)

1. We completed our photon counting quantum light spectroscopy (PCQLS) experiment to study the absorption of single photons by photosynthetic systems under controlled conditions of incident single photons, removing a detection loophole by making conditional measurements of fluorescent photons. We also measured the second order coherence functions of both herald and fluorescent photons to confirm that both the incident and fluorescent light is composed of single photons. The experiment was carried out on the light-harvesting 2 (LH2) complex from the purple bacterium *Rhodobacter sphaeroides* under ambient conditions. This work, published in Nature in June 2023 (see below), provides the first experimental demonstration of the cycle of absorption and emission of single photons by a complex biomolecular photosynthetic system, validating the notion of quantum efficiency that is usually measured with bulk kinetics at the microscopic single photon level and revealing that one photon with a single absorption event can initiate photosynthesis. It also demonstrates the controlled generation of a single excited state in such a complex system and showcases the capabilities and potential of the PCQLS technique that we have developed under this DOE support.

2. We reconfigured the PCQLS to enable different measurement conditions. This enabled us to carry out the experiment in 1. with an incident thermal state source to compare the effect of different incident photon statistics (single photon vs thermal) on the lifetime, efficiency, and photon statistics of fluorescence. This provides a bridge between in vitro and in vivo studies of photosynthesis and is also relevant to discussions of the differences between sunlight, a weak thermal light source, and the laser light used in ultrafast spectroscopic studies of photosynthesis. The fluorescence lifetime and quantum efficiency are found to be the same and independent of photon statistics, while the fluorescence photon statistics are different and reflect the statistics of the incident light in each case. This implies that averaged dynamical properties of photosynthesis are not affected by photon statistics of incident light under weak illumination. This work is now being prepared for publication. We have also reconfigured

the PCQLS to measure the second order coherence of fluorescence with polarization selectivity and frequency resolution, which has been predicted to provide a probe of excitonic coherence.

3. On the theoretical side, we completed and published (see below) our conditioned quantum trajectory approach to study of absorption of single photons by photosynthetic systems including exciton-phonon coupling, using this to estimate the quantum efficiency for PSII under single-photon excitation and arriving at good agreement with the value obtained from bulk in vivo measurements. We also developed a new conditioned quantum trajectory approach allowing theoretical simulation of the excitonic dynamics with more realistic non-Markovian coupling to vibrational degrees of freedom, under monitoring of fluorescent photon emission.

4. Following estimates of feasibility for pump-probe experiments with single photon pump pulses and single photon probe pulses that showed such ideal quantum light experiments are not realistic, we have now focused on experiments with classical (laser) pump pulses and either single photon probe pulses or single photon probes from an entangled pair. Here we have derived an unexpected and key new result demonstrating that for a broad class of quantum light spectroscopy (QLS) experiments using n = 0,1,2,$\cdots$ classical light pulses and an entangled photon pair (a biphoton state) where one photon acts as a reference without interacting with the matter sample, identical signals can be obtained by replacing the biphotons either by single photons, or – more importantly – by replacing the biphotons with classical-like coherent states of light (laser light). The relevant classical-like coherent states are defined explicitly in terms of the parameters of the biphoton states and can be readily generated with pulse shapers such as Dazzlers. Thus two-photon entanglement offers no true quantum advantage in these QLS experiments. This analysis shows that understanding the equivalence between entangled biphoton probes and carefully designed classical-like coherent state probes leads to quantum-inspired classical experiments that are significantly easier to realize and provides insights for future design of QLS experiments that could provide a true quantum advantage. This work was also published (see below).

Science priorities for the next year (2023-2024):

- Implement frequency resolved and polarization selected $g^{(2)}(0)$ measurements for coherence witness
- Emulation of classical pump – biphoton probe spectroscopy by specially designed classical coherent light pulses, i.e., pump quantum-inspired probe (PQIP) spectroscopy
- Identify QLS experiments that cannot be emulated by quantum-inspired classical pulses and that show a true quantum advantage for probing dynamics of photosynthetic systems
- Developing a more realistic treatment of phonons within conditional quantum trajectories and analyzing the role of phonon transitions

My major scientific area(s) of expertise is/are: Quantum Physics (qu. dynamics, qu. optics, open qu. systems, qu. information, qu. computation, superfluids), Quantum Biology (excitonic energy transfer, photosynthesis).

