Meeting Book for the Photosynthetic Systems Principal Investigators Meeting

Held Virtually by Video Conference, November 16-18, 2021

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 efficiencies. 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 with Linda Severson and Tia Moua 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
B. Gail Mclean, Team Lead, Photochemistry and Biochemistry Team, DOE BES

Acknowledgement and Disclaimer

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2021 Photosynthetic Systems Principal Investigators Meeting

AGENDA

Tuesday, November 16
(All times Eastern)

12 noon – 12:10PM	Welcome **Steve Herbert,** Program Manager, Photosynthetic Systems program
	Section I: Light Harvesting and Charge Separation *Moderators:* ***Gabriela Schlau-Cohen****, MIT* ***Rick Debus****, UC, Riverside*
12:10 – 12:40PM	Single Photon Studies of Photosynthetic Light Harvesting **Birgitta Whaley,** University of California, Berkeley
12:40 – 1:10PM	Energy Transfer and Radiationless Decay in Light-Harvesting Proteins **Warren Beck**, Michigan State University
1:10 – 1:40PM	Elucidating the Mechanism of OCP-mediated Antenna Quenching **Cheryl Kerfeld**, Michigan State University
1:40 – 2:10PM	Photoprotective Structural Change Led by Intramolecular vibrational switching **Andrea Markelz**, University of Buffalo
2:10 – 2:40PM	Vibronic Coupling Steers Energy Transfer within FMO in Response to Oxidative Conditions Driving Changes in Excited State Quantum Beating Signatures **Greg Engel**, University of Chicago
2:40 – 3:10PM	*Break*
3:10 – 4:00PM	Regulation of Photosynthesis **Graham Fleming**, UC, Berkeley & Lawrence Berkeley National Lab **Kris Niyogi**, UC, Berkeley & Lawrence Berkeley National Lab
4:00 – 4:30PM	Multidimensional Spectroscopies for Probing Coherence and Charge Separation in Photosynthetic Reaction Centers **Jennifer Ogilvie**, University of Michigan
4:30 – 5:00PM	Energy Conversion in Photosynthesis: Untangling the Sequence of Events During the $S_2 \rightarrow S_3$ Transition in Photosystem II **Vittal Yachandra**, Lawrence Berkeley National Lab

Tuesday, November 16, cont'd
(All times Eastern)

5:00 – 5:30PM	Time Resolved Infrared Difference Spectroscopy for the Study of the Cofactors Involved in Electron Transfer in Photosystem I **Gary Hastings**, Georgia State University
5:30 – 6:30PM	*Break*
6:30 – 9:00PM	*Poster Session 1* Presenters and titles for Poster Session 1 are listed after the agenda in the meeting book.

Wednesday, November 17
(All times Eastern)

12 noon – 12:10PM	Announcements **Steve Herbert,** Program Manager, Photosynthetic Systems
	Section II: Electron Transport, H_2, Fe, and CO_2 *Moderators:* ***Kate Brown,*** *National Renewable Energy Lab* ***Rebecca Roston****, University of Nebraska, Lincoln*
12:10 – 12:40PM	Biohybrid and Selective Deuteration Approaches for Investigating Photosynthetic Electron Transfer and Charge Accumulation **Lisa Utschig**, Argonne National Lab
12:40 – 1:10PM	A PSI-[FeFe] Hydrogenase Chimera: Activation for *In Vitro* Mechanistic Studies **David Britt**, University of California, Davis
1:10 – 1:40PM	Photosynthetic Energy Transduction Core Program: Dissecting the Electron Transfer Mechanism of Photosynthetic Flavodiirons **David Mulder**, National Renewable Energy Lab
1:40 – 2:10PM	The Dynamics of Cyclic Electron Flow around PS I under Moderate and Acute High Temps in the Green Alga *Chlamydomonas reinhardtii* **Ru Zhang**, Donald Danforth Center, St. Louis
2:10 – 2:40PM	Dissecting Mechanisms of Reductant Flow from the Chloroplast **Matt Posewitz,** Colorado School of Mines
2:40 – 3:10PM	*Break*

Wednesday, November 17, cont'd
(All times Eastern)

3:10 – 3:40PM	Iron Economy in Photosynthesis **Sabeeha Merchant**, University of California, Berkeley
3:40 – 4:10PM	Structure and Function of the CO_2 Uptake NDH-1 Complexes in Cyanobacteria **Rob Burnap**, Oklahoma State University Photosynthetic Reduction of
4:10 – 4:40PM	Carbon Dioxide: Kinetic Trade-offs for Rubisco and Phospho*enol*pyruvate Carboxylase **Asaph Cousins**, Washington State University Characterizing Rubisco by
4:40 – 5:20PM	Phylogeny-Informed Mutagenesis **Maureen Hanson**, Cornell University **Myat Lin**, Cornell University
5:20 – 5:50PM	Exploring Rubisco Activase Limitations to C4 Carbon Capture **David Stern**, Boyce Thompson Institute, Ithaca, NY
5:50 – 6:30PM	*Break*
6:30 – 9:00PM	*Poster Session 2* Presenters and titles for Poster Session 2 are listed after the agenda in the Meeting Book.

Thursday, November 18
(All times Eastern)

Section III: *Architecture, Self-Assembly, Self-Repair*
Moderator: *Steve Herbert*

12:00 – 12:40PM	Photosynthetic Energy Capture, Conversion and Storage: From Fundamental Mechanisms to Modular Engineering. **Christoph Benning,** Director, Plant Research Lab, Michigan State U.
12:40 – 1:10PM	Engineering Heterologous Metabolism in Cyanobacteria and Interrogating Impacts on Photosynthetic Performance **Danny Ducat**, Plant Research Lab, Michigan State University
1:10 – 1:40PM	Growing and Maintaining Thylakoids in Chloroplasts of Land Plants **Rebecca Roston**, University of Nebraska, Lincoln

Thursday, November 18, cont'd
(All times Eastern)

1:40 – 2:10PM	Protein Targeting to the Chloroplast Thylakoid Membrane: Structure and Function of a Targeting Complex **Colin Heyes**, University of Arkansas
2:10 – 2:40PM	A dual-function chaperone coordinates biogenesis of the light harvesting complex **Shu-ou Shan**, Cal Tech
2:40 – 3:00PM	Final Comments **Steve Herbert, Gail McLean**

POSTER PRESENTATIONS

Poster Session 1
6:30 to 9:00PM, Tuesday, November 16
(Posters are listed alphabetically by PI}

Elucidating Photoinduced Processes of Photosystem I Via Multidimensional Electronic and Vibrational Spectroscopies
Jessica Anna, University of Pennsylvania

Structural and Mechanistic Studies of O_2-Dependent and O_2-Independent Enzymes of Chlorophyll Biosynthesis
Jennifer Bridwell-Rabb, University of Michigan

Structure and Function of Rubisco Activase
Po-Lin Chiu, Arizona State University

Insight into O_2 Formation in Photosystem II from Substrate Water Exchange Kinetics, IR, EPR, and cryo-EM
Rick Debus, University of California, Riverside

Understanding PS II Energy Conversion in PS IIX Crystals and *In Vivo*
Chuck Dismukes, Rutgers University