To take my project to the next level, my ideal collaborator would have expertise in: quantum optics, molecular spectroscopy of photosynthetic systems, broad and in-depth knowledge of photosynthetic systems and their organization and all aspects of their functioning in vivo.

Publications supported by this project :

1. L. Ko, R. L. Cook, K. B. Whaley, "Dynamics of photosynthetic light harvesting systems interacting with N-photon Fock states", J. Chem. Phys. 156, 244108 (2022).
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Signal Transduction Pathways of Chloroplast Quality Control

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Overall research goals: The goal of this project is to understand the signaling mechanisms behind chloroplast quality control pathways that initiate chloroplast repair or degradation in response to photo-oxidative stress. We hypothesize reactive oxygen species (ROS) that are produced naturally during photosynthesis have the ability to trigger these pathways, allowing individual chloroplasts the ability to control their own fate under dynamic conditions. Such signaling may allow cells to maintain healthy populations of chloroplasts performing efficient photosynthesis. While chloroplast disassembly is a process that occurs naturally in a wide range of plant species, the mechanisms regulating and controlling these processes are not well understood.

To identify and characterize genes and proteins in chloroplast quality control pathways, we have used the *plastid ferrochelatase two* (*fc2*) mutant of the model plant *Arabidopsis thaliana*. In this mutant, accumulation of the ROS singlet oxygen ($^1O_2$) in chloroplasts can be induced non-invasively by growing plants in diurnal light cycles, triggering chloroplast degradation and eventually cell death. A forward genetics screen identified suppressors of chloroplast degradation in *fc2* mutants demonstrating this degradation is a genetically controlled response to chloroplast $^1O_2$. Two key processes important for initiating and propagating the signal were also identified through this screen. First, we demonstrated chloroplast gene expression is necessary to initiate signaling, suggesting a product of the chloroplast genome is a signaling factor. Second, a suppressor mutation affecting a cytoplasmic E3 ubiquitin ligase has demonstrated the ubiquitination of chloroplast proteins is an important step in allowing the cell to identify damaged chloroplasts. Therefore, we hypothesize chloroplast-encoded factors allow $^1O_2$ to "mark" specific chloroplasts for repair or degradation by ubiquitinating chloroplast proteins. Such a signaling system may allow individual chloroplasts to control their own maintenance in stressful and dynamic environments. Here we propose to test and expand on these models of chloroplast quality control. The proposed studies will integrate genetic, molecular, and biochemical approaches in Arabidopsis in an effort to: (1) Understand the mechanism(s) by which $^1O_2$ triggers chloroplast degradation; (2) Explore the roles of ubiquitination in chloroplast quality control; (3) Determine the role of chloroplast quality control under natural light stress.

Significant achievements (2021-2023):

- Through mutational mapping, we identified three new genes involved in chloroplast quality control pathways. These genes encode proteins necessary for efficient plastid gene expression, allowing us to hypothesize that the plastid genome encodes an important factor involved in such chloroplast stress signaling.
- We finished a large forward genetic screen for dominant gain-of-function suppressors of chloroplast quality control pathways. We have identified the causative gene in three of these mutants and have begun their initial characterization.

- We demonstrated that singlet oxygen-induced degradation of chloroplasts is independent of macroautophagy. We hypothesize that a fission-type micoautophagy pathway may be utilized instead. Such degradation may affect multiple chloroplasts within a single cell.
- By using a reverse genetics approach, we have demonstrated that multiple singlet oxygen signaling pathways are utilized by stressed chloroplasts. We hypothesize that the singlet oxygen signal in *fc2* mutants is also initiated by natural excess light stress.

Science priorities for the next year (2023-2024):

- Continue the characterization of new dominant gain-of-function mutations that block chloroplast quality control pathways. Such work will identify new signaling factors.
- Test the hypothesis that expression of plastid-encoded factors is involved in chloroplast stress signaling. We will also aim to identify the genes encoding these factors.
- Continue investigating the role of chloroplast ubiquitination on chloroplast stress signaling and chloroplast turnover.
- Test the roles of chloroplast quality control pathways under natural excess light stress.

My major scientific area(s) of expertise is/are: Genetics, Cell Biology, Molecular Biology, Plant physiology.

To take my project to the next level, my ideal collaborator would have expertise in: Optical Tweezers, Cryogenic electron microscopy.

Publications supported by this project: 2018-2023

1. M. D. Lemke, Jesse D. Woodson. "A genetic screen for dominant chloroplast reactive oxygen species signaling mutants reveals life stage-specific singlet oxygen signaling networks" *bioRxiv*. (2023) https://www.biorxiv.org/content/10.1101/2023.10.26.564295v1.abstract
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8. M. D. Lemke, K. E. Fisher, M. A. Kozlowska, D. W. Tano, Jesse D. Woodson. "The core autophagy machinery is not required for chloroplast singlet oxygen-mediated cell death in the Arabidopsis thaliana plastid ferrochelatase two mutant" *BMC Plant Biology*. 21:342. (2021) https://doi.org/10.1186/s12870-021-03119-x
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10. K. Alamdari, K. E. Fisher, A. B. Sinson, J. Chory, and Jesse D. Woodson. "Roles for the chloroplast-localized PPR Protein 30 and the 'Mitochondrial' Transcription Termination Factor 9 in chloroplast quality control" *The Plant Journal*. 104:735-51. (2020) https://doi.org/10.1111/tpj.14963
11. Y. Kikuchi, S. Nakamura, Jesse D. Woodson, H. Ishida, Q. Ling, J. Hidema, R. P. Jarvis, S. Hagihara, M. Izumi. "Chloroplast Autophagy and Ubiquitination Combine to Manage Oxidative Damage and Starvation Responses" *Plant Physiology*. 183:1531-44. (2020) https://doi.org/10.1104/pp.20.00237
12. Jesse D. Woodson. "Chloroplast stress signals: regulation of cellular degradation and chloroplast turnover". *Current Opinion in Plant Biology*. 52:30–37. (2019) https://doi.org/10.1016/j.pbi.2019.06.005

Electronic Structure of the Mn Cluster in Photosystem II Using an XFEL

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Overall research goals:

The objective of this proposal is to investigate the molecular and electronic structure and dynamics of PS II and the OEC, during the light-driven process, and understand the mechanism of solar energy conversion, by which water is oxidized to dioxygen. We approach this objective by developing and utilizing methodologies of X-ray spectroscopy and crystallography. The specific questions are as follows: What is the chemical and structural sequence of events towards the O-O bond formation and the recovery of the catalytic center, and is the reaction initiated by an oxo-oxyl coupling, or by a nucleophilic attack involving a high-valent Mn(V), or by some other mechanism? How does the electronic structure at the OEC change and to what extent charge is distributed between metals and ligands? Can we spatially resolve electronic changes of each Mn, and what are the stepwise redox changes of Mn and its kinetics during S_3 to S_0? How does the environment (protein and hydrogen bonding network) accommodate the changing energy landscape such as what are the substrate intake and proton release pathways and how mobile quinones are exchanged during the cycle? And finally, how the light-driven assembly of the OEC occurs, and in what sequence, and what is the role of Ca during the OEC assembly?

Significant achievements (Current Funding, 2021-2023):

We made progress in the data collection method for X-ray absorption spectroscopy (XAS) from PS II in the S_1 state during the most recent XFEL beamtimes. We built a HERFD-XAS setup, with our drop-on-tape (DOT) sample delivery method (**Fig. 1a** and **b**). Typically, time-dependent XAS data collection at XFELs is carried out by directly collecting fluorescence photons (Mn Kα) from the sample on a pixel array detector. In HERFD-XAS, a Bragg reflection from an analyzer crystal is used to focus the metal Kα signal into the detector (Fig. 1a). This method allows discriminating the signal from the metal of interest from the contribution of elastic photons on the XAS detector. In our experiment, we used a spherical Lithium Niobium oxide cut crystal with Rowland geometry (200 mm bent radius and Bragg angle is ~79.4 deg for the Mn Kα energy) (Fig. 1b).