Towards Structure Determination of the of the Photosynthetic Supercomplex to understand the Regulation of sustained Cyclic Electron Flow (CEF) in the Antarctic photopsychrophile Chlamydomonas sp. UWO241
Petra Fromme, Arizona State University

Poster Session 1, continued
6:30 to 9:00PM, Tuesday, November 16
Posters are listed alphabetically by PI

Involvement of Pyrenoid Putative Methyltransferase in Photosystem I Biogenesis in *Chlamydomonas reinhardtii*
Arthur Grossman, Carnegie Institution for Science, Dept. of Plant Biology, Stanford , CA

Two disulfide-reducing pathways are required for plastid cytochrome *c* assembly
Patrice Hamel, The Ohio State University

Control of the Thylakoid Ultrastructure by K^+ and Cl^- transporters/channels
Helmut Kirchhoff, Washington State University

Diversification and Function of Bilin Chromophores in Oxygenic Photosynthesis
Clark Lagarias, University of California, Davis

Elucidating the Principles that Control Electron and Proton-Coupled Electron Transfer Reactions in the Photosynthetic Reaction Centers
KV Lakshmi, Rennselaer Polytechnic Institute

Cyclic electron flow and thylakoid ultrastructure during long term stress in the photopsychrophile, *Chlamydomonas* sp. UWO241
Rachael Morgan-Kiss, Miami University, Ohio

CRISPR-based Manipulation of Photosystems in Cyanobacteria
Himadri Pakrasi, Washington University, St. Louis

Genetic and Biophysical Dissection of Electron Transport in Heliobacteria Driven by the Heliobacterial Reaction Center (HbRC)
Kevin Redding, Arizona State University

Spectroscopic Studies of Protein-Protein Association in Model Membranes
Gabriela Schlau-Cohen, Massachusetts Institute of Technology

Hydrophobic Mismatch Effect is a Key Factor in Protein Transport on the Tat Pathway
Steve Theg, University of California, Davis

Early Steps of PS II O_2-Evolving Complex Assembly are Limited by Proton Release
David Vinyard, Louisiana State University

Poster Session 2
6:30 to 9:00PM, Wednesday, November 17
(Posters are listed alphabetically by PI)

Elucidating Mechanisms of Photosystem I Assembly and Repair
Alice Barkan, Oregon State University

New Insights from Cryo-EM Structures of Photosystem I and II
Gary Brudvig, Yale University

Isothermal Titration Calorimetry of Membrane Protein Interactions: FNR and Cytochrome b_6f Complex
William Cramer, Purdue University

Resolving Proteins-Semiquinone Interactions by Advanced EPR Spectroscopy
Sergei Dikanov, University of Illinois, Urbana-Champaign

Multidisciplinary Tools for Illuminating the Details of Photosynthetic Light Harvesting Systems
Matt Francis, Lawrence Berkeley National Lab

Function of the Low Molecular Mass Dicluster Ferredoxins in *Heliobacterium modesticaldum*
John Golbeck, Pennsylvania State University

Studies of Photosynthetic Reaction Centers and Biomimetic Systems
Marilyn Gunner, City University of New York

Structural Dynamics in Photosynthetic Reaction Centers
Jan Kern, Lawrence Berkeley National Lab

The Dynamic Energy Budget of Photosynthesis
David Kramer, Michigan State University

Controlling Electron Transfer Pathways in Photosynthetic Reaction Centers
Philip Laible, Argonne National Lab
Christine Kirmaier, Washington University, St. Louis

Probing Molecular Mechanism of Action of the Cyanobacterial Orange Carotenoid Protein by using Structural Mass Spectroscopy
Haijun Liu, Washington University, St. Louis

Exploring Structure-Function Relationships Governing Transport by the Major Cyanobacterial Bicarbonate Transporters SbtA and BicA
David Nielsen, Arizona State University

Poster Session 2, cont'd
6:30 to 9:00PM, Wednesday, November 17
(Posters are listed alphabetically by PI)

Molecular Mechanisms of Photosystem II Disassembly
Sujith Puthiyaveetil, Purdue University

Accessing Structure and Dynamics of Photosynthetic Pigment-Protein Complexes by Time-Resolved Circular Dichroism Spectroscopy
Sergei Savikhin, Purdue University

Fundamental Research Aimed at Diverting Excess Reducing Power in Photosynthesis to Orthogonal Metabolic Pathways
Alexey Silakov, Pennsylvania State University

Mechanisms for Tuning Protein Electron Transfer Investigated via Site-Specific Linear and Two-Dimensional Infrared Spectroscopy
Megan Thielges, Indiana University

Signal Transduction Pathways of Chloroplast Quality Control
Jesse Woodson, University of Arizona

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

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Overall goals of the project: The overarching goal of the project is to elucidate the mechanism of energy and electron transfer that governs the efficient charge separation in cyanobacterial photosystem I (PSI) complexes through 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, PSI with reaction center mutations, and WT PSI complexes, we are able investigate different aspects of energy transfer and charge separation. Progress towards the overarching goal will be made 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 long wavelength Chl f states of FRL-PSI; and (3) probing charge separation in PSI through ultrafast vibrational spectroscopies.

Significant achievements of the past 2 years: *Energy Transfer in Chl f Containing PSI Complexes:* We have applied two-dimensional electronic spectroscopy (2DES) to WT and engineered Chl f containing PSI complexes from PCC 7002. We applied a global analysis to the 2D spectra, finding differences in the trapping and non-decaying components of Chl f containing PSI when compared to WT PSI. We will expand on these studies to obtain 2DES spectra on longer timescales, investigate how the spectral profile of the incoming pump pulses impacts the trapping, and perform an additional analysis of the timescales through 2D Lifetime Density Maps. *Using Vibrational Spectroscopies to Probe Charge Transfer:* We have completed the construction of our two-dimensional electronic vibrational (2DEV) spectrometer and obtained 2DEV spectra of Chl a molecules in different solvents to prepare for 2DEV experiments on PSI. In addition, we have performed 2DIR spectroscopy on phylloquinone (PhQ) in different solvents to determine how changes in the local environment present in the 2DIR spectra. We will use these results to help interpret 2DEV and T-2DIR based measurements performed on PSI.

Objectives for the coming year:

- We will expand our global analysis toolbox to analyze 2DES spectra with 2D Lifetime Density Maps. We will apply this technique to the 2DES spectra of Chl f PSI and WT PSI from PCC 7002 to further understand the differences in trapping and energy transfer involving the Chl f molecules.
- We will obtain 2DES spectra of Far Red Light PSI. To obtain these 2DES spectra, we have completed the construction of a DOPA for generating tunable pulses in the 700-900 nm region. Over the next year we will compress the DOPA pulses and obtain 2DES spectra of Far Red Light PSI, using two different spectral tunings for the incoming pulses. Using a broader band DOPA, we will obtain 2DES spectra to map pathways of energy transfer among the Chl f, Red Chl, Bulk Chl

and RC Chl. Using a more narrowband red-shifted DOPA centered at ~780 nm, we will obtain 2DES spectra with finely stepped waiting times to characterize the nature of the 780 nm Chl f state. The different 2DES spectra will be analyzed through extracting 2D-Decay Associated Spectra and 2D Lifetime Density Maps. For the 2DES spectra of the 780 nm Chl f state we will also extract oscillatory components through analysis of residuals and perform a line shape analysis to understand how the 780 nm Chl f's couple to the local environment.

- We will perform initial 2DEV measurements on PSI complexes. We have already confirmed that the 2DEV spectrometer is functioning properly by obtaining visible pump mid-IR probe spectra and 2DEV spectra of Chl a molecules in different solvents. We will expand on these studies to perform visible pump – mid-IR probe spectroscopy and 2DEV spectroscopy on WT PSI complexes focusing on understanding the ultrafast formation of the cationic bands. Again, the spectra will be interpreted through global analysis procedures by extracting Decay Associated Spectra and Lifetime Density Maps.
- A thorough characterization of the vibrational potential energy surface of PhQ in hydrogen bonding solvents will aid in the interpretation of the visible pump mid-IR probe based experiments (2DEV and T-2DIR) to be performed on the PSI complexes. We have used 2DIR spectroscopy to characterize PhQ in different solvents and will submit a manuscript to report our results.

Publications of the last 2 years supported by the award:

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. R. Gera, S.L. Meloni, J.M. Anna*, Unraveling Confined Dynamics of Guests Trapped in Self-Assembled Pd_6L_4 Nanocages by Ultrafast Mid-IR Polarization-Dependent Spectroscopy, *J. Phys. Chem. Lett.*, 2019, *10* (3), 413–418. https://doi.org/10.1021/acs.jpclett.8b03485
3. A. Jain, A. Petit, J.M. Anna, J.E. Subotnik*, Simple and Efficient Theoretical Approach to Compute 2D Optical Spectra, *J. Phys. Chem. B*, 2019, *123* (7), 1602–1617. https://doi.org/10.1021/acs.jpcb.8b08674

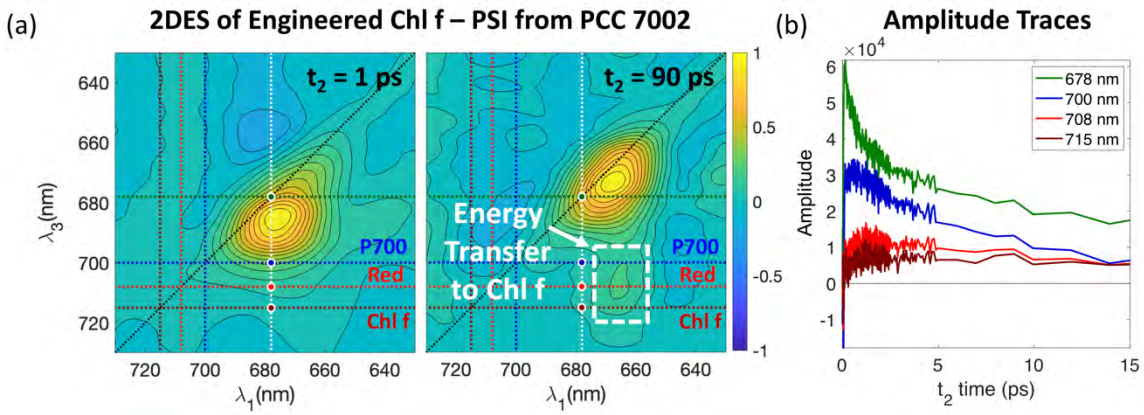

Figure: (a) Representative 2DES spectra of engineered Chl f containing PSI complexes at t_2 = 1 and 90 ps. The cross peak indicates energy transfer to the Chl f molecules on longer timescales and is not present in WT PSI. (b) Amplitude traces are plotted for different λ_1, λ_3 coordinates.

Elucidating mechanisms of Photosystem I assembly and repair

Alice Barkan
Jie Shen, Margarita Rojas, Rosalind Williams-Carrier, Susan Belcher, Prakitchai Chotewutmontri, Simon Oh, Emily Boyce, Alex Wong
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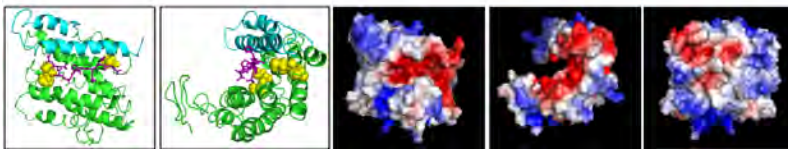
Overall goals of the project – The overarching goal of this project is to elucidate how the assembly of Photosystem I (PSI) is orchestrated and how the integrity of mature PSI is maintained. Impressive advances have been made in elucidating PSI structure and photochemical mechanisms. However, mechanisms underlying PSI biogenesis are just starting to emerge. Given the hydrophobic nature of most of its subunits and the oxidation sensitivity of its prosthetic groups, it is anticipated that accessory proteins orchestrate PSI assembly to promote productive interactions, prevent or resolve non-productive interactions, and prevent deleterious reactions involving prosthetic groups. Indeed, seven proteins have been identified that promote the assembly of PSI. A handful of interactions among these proteins and PSI subunits have been reported, but in no case has the biochemical role of a PSI assembly factor been established.

This project centers on a protein called PSA3, which we previously implicated in the assembly and/or repair of PsaC. PsaC binds the two [4Fe-4S] clusters that serve as the terminal electron acceptors of PSI. PsaC is the core of PSI's stromal ridge and forms an intimate connection with stroma-exposed loops of the membrane-embedded PsaA/B reaction center. PsaC undergoes a conformational change to establish these contacts, and PsaC's Fe-S clusters are particularly prone to oxidative damage. We showed previously by BiFC that PSA3 is in close proximity to PsaC *in vivo,* whereas other subunits of the stromal ridge did not score positive in the same assay. We hypothesized that PSA3 mediates the incorporation of PsaC into PSI, that it does so by facilitating the conformational change in PsaC and/or by shielding its Fe-S clusters from oxidation, that PSA3's activity involves the redox-dependent binding of a peptide to an acidic groove on its surface, and that its role might extend to post-assembly PSI protection or repair. The primary goal of this project is to test and elaborate on that model.

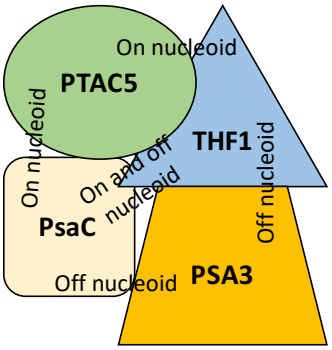


Predicted PSA3 structure (I-TASSER). Predicted peptide binding pocket flanked by cysteine pairs (yellow) is shown to the left. Electrostatic surface representation (right) shows the acidic groove (red).

Significant achievements of the past 2 years –

1. Testing the in vivo function of PSA3's acidic groove and flanking cysteine pairs. Transgenes expressing mutants of PSA3 in which each of these features is disrupted were introduced into Arabidopsis and crossed into a *psa3* background. Each mutant protein partially rescued the *psa3* phenotype. However, the mutant proteins accumulated to levels that were considerably lower than that of native PSA3, making it impossible to distinguish effects of the mutations from effects resulting from low protein abundance. We therefore stopped this line of inquiry.