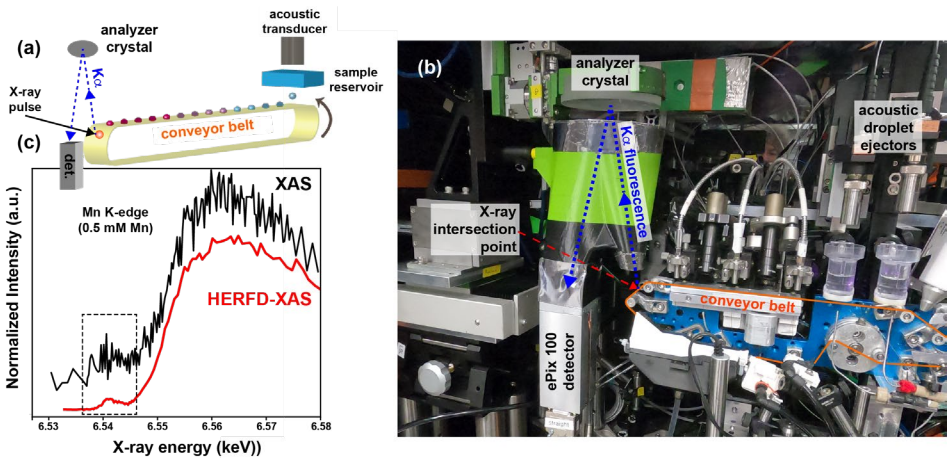

Figure 1: HERFD (High Energy Resolved Fluorescence Detected)-XAS data collection with Drop-on-Tape method. (a, b): HERFD-XAS data collection scheme was incorporated into the Drop-on-Tape (DOT) sample delivery method. **(c):** Mn XAS collected from PS II S_1 state. The black line shows the XAS data collected with the traditional approach (2hr), and the red line shows HERFD-XAS (15 min). The significant improvement is clear in the data quality of the HERFD-XAS, with even the 1s to 3d pre-edge transition visible at ~6.538-6.545 keV (dotted square).

The improvement in data quality and collection efficiency of the HERFD-XAS is significant (**Fig. 1c**), and it clearly demonstrates the importance of reducing the background elastic signal. Within ~ 15 min of data collection time, even the signal in the pre-edge region (Fig. 1c, dotted box) becomes visible. The result is highly encouraging, and we plan to move on to time-resolved data between the S_3 and S_0 states.

Science priorities for the next year (2023-2024):

- Room temperature EXAFS and XANES methods that we developed for PS II at XFELs, in addition to the Mn $K\beta_{1,3}$ and the ligand to valence $K\beta_{2,5}$ emission studies that are ongoing.
- Mn L-edge spectra at RT for capturing the electronic structural changes during the $S_3 \rightarrow [S_4] \rightarrow S_0$ transition of the Mn complex.
- Continue our efforts with developing non-linear X-ray spectroscopy methods for dilute transition metal solution studies.

Our major scientific area(s) of expertise is/are: XFELs and the use of spectroscopy and crystallography for time-resolved studies.

To take our project to the next level, my ideal collaborator would have expertise in: simulating the spectra obtained using the XFEL data using advanced methods in quantum chemistry, time-dependent density functional theory, and molecular dynamics.

Publications supported by this project: Past 3 Years, 2021-2023:

1. Bhowmick, A., Hussein, R., Bogacz, I., Simon, P. S., Kern, J., Yano, J., Yachandra, V. K., et al. (2023) Structural evidence for intermediates during O_2 formation in Photosystem II, *Nature,* **617**, 629-636. doi.org/10.1038/s41586-023-06038-z.
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Energy Conversion in Photosynthesis: Capturing the Sequence of Events During the $S_3 \rightarrow S_0$ Transition in Photosystem II

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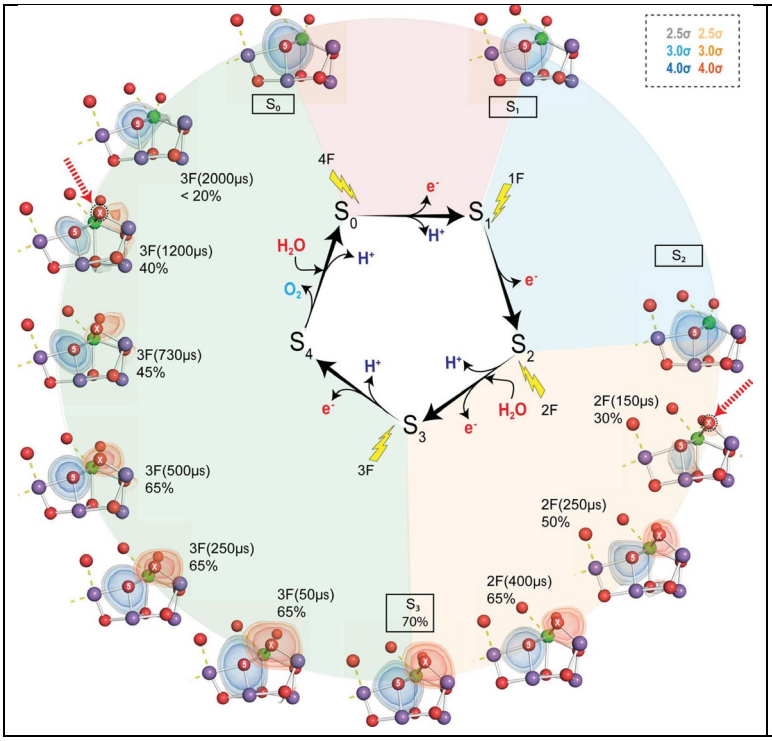
Overall research goals:

The objective of this proposal is to investigate the molecular and electronic structure and dynamics of PS II and the OEC, during the light-driven process, and understand the mechanism of solar energy conversion, by which water is oxidized to dioxygen. We approach this objective by developing and utilizing methodologies of X-ray spectroscopy and crystallography. The specific questions are as follows: What is the chemical and structural sequence of events towards the O-O bond formation and the recovery of the catalytic center, and what is the reaction mechanism? How does the electronic structure at the OEC change and to what extent charge is distributed between metals and ligands? Can we spatially resolve electronic changes of each Mn, and what are the stepwise redox changes of Mn and its kinetics during S_3 to S_0? How does the environment (protein and hydrogen bonding network) accommodate the changing energy landscape such as what are the substrate intake and proton release pathways and how mobile quinones are exchanged during the cycle? And finally, how the light-driven assembly of the OEC occurs, and in what sequence, and what is the role of Ca during the OEC assembly?

Significant achievements (Current Funding, 2021-2023):

- We have collected the room temperature crystal structures and $K\beta_{1,3}$ spectra from several timepoints between the S_3 and S_0 states. The O_X density which appeared during the S_2 to S_3 transition disappears during the S_3 to S_0 transition. There are some changes in the O5 density during this transition and the Mn1-Mn4 distance contracts at 2000 μs, returning to 4.97 Å, similar to the S_0 state. There are several changes in the structures of residues around the complex, and the proton gate identified during the S_2 to S_3 transition, shows changes at two different times for the S_3 to S_0 transition, indicating the release of the two protons during this step. The overall changes seem to indicate the presence of an intermediate/transient state during this transition.



Changes in the electron density at the OEC during the Kok cycle. The omit map of the oxygen atom O_X, which is inserted during the $S_2 \rightarrow S_3$ transition as a bridging oxygen between Mn1 and Ca is shown in orange. For reference, omit maps constructed by separately omitting the O5 atom are overlaid in blue at the same density levels. The density of O_X increases gradually in the $S_2 \rightarrow S_3$ transition and starts to decrease around 250-500 μs in the $S_3 \rightarrow S_0$ transition with the density below noise level by 2000 μs. The red arrows show the first and last timepoint where we observe significant Ox density. The populations shown are modelled O_X occupancies (except at 3F(2000 μs)) in the primary component (see text). Note that because we show the primary component here, the O5 and OEC population is also changing. Manganese (Mn) atoms are shown in purple, calcium (Ca) in green and oxygen atoms in red spheres. Color legend for the various levels of the omit maps shown are provided in the top-right box.

- We have examined the structures of waters and identified the proton and water channels, and crystalline MD calculations have provided insight into the directionality and structure of the H-bonds.

Science priorities for the next year (2023-2024):

- We will collect simultaneous XRD/XES at a resolution of ~1.9 Å and Mn Kβ XES to follow the steps between the S_3 to S_0 transition, to study Mn oxidation state changes and the O-O bond formation chemistry.
- We have also started using the spatially resolved anomalous diffraction (SPREAD) technique to follow the oxidation state of Mn atoms from the diffraction data.
- We started studies on Sr-PS II, and flash-induced photo-assembly of the Mn cluster.