2. Elucidation of the network of protein-protein interactions involving PSA3. Previously we analyzed proteins that coimmunoprecipitate with PSA3 from solubilized thylakoid membranes, and from pre-immune controls performed in parallel. During this period we performed the analogous experiment with antibody to HCF244, which also resides on the stromal face of the thylakoid membrane but functions in PSII biogenesis. The HCF244 data help to distinguish contaminants that are abundant in stromal thylakoids from likely interactors. These combined data sets provide strong evidence that proteins called THF1 and PTAC5 associate with PSA3 *in vivo*: both proteins were highly enriched in replicate PSA3 experiments and were not detected in any of the control experiments. Several ClpR proteins behaved similarly, but were slightly less enriched.



We then assessed pairwise interactions among PSA3, THF1, PsaC, and PTAC5 both *in vitro* and *in vivo. In vitro* pulldown experiments under reducing conditions did not detect any direct interactions. However, *in vivo* BiFC assays suggest the network of interactions diagrammed to the right. The nucleoid location of some of these interactions suggest a cotranslational role during PsaC biogenesis. The functions of PTAC5 and THF1 are not well understood, but current data suggest that both are involved in cotranslational proteostasis events. *Together, these data suggest that PSA3 functions in folding, disulfide transactions or quality control of nascent PsaC, and that it acts in coordination with PTAC5 (on the nucleoid) and THF1 (on and off the nucleoid).*

3. Initiating studies of the coupling of photosystem subunit synthesis and assembly in photosynthetic microorganisms. Results above in conjunction with advances in understanding PSII biogenesis suggest that cotranslational events play important roles in PSI and PSII assembly. To provide a tool to examine these phenomena in photosynthetic microorganisms, we are optimizing methods for ribosome profiling in *Chlamydomonas* and *Synechocystis*, the latter in collaboration with Josef Komenda's group.

Objectives for the coming year –

1. PSA3's predicted peptide binding groove may bind ligand only when the flanking cysteine pairs are oxidized. Therefore, we will repeat the *in vitro* pulldown experiments involving PSA3, PsaC, THF1, and PTAC5 under oxidizing conditions.
2. We will publish a manuscript describing the PSA3 interactome.
2. PTAC5 is a predicted disulfide isomerase. We will explore whether disulfide bonds involving PSA3 or PsaC are altered in *ptac5* mutants.
3. To determine whether PTAC5 binds co-translationally to PsaC (and other proteins), we will determine whether antibody to PTAC5 coprecipitates radiolabeled proteins from seedlings that had been pulse labeled with radioactive amino acids.
4. We will use ribosome profiling in conjunction with the rich collection of PSI and PSII assembly mutants in Chlamydomonas and Synechocystis to address the coupling of photosystem subunit synthesis and assembly.

Publications of the last 2 years supported by the award – No Publications

Energy Transfer and Radiationless Decay in Light-Harvesting Proteins

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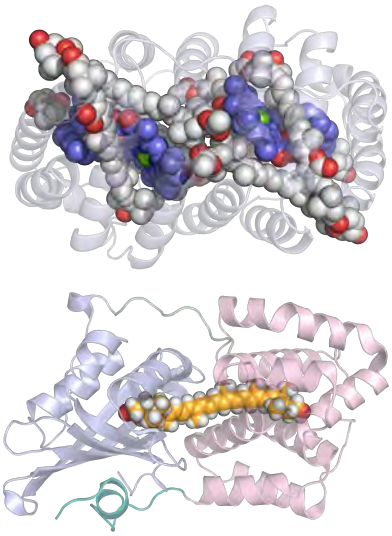

Overall research goals: This project has focused so far on the photophysical and photochemical mechanisms that are involved in excitation energy transfer and photoprotection in two systems that bind ketocarotenoids:

1. the **peridinin–chlorophyll protein** (PCP), a mid-visible light-harvesting complex in marine dinoflagellates in which the ketocarotenoid peridinin serves as the principal light absorber and as an excitation energy donor to chlorophyll *a*.
2. the **orange carotenoid protein** (OCP), which mediates nonphotochemical quenching (NPQ) mechanisms in cyanobacteria using the ketocarotenoids 3'-hydroxyechinenone or canthaxanthin as quenchers of bilin excited states in the phycobilisome.

We are examining the functional role of the enhanced intramolecular charge-transfer (ICT) character in ketocarotenoids, which is derived from activated out-of-plane motions of the conjugated isoprenoid backbone. Two-dimensional electronic spectroscopy (2DES) with broadband (<8 fs) excitation pulses is used to characterize nonradiative decay and excitation energy transfer mechanisms in PCP, OCP, and in solutions of carotenoids. Additionally, fluorescence line shape, quantum yield, and anisotropy measurements as a function of temperature are employed to characterize out-of-plane conformational motions in the S_2, S_x and S_1 states of carotenoids in solution and in OCP.

Significant achievements in 2019–2021:

- Fluorescence anisotropy studies of β-carotene, detecting large-amplitude out-of-plane conformational motions after barrier crossing from the S_2 state to the S_x intermediate and persisting after nonradiative decay to the S_1 state.
- 2DES studies of the peridinin–chlorophyll protein, showing that excitation energy is transferred between the peridinin and chlorophyll excitons over an initially delocalized, two-step pathway.
- 2DES/3DES studies of canthaxanthin, showing that nonradiative decay from S_2 to S_1 via the S_x intermediate is vibrationally coherent, involving modes principally with respect to out-of-plane vibrations of the isoprenoid backbone.
- 2DES measurements of excitation energy transfer in the intact phycobilisome from *Fremyella diplosiphon*, revealing excitation energy transfer pathways down the rods to the terminal emitting allophycocyanin segments in the core.

Science objectives for 2021–2022:

- 2DES/3DES studies of OCP–canthaxanthin to characterize photoinduced formation of a nonequilibrium form of the ground state, S_0*.
- Analysis of the photoactivation action spectrum for OCP–canthaxanthin.
- 2DES/3DES studies of vibronic coherence in allophycocyanin trimers.

References to work supported by this project 2019-2021:

Gurchiek, J. K.; Rose, J. B.; Guberman-Pfeffer, M. J.; Tilluck, R. W.; Ghosh, S.; Gascón, J. A.; Beck, W. F. Fluorescence Anisotropy Detection of Barrier Crossing and Ultrafast Conformational Dynamics in the S_2 State of β-Carotene. *J. Phys. Chem. B* **2020**, *124*, 9029–9046, DOI: 10.1021/acs.jpcb.0c06961.

Pigni, N. B.; Clark, K. L.; Beck, W. F.; Gascón, J. A. Spectral Signatures of Canthaxanthin Translocation in the Orange Carotenoid Protein. *J. Phys. Chem. B* **2020**, *124*, 11387–11395, DOI: 10.1021/acs.jpcb.0c08756.

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.

Mohan T. M., N.; Leslie, C. H.; Sil, S.; Rose, J. B.; Tilluck, R. W.; Beck, W. F. Broadband 2DES Detection of Vibrational Coherence in the S_x State of Canthaxanthin. *J. Chem. Phys.* **2021**, *155*, 035103, DOI: 10.1063/5.0055598.