Our major scientific area(s) of expertise is/are: XFELs and the use of spectroscopy and crystallography for time-resolved studies.

To take our project to the next level, my ideal collaborator would have expertise in: computational biology who can use AI/ML to parse the enormous amount of XFEL data collected during the Kok cycle to unravel the time-evolution of a multi-component system, with each component evolving in time at different rates.

Publications supported by this project: Past 3 Years, 2021-2023:

1. Bhowmick, A., Hussein, R., Bogacz, I., Simon, P. S., Kern, J., Yano, J., Yachandra, V. K., et al. (2023) Structural evidence for intermediates during O_2 formation in Photosystem II, *Nature,* **617**, 629-636. doi.org/10.1038/s41586-023-06038-z.
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3. Bogacz, I., Makita, H., Simon, P. S., Zhang, M., Doyle, M. D., Yachandra, V. K., Kern, J., Yano, J. (2023) Room temperature X-ray absorption spectroscopy of metalloenzymes with drop-on-demand sample delivery at XFELs, *Pure Appl. Chem.* In Press. doi.org/10.1515/pac-2023-0213.
4. Doyle, M. D., Bhowmick, A., Yachandra, V. K., Kern, J. F., Yano, J., and Wall, M. E. (2023) Water Networks in Photosystem II Using Crystalline Molecular Dynamics Simulations and Room-Temperature XFEL Serial Crystallography, *J. Am. Chem. Soc.* **145**, 14621-14635. doi.org/10.1021/jacs.3c01412.
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water channels on the oxidizing side of photosystem II and its relevance to function, *Photosynth. Res.*, In Press.

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7. Simon, P. S., Makita, H., Bogacz, I., Kern, J. F., Yachandra, V. K., Yano, J., et al. (2023) Capturing the sequence of events during the water oxidation reaction in photosynthesis using XFELs, *FEBS Lett*., **597**, 30-37, doi.org/10.1002/1873-3468.14527.
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9. Fransson, T., Yachandra, V. K., Yano, J., Kern, J., Bergmann, U., et al. (2021) Effects of X-ray free-electron laser pulse intensity on the Mn $K\beta_{1,3}$ x-ray emission spectrum in photosystem II - A case study for metalloprotein crystals and solutions. *Struct. Dynamics* **8**, 064302, doi.org/10.1038/s41598-021-00236-3.
10. Hussein, R., Ibrahim, M., Bhowmick, A., Simon, P. S., Kern, J., Yachandra, V. K., Yano, J., et al. (2021) Structural dynamics in the water and proton channels of photosystem II during the S_2 to S_3 transition. *Nature Commun.* **12**, 6531, doi.org/10.1038/s41467-021-26781-z.
11. Butryn, A., Simon, P.S., Yachandra, V.K., Yano, J.,, Kern, J., Orville, A.M., et al. (2021) An on-demand, drop-on-drop method for studying enzyme catalysis by serial 1 crystallography, *Nature Comm*. **12***,* 4461, https://doi.org/10.1038/s41467-021-24757-7.
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13. Bergmann, U., Kern, J., Schoenlein, R. W., Wernet, P., Yachandra, V. K., Yano, J. (2021) Using X-ray free-electron lasers for spectroscopy of molecular catalysts and metalloenzymes, *Nature Reviews Physics*, **3**, 264-282, doi.org/10.1038/s42254-021-00289-3.

Collaborative Project: Dynamics and consequences of PSI Supercomplexes

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Overall research goals:

[The overall research goal of this project is to understand the diversity of PSI supercomplexes (PSI-SC), their contribution to PSI-driven cyclic electron flow (CEF), and the roles of CEF in stress acclimation over short- and long-term time scales. Our major objectives are: **(O1)** Determine the structure and localization of the *C priscuii* (formerly *Chlamydomonas* sp. UWO241) PSI-SC using single particle and in situ imaging; **(O2)** Determine the contribution of PSI-SC variability and CEF to supporting growth and stress response during long-term stress; **(O3)** Consider the contributions of high CEF to supporting growth and stress response during long-term stress.