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

PI: Christoph Benning. Co-Investigators: Federica Brandizzi, Danny D. Ducat, Gregg A. Howe, Jianping Hu, Cheryl A. Kerfeld, David M. Kramer, Beronda L. Montgomery, Thomas D. Sharkey, Josh Vermaas, Berkley Walker.
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Overall goals of the project – The MSU-DOE Plant Research Laboratory (PRL) is a joint venture between the DOE-BES and MSU. Currently, 11 PRL faculty members participate in this endeavor, covering a comprehensive spectrum of expertise in molecular, synthetic and cell biology, biophysics, biochemistry, plant and microbial physiology, genomics and genetics. Collectively, we are exploring photosynthetic processes covering the scales of biological organization from photoactive compounds, enzymes, protein complexes and bacterial micro compartments (BMCs), the thylakoid membrane, to the overall integration of photosynthesis in cells and organisms in their environments. Importantly, we strive to gain a comprehensive understanding of basic mechanisms of "real life photosynthesis", *i.e.*, its limitation and regulation under stochastic conditions in the natural environment and in response to environmental challenges. The long-term goal of these scientific endeavors is to explore basic mechanisms of energy storage by oxygenic photosynthesis, its use in the fixation of carbon, and directing it into energy storage and the building and maintenance of the biological solar panels in cyanobacteria, algae, and plants. Gaining multiscale mechanistic photosynthetic knowledge will allow us to improve photosynthetic efficiency and, therefore, plant productivity. It will enable us to develop photosynthetic modules that can be recombined in novel ways to expand the production of photosynthesis-based bioproducts. Two representative projects are highlighted that show the interdisciplinary nature and collaborative spirit of work conducted under this grant: 1. Exploring the function of thylakoid membrane lipids under dynamic conditions (D. Kramer and C. Benning) and 2. Introducing BMCs into plants to manipulate carbon fixation (C. Kerfeld, F, Brandizzi, B. Walker, C. Benning).

Significant achievements of the past 2 years
Example 1:

- Discovered peroxiredoxin requirement for the biosynthesis of 16:1t, a fatty acid specifically found attached to phosphatidylglycerol in thylakoid membrane linking the redox state of the chloroplast to the synthesis of this lipid molecular species.
- We showed evidence that natural variations in temperature responses of photosynthesis are modulated by specific lipid species, such as 16:1t, rather than bulk lipid properties, with implications for improving photosynthetic energy storage under changing environments.
- We phenotyped 59 Arabidopsis lipid mutants under dynamic conditions at different temperature regimes for photosynthetic performance, lipid composition and relative growth rate and applied novel analytical tools to explore this complex data set.

Example 2:

- Developed strategies for stable introduction of BMC components into Arabidopsis chloroplasts and encapsulation of proteins into synthetic BMC shells.
- Produced different tagged versions of BMC shell components for direct introduction into Camelina seedlings.
- Characterized different Arabidopsis carbonic anhydrase isoforms through mutant analysis, assay development using a membrane inlet mass spectrometer approach, and crystallization of isoforms, with the goal to encapsulate an optimized version into BMCs in plants.

Objectives for the coming year –

Example 1:

- Continue to investigate how the redox state of the chloroplast affects 16:1t levels and whether this serves as a signal to adjust photosynthesis.
- Build and test new hypotheses based on the lipid/phenomics study.

Example 2:

- Establish and analyze transgenic plants producing multiple BMC components in chloroplasts.
- Identify a carbonic anhydrase suitable for BMC encapsulation and initiate encapsulation in vitro and in plants.
- Continue developing a seedling-based system for uptake and study of BMC components.

Representative Publications of 37 total Publications (last 2 years) Supported by the Award

1. Horn, P. J., M. D. Smith, T. R. Clark, J. E. Froehlich, and C. Benning. 2020. PEROXIREDOXIN Q stimulates the activity of the chloroplast 16:1$^{\Delta 3trans}$ FATTY ACID DESATURASE4. *Plant J* 102: 718-729. https://doi.org/10.1111/tpj.14657.
2. Major, I. T., Q. Guo, J. Zhai, G. Kapali, D. M. Kramer, and G. A. Howe. 2020. A phytochrome b-independent pathway restricts growth at high levels of jasmonate defense. *Plant Physiol* 183: 733-749. https://doi.org/10.1104/pp.19.01335.
3. Plegaria, Jefferson S., Matthew D. Yates, Sarah M. Glaven, and Cheryl A. Kerfeld. 2020. Redox characterization of electrode-immobilized bacterial microcompartment shell proteins engineered to bind metal centers. *ACS Applied Bio Materials* 3: 685-92. https://doi.org/10.1021/acsabm.9b01023.
4. Rohnke, B. A., K. J. Rodriguez Perez, and B. L. Montgomery. 2020. linking the dynamic response of the carbon dioxide-concentrating mechanism to carbon assimilation behavior in *Fremyella diplosiphon*. *mBio* 11: https://doi.org/10.1128/mBio.01052-20.
5. Santos-Merino, M., A. Torrado, G. A. Davis, A. Rottig, T. S. Bibby, D. M. Kramer, and D. C. Ducat. 2021. Improved photosynthetic capacity and photosystem I oxidation via heterologous metabolism engineering in cyanobacteria. *PNAS* 118: https://doi.org/10.1073/pnas.2021523118.
6. Xu, Y., X. Fu, T. D. Sharkey, Y. Shachar-Hill, and B. J. Walker. 2021. The metabolic origins of non-photorespiratory CO_2 release during photosynthesis: A metabolic flux analysis." *Plant Physiol*. 186:297-314. https://doi.org/10.1093/plphys/kiab076.

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

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Overall goals of the project – 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 focus on the parallel (metallo)enzymes that catalyze equivalent O_2-dependent and O_2-independent reactions in chlorophyll biosynthesis, which is part of a larger biosynthetic pathway that forms the "pigments of life" (Figure 1). Towards understanding how these parallel photosynthetic 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 fundamental information regarding how diverse photosynthetic organisms build the pigments needed for facilitating conversion of sunlight into chemical energy.

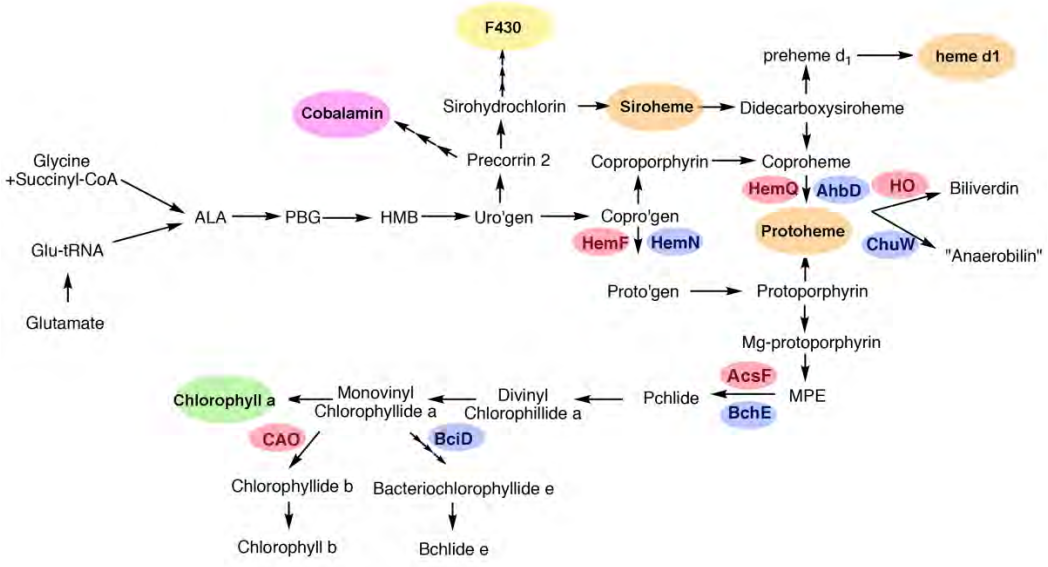

Figure 1. The biosynthesis of the "pigments of life" (chlorophyll, heme, siroheme, heme d_1, cobalamin, and coenzyme F_{430}), involves many steps that are catalyzed by parallel enzymes[1-2]. The enzymes involved in these steps are highlighted with red (O_2-dependent) and blue (O_2-independent) circles. 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).

Significant achievements – Over the past year, we have (i) identified chlorophyll biosynthetic enzyme homologs that are amenable to our sought-after biochemical work and (ii) developed techniques to isolate, purify, solubilize, and characterize the required photosynthetic pigment substrates and products of the enzymes under investigation. Importantly, thus far, we have recombinantly expressed, purified, and reconstituted several of the parallel enzymes that are involved in chlorophyll biosynthesis. We have had success generating crystals of some of the enzyme targets and have collected initial data sets on these crystals. In addition, we have isolated several chlorophyll biosynthetic pathway enzymes that we hypothesize are important for the activity of the enzymes under investigation. We have elucidated how a transcription factor senses and responds to changes to cellular redox potential (*see publication 3*). Finally, we have done extensive work investigating the structure-function relationships of cobalamin-dependent radical SAM enzymes to guide studies on the O_2-independent chlorophyll biosynthetic enzyme BchE (*see publication 1*).

Objectives for the coming year – In the coming year, we will be focused on optimizing our initial crystal conditions and phasing our initial datasets. We will demonstrate the in vitro activity of the O_2-dependent and O_2-independent enzymes that form the fifth ring of the chlorophyll scaffold. These enzymes either use a diiron metallocluster to activate O_2, or a combination of an Fe-S cluster, *S*-adenosylmethionine, and cobalamin to catalyze a parallel reaction. We will capitalize on the spectroscopic data that we have collected on these enzymes to interrogate the roles of the metallocofactors in the reaction cycles. We will probe protein-protein interactions between the targets of this project and other biosynthetic pathway members. Finally, we will investigate the structural differences between annotated O_2-dependent enzyme homologs that are involved in formylating the chlorophyll macrocycle (chlorophyllide *a* oxygenase and protochlorophyllide *a* oxygenase) from bacteria, algae, and plants.

Publications supported by the award –

1. Bridwell-Rabb, J. Li, B. Drennan, C.L., (2021) Cobalamin-dependent Radical *S*-adenosylmethionine Enzymes: Capitalizing on Old Motifs for New Functions, *Submitted.*

2. Dill, Z., Li, B., and Bridwell-Rabb, J. (2021) Purification and Structural Elucidation of a Cobalamin-dependent radical SAM enzyme OxsB, Coenzyme B_{12} Enzymes – Methods in Enzymology, *Submitted.*

3. Li, B., Jo. M., Liu, J., Canfield, R., and Bridwell-Rabb, J. (2021) A Repurposed Metalloregulatory Scaffold for Redox Sensing in Photosynthetic Organisms, *Under Revision.*

References:

1. Li, B., et al., *Biochemistry* **2019,** *58* (2), 85-93.
2. Fujita, Y., et al., *Life (Basel)* **2015,** *5* (2), 1172-203.

A PSI-[FeFe] hydrogenase chimera: activation for in vitro mechanistic studies

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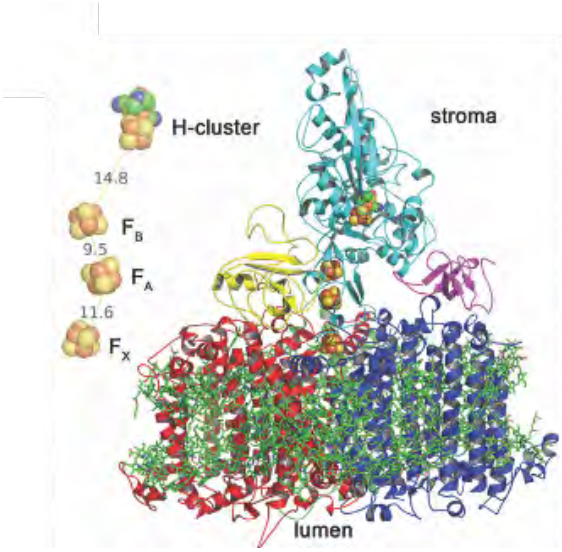
Overall goals of the project – Recently the Kevin Redding laboratory at ASU has engineered a photosystem I - [FeFe] hydrogenase chimeric construct (Fig 1) that produces solar hydrogen *in vivo* (1). This approach offers the advantage that the entire H_2 photogeneration complex is simply expressed in the organism and can also be repaired *in vivo*. This construct has been well characterized in its *in vivo* H_2 production.



(1) A model of the PSI [Fe-Fe] hydrogenase chimera (from Kanygin et al. (1)) To the side is shown the train of PSI [4Fe-4S] clusters leading to the catalytic H-cluster of the [Fe-Fe] hydrogenase domain

Our goals are to characterize the *in vitro* performance of this chimera using EPR and other spectroscopic techniques. All of the *in vitro* characterization will require the reactivation of the hydrogenase domain after isolation, as so far in the Redding lab attempts at isolating the chimeric construct have resulted in loss of hydrogenase activity. Fortunately, the Britt laboratory has great expertise in assembling the catalytic H-cluster of this [FeFe] hydrogenase using either (1) the set of native Fe-S enzymes HydG, HydE, and HydF that assemble the organometallic dinuclear $[2Fe]_H$ component where the catalysis is localized, or (2) replacing one, two, or all of these enzymes with synthetic organometallic constructs that functionally replace the different enzymes' products (2-9). Both of these approaches will provide a high degree of isotopic editing of the active site that will be very useful in spectroscopic studies (EPR, FTIR, etc) of light driven catalysis in the activated chimera (8).

Significant achievements of the past 2 years – this is a brand new project.