Significant achievements ([2022-2023]):

1. We identified conditions where the model green alga *Chlamydomonas reinhardtii* (*C. reinhardtii*) has sustained CEF, which was with the treatment of moderate (35°C) and acute (40°C) high temperatures (Fig. 1). We cultivated *C. reinhardtii* under well-controlled conditions in photobioreactors at 25°C first and then switched the culture temperature to 35°C or 40°C for 24-h, followed by 24-h recovery at 25°C. The heat treatments we used are physiologically relevant high temperatures that outdoor algal ponds often experience. In collaboration with the Kramer lab, we used P700 measurement to monitor CEF activities in algal cultures before, during, and after high temperature treatments. Our data showed that both 35°C and 40°C induced sustained CEF but with different dynamics and reversibility. CEF was induced slowly at 35°C and reversible after returning to 25°C. In contrast, CEF was induced fast at 40°C and irreversible after returning to 25°C for 24-h. Combing our data of photosynthesis, cell physiology, and omics analyses, we hypothesized that the slowly-induced and reversible CEF with the 35°C treatment was for increased growth and metabolism, while the fast-induced and irreversible CEF with the 40°C treatment may be mainly for photoprotection. Additionally, our transmission electron microscopy images showed that the 35°C treatment affected thylakoid structures moderately, however, the 40°C treatment resulted in destacked and disorganized thylakoids. We further hypothesized that different dynamics and functions of CEF during 35°C or 40°C treatments were associated with variations in the assembly, components, abundance, location of CEF-supporting PSI-SC and/or surrounding thylakoid structures for the need of energy or photoprotection in *C. reinhardtii*. Our paper about this work was published in Communications Biology (2022).

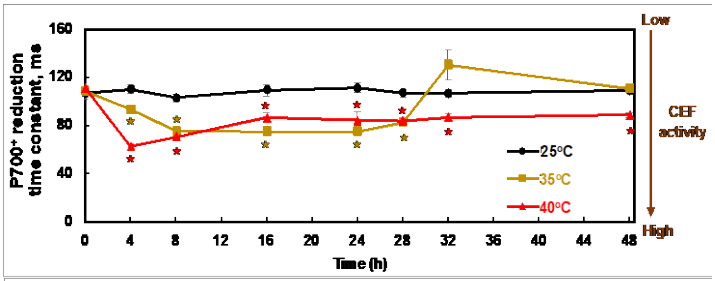

Fig. 1. Moderate (35°C) and acute (40°C) high temperatures induced CEF with different dynamics in *C. reinhardtii*. Algal cultivation and heat treatments were conducted in tightly controlled photobioreactors. P700+ reduction to measure CEF with 10 μM DCMU. The red shaded area depicts the duration of the high temperatures. *, significant differences as compared to 25°C at the same time point. Colors of * match the heat conditions. Mean ± SE, n = 5. Figure from (Zhang, Mattoon et al., 2022).

2. In collaboration with the lab of Dr. Rachael Morgan-Kiss, we have been working on to isolate PSI-SC from *C. reinhardtii* with moderate and acute high temperature treatments as mentioned above. We are working on to optimize the PSI-SC isolation protocol with different detergents, alpha- or beta-DDM (n-Dodecyl-Maltoside). We were able to get a strong PSI-SC band in sucrose density gradient using alpha-DDM in *C. reinhardtii* cells treated with 8-h heat at 35°C. We will continue to optimize the isolation protocol to improve the isolation yield and PSI-SC stability. We next will use the isolated PSI-SC for western and proteomic analysis to identify its components.
3. Cryo-volume electron microscopy (cryo-vEM) and cryo-electron tomography (cryo-ET) are emerging molecular imaging techniques for visualization of the 3D spatial organization of cellular structures, which are