Objectives for the coming year – the first important goal is to generate active PSI-hydrogenase chimeric constructs from the inactive form isolated by the Redding laboratory. We will use the same cell-free synthetic approach that works well for the isolated HydA hydrogenase, incubating the chimeric construct anaerobically with HydG, HydE, and HydF along with a well honed cocktail of enzyme substrates and cofactors. A second approach is to bypass the enzymatic synthesis entirely by incubating the construct with a synthetic binuclear cluster of the form $[Fe_2S_2(CO)_4(CN)_2(adt)]$, where adt is the required azadithiolate bridge of the $[2Fe]_H$ subcluster. We will then be able to characterize the *in vitro* H_2 production via saturating light flashes, measuring kinetics and quantum yield. This is a required step to characterizing the H-cluster catalytic intermediates as driven by light in this "hard-wired" photocatalytic system. The use of isotope editing via activation routes (1) or (2) will provide important spectroscopic insights into the light driven catalysis via isotope sensitive spectroscopies such as FTIR and EPR/ENDOR.

Publications of the last 2 years supported by the award – none yet!

(1) A. Kanygin, *et al.*, *Energy & Environmental Science* 13, 2903 (2020).
(2) Kuchenreuther, J. M. *et al., Science* 342:472-475. (2013)
(3) Kuchenreuther, J. M. *et al., Science* 343:424-427. (2014)
(4) Rao, G. *et al.***,** *Proc. Natl. Acad. Sci. U.S.A.,* 116:20850-20855. (2019)
(5) Britt, R.D *et al., Chem. Sci.* 11:10313-10323. (2020)
(6) Tao, L.Z *et al., J. Am. Chem. Soc*. 142:10841-10848. (2020)
(7) Rao, G. *et al., Chem. Sci.* 11:1241-1247. (2020)
(8) Britt, R.D. *et al., Nat. Rev. Chem*. https://doi.org/10.1038/s41570-020-0208-x (2020)
(9) Britt, R.D. *et al., ACS Bio & Med Chem Au,* doi.org/10.1021/acsbiomedchemau.1c00035 (2021)

New insights from cryo-em structures of photosystems I and II

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Overall goals of the project:
The specific aims of the project are to probe the structure and function of photosystem II (PSII) by: (i) measuring $^{16}O/^{18}O$ kinetic isotope effects, (ii) investigating D1-S169A mutated PSII, (iii) probing the functional role of Na^+ that we recently discovered to bind near the O_2-evolving complex (OEC); (iv) determining the cryogenic electron microscopy (cryo-em) structure of wild-type *Synechocystis* sp. PCC 6803 PSII, (v) determining the cryo-em structures of D1-S169A and D1-N181A mutated *Synechocystis* sp. PCC 6803 PSII; and (vi) determining the cryo-em structure of far-red light photoacclimated PSII from *Fischerella thermalis* sp. PCC 7521.

Significant achievements of the past 2 years:
(i) Analysis of site-directed mutations of the D1-S169 residue in the "narrow" channel. We find that the D1-S169A mutation inhibits water oxidation and perturbs the H-bonding network around the OEC. We also find that NH_3 does not affect the S_2-state EPR signal in D1-S169A PSII, indicating that NH_3 does not bind to the OEC as it does in wild-type PSII. A QM/MM analysis indicates that an additional water molecule binds to Mn4 and displaces O5 in the S_2 state of D1-S169A PSII, thereby blocking the NH_3-binding site. These findings suggest that a similar water-binding reaction may occur during the S_2-to-S_3 state transition in wild-type PSII.
(ii) Identification of a Na^+-binding site near the OEC of spinach PSII. A comparison of X-ray crystal and cryo-em structures of PSII revealed an unexplained positive peak adjacent to deprotonated D1-H337 that is assigned to a Na^+ ion. We suggest that Na^+ binding upon deprotonation of D1-H337 allows for the conservation of charge near the OEC and helps to optimize O_2-evolution activity over a wide pH range.
(iii) Analysis of chlorophylls (Chls) in cryo-em structures. We analyzed the recent cryo-em structures of far-red light photoacclimated PSI (FRL-PSI) in which ~7 of the Chl *a* molecules have been replaced by Chl *f*. Owing to the lack of sufficient resolution, it is difficult to distinguish Chl *f* from Chl *a*. By using a "cone scan" method, we identify the Chl *f*-binding sites.
(iv) Cryo-em structures of PSI and PSII. We solved structures at 2.58 and 2.91 Å resolution, respectively, of apo-PSII from *Synechocystis* sp. PCC 6803 and FRL-PSI from *Synechococcus* 7335. The apo-PSII complex is proposed to be similar to an intermediate assembly state of PSII and provides insight into photoactivation of PSII. Based on a quantitative "cone scan" assessment of the FRL-PSI cryo-em map, we assign six high specificity Chl *f*-binding sites, and three low specificity Chl *f*-binding sites per FRL-PSI monomer.

Objectives for the coming year:
Ongoing work involves the use of cryo-em to determine structures of site-directed mutated *Synechocystis* sp. PCC 6803 PSII, including D1-S169, D1-D61 and D1-N181 mutations, KIE measurements and biophysical studies of site-directed mutated PSII to probe 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 of the last 2 years supported by the award:

1. "Opportunities and Challenges for Assigning Cofactors in Cryo-EM Density Maps of Chlorophyll-Containing Proteins", C. J. Gisriel, J. Wang, G.W. Brudvig and D.A. Bryant (2020) *Communications Biology 3*, 408.
2. "Identification of a Na^+-Binding Site Near the Oxygen-Evolving Complex of Spinach Photosystem II", J. Wang, J. M. Perez-Cruet, H.-L. Huang, K. Reiss, C.J. Gisriel, G. Banerjee, D. Kaur, I. Ghosh, A. Dziarski, M.R. Gunner, V.S. Batista and G.W. Brudvig (2020) *Biochemistry 59*, 2823-2831.
3. "D1-S169A Substitution of Photosystem II Reveals a Novel S_2-State Structure", I. Ghosh, G. Banerjee, K. Reiss, C.J. Kim, R.J. Debus, V.S. Batista and G.W. Brudvig (2020) *Biochim. Biophys. Acta 1861*, 148301.
4. "Cryo-EM Structure of Monomeric Photosystem II from *Synechocystis* sp. PCC 6803 Lacking the Water-Oxidation Complex", C.J. Gisriel, K. Zhou, H.-L. Huang, R.J. Debus, Y. Xiong and G.W. Brudvig (2020) Joule 4, 2131-2148.
5. "Oxygen Evolution of Photosystem II", H.-L. Huang and G.W. Brudvig (2021) in: "Comprehensive Coordination Chemistry III", Volume 8 on Bioinorganic Chemistry (E. Constable, G. Parkin and L. Que, Jr., eds.) Elsevier, Amsterdam, The Netherlands, pp. 569-588.
6. "Is Deprotonation of the Oxygen-Evolving Complex of Photosystem II during the $S_1 \rightarrow S_2$ Transition Suppressed by Proton Quantum Delocalization?", K. R. Yang, K.V. Lakshmi, G.W. Brudvig and V.S. Batista (2021) *J. Am. Chem. Soc. 143*, 8324-8332.
7. "Proton Exit Pathways Surrounding the Oxygen Evolving Complex of Photosystem II", D. Kaur, Y. Zhang, K.M. Reiss, M.Mandal, G.W. Brudvig, V.S. Batista and M.R. Gunner (2021) *Biochim. Biophys. Acta 1862*, 148446.
8. "Kinetic Modeling of Substrate-Water Exchange in Photosystem II", H.-L. Huang and G.W. Brudvig (2021) *BBA Advances 1*, 100014.
9. "Quantitative Analysis of Chlorophyll Types in Cryo-EM Maps of Photosystem I Adapted to Far-red Light", C.J. Gisriel, H.-L. Huang, K.M. Reiss, D.A. Flesher, V.S. Batista, D.A. Bryant, G.W. Brudvig and Jimin Wang (2021) *BBA Advances 1*, 100019.
10. "Toward Understanding the S_2-S_3 Transition in the Kok Cycle of Photosystem II: Lessons from the Sr-Substituted Structure", M. Amin, D. Kaur, M.R. Gunner and G.W. Brudvig (2021) *Inorg. Chem. Comm. 133*, 108890.
11. "Heterogeneous Composition of Oxygen-Evolving Complexes in Dark-Adapted Crystal Structures of Photosystem II", J. Wang, C.J. Gisriel, K. Reiss, H.-L. Huang, G.W. Brudvig and V.S. Batista (2021) *Biochemistry 60*, in press.
12. "Structure of a Photosystem I-Ferredoxin Complex from a Marine Cyanobacterium Provides Insights into Far-Red Light Photoacclimation", C.J. Gisriel, D.A. Flesher, G. Shen, J. Wang, M.-Y. Ho, G.W. Brudvig and D.A. Bryant (2021) *J. Biol. Chem. 296*, in press.