embedded in vitreous ice. Cryogenic imaging methods preserve cells in their near-native environment without traditional chemical fixation, heavy metal staining, or resin embedding. In July 2023, our Donald Danforth Plant Science Center Advanced Bioimaging Laboratory (ABL) installed a Thermo Scientific Cryo Helios 5 Hydra cryo-plasma focused ion beam (FIB) equipped with 3D cryo-vEM, including integrated cryo-fluorescence microscopy and on-grid/lift-out lamella capabilities, led by our ABL director, Dr. Kirk Czymmek. The Helios 5 Hydra has superior resolution and unmatched contrast for visualizing unstained vitrified cells along with improved plasma-FIB sample milling. Dr. Czymmek formerly led a ZEISS team developing cryo light microscopy, cryo-vEM, and cryo-ET workflows with customers in a variety of organisms, including algae, and already has worked with collaborator Dr. Wah Chiu at the Stanford University on cryo-ET in whole heat-shocked yeast cells. Cryo-vEM data of *C. reinhardtii* cells grown under the control temperature were obtained during our demonstration of the Cryo Helios 5 Hydra, approaching 5-nm resolution. We will use cryo-vEM to investigate the 3D status of the thylakoid membranes and even entire *C. reinhardtii* cells in native status in situ with control and high temperature treatments. Select, frozen, thin lamellae will be sent to the Chiu Lab for cryo-ET data acquisition/analysis and in situ localization of PSI-SC.

Science priorities for the next year (2023-2024):

- Isolate stable PSI-SC from *C. reinhardtii* cells with heat treatments and identify PSI-SC components using western and proteomic analysis (in collaboration with Drs. Rachael Morgan-Kiss and Xin Wang);
- Employ 3D cryo-vEM and cryo-ET to reveal the changes of thylakoid structures, the distribution and abundance of PSI-SC in *C. reinhardtii* cells with heat treatments (in collaboration with Drs. Kirk Czymmek, Wah Chiu, Jianlin Cheng, and Ben Eagle);
- Use isolated PSI-SC from *C. reinhardtii* cells with heat treatments for single particle cryo electron microscopy (cryo-EM) analysis to reveal its structure (in collaboration with Drs. Wah Chiu, Kirk Czymmek, Jianlin Cheng)

My major scientific area(s) of expertise is/are: algal heat response, photosynthesis, cultivation, high-throughput genotyping and phenotyping.

To take my project to the next level, my ideal collaborator would have expertise in: photosynthesis, proteomics, cryo-vEM, cryo-ET, cryo-EM data analysis, and modeling of thylakoid dynamics.

Publications supported by this project [Enter Publications Supported by This Grant/FWP Over The Past 3-5 Years] : (Zhang Lab mentees with underlines)

(1) Ningning Zhang[#], Erin M. Mattoon[#], Will McHargue, Benedikt Venn, David Zimmer, Kresti Pecani, Jooyeon Jeong, Cheyenne M. Anderson, Chen Chen, Jeffrey C. Berry, Ming Xia, Shin-Cheng Tzeng, Eric Becker, Leila Pazouki, Bradley Evans, Fred Cross, Jianlin Cheng, Kirk J. Czymmek, Michael Schroda, Timo Mühlhaus, **Ru Zhang*** (2022) Systems-wide analysis revealed shared and unique responses to moderate and acute high temperatures in green alga *Chlamydomonas reinhardtii*. ***Communications Biology***, 5: 460. https://www.nature.com/articles/s42003-022-03359-z

(2) Ningning Zhang[#], Leila Pazouki[#], Huong Nguyen[#], Sigrid Jacobshagen, Brae Bigge, Ming Xia, Erin M. Mattoon, Anastasiya Klebanovych, Maria Sorkin, Dmitri A. Nusinow, Prachee Avasthi, Kirk J. Czymmek, **Ru Zhang*** (2022) Comparative phenotyping of two commonly used *Chlamydomonas reinhardtii* background strains: CC1690 (21gr) and CC5325 (the CLiP library background). ***Plants***, 11(5), 585. https://pubmed.ncbi.nlm.nih.gov/35270055/

(3) Isha Kalra, Xin Wang, **Ru Zhang**, Rachael Morgan-Kiss. (2023) High salt-induced PSI-supercomplex is associated with high CEF and attenuation of state-transitions. ***Photosynthesis Research***, 157: 65-84. https://pubmed.ncbi.nlm.nih.gov/37347385/

(4) Isha Kalra, Xin Wang, Marina Cvetkovska, Jooyeon Jeong, William McHargue, **Ru Zhang**, Norman Hüner, Joshua S Yuan, Rachael Morgan-Kiss (2020) *Chlamydomonas* sp. UWO241 exhibits constitutively high cyclic electron flow and rewired metabolism under high salinity. Plant Physiology. 183: 588-601. https://pubmed.ncbi.nlm.nih.gov/32229607/

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