Structure and Function of the CO_2 uptake NDH-1 Complexes in Cyanobacteria

DE-FG02-08ER15968

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Overall research goals: Cyanobacteria have evolved a mechanism for scavenging CO_2 that enables them to operate at very low ambient inorganic carbon levels and could be exploited in engineering efforts aimed at the reduction of CO_2 emissions and other biotechnological applications. This project is basic research 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 by concentrating it within the cell, thereby saturating the active sites of the CO_2-fixation enzyme, Rubisco, thereby increasing the efficiency of photosynthesis. The project focus is on the **NDH-1 complexes** that mediate cyclic electron flow including specialized forms of **NDH-1** function

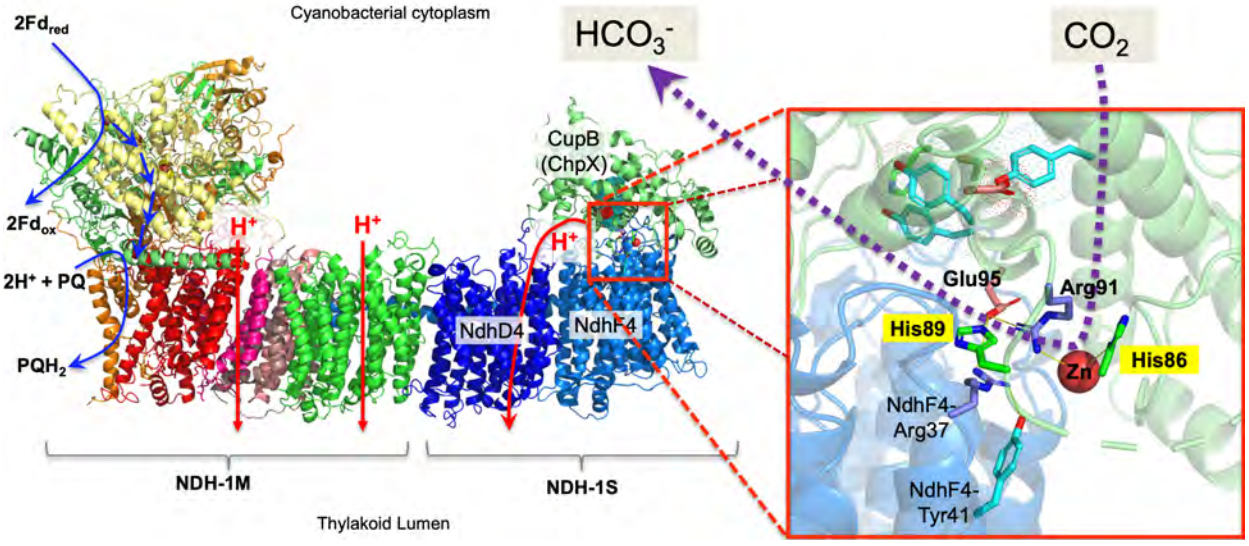

Figure 1. Homology modelling of the *Synechococcus* **NDH-1 complex** CupB involved in CO_2 uptake by a poorly understood redox-powered carbonic anhydrase. The published coordinates of the homologous *Thermosynechococcus* cryoEM structure (PDB ID=6TJV) were utilized.

Significant achievements 2019-2021:

Homology modelling Syn7942 NDH-MS complex and site-directed mutagenesis of the CupB protein: The new CryoEM structure, though breath-taking, raises more questions than it answers. For testable structure-based hypotheses in the model cyanobacterium Syn7942, we developed a homology model (**Fig. 1**). Unusually, the only two Zn ligands CupA-His130 and CupA-Arg135, are observed in *Thermosynechococcus*. These appear to be highly conserved in both CupA and CupB, corresponding to residues CupB-His86 and CupB-Arg91 in CupB of *Synechococcus* sp. PCC7942 (Syn7942).

Proton pumping by NDH-1 complexes: Understanding proton-pumping by NDH-1 complexes will be critical to testing hypotheses regarding the redox-powered CO_2-hydration mechanism. This question intersects with cyclic electron flow (CEF), since it is critical for CO_2 uptake and modulation of ATP/NADPH production ratio. Despite this significance, critical questions on CEF have remained unresolved including: *1.) which complexes have the major role in mediating the return flow of electrons to the PQ pool and 2.) to what extent does CEF contributes to photosynthetic proton pumping*. By modifying the optics of our BioLogic kinetic spectrofluorometer and developing the use pH-sensitive fluorescence reporters, it was shown that CEF via NDH-1 complexes is the main circuit for proton pumping and contributes nearly 40% of the pumping overall, a figure much higher than previous estimates. Additionally, NDH-1 mediated CEF also maintains high levels of the proton pumping rate in the absence of linear flow, compensating for loss of PSII, which likely accounts for robustness during photoinhibition.

Site-directed mutagenesis of the CupB protein: The new CryoEM structure raises many questions. Typically, CA active site Zn ions are coordinated by three amino acids, often His or a combination of His and Cys residues. Unusually, the two Zn ligand residues CupB-His86 and CupB-Arg91 in CupB of the model cyanobacterium Syn7942. Site-directed mutagenesis targeting some of the conserved residues were produced and studied. Growth and CO_2 uptake assays show that the most severe defects in activity upon substitution of the predicted Zn ligand, CupB-His86. Mutations at other sites produced intermediate effects. Completion of this work was hampered by lack of antibodies to CupB, which remains a problem despite strenuous efforts. Instead, we have this last year developed Mass-Spec proteomic methods to evaluate potential alterations in the accumulation of the mutant proteins. This revealed that the CupB site-directed mutants had upregulated bicarbonate transport proteins despite growth under CO_2-enriched conditions, whereas, surprisingly, the deletion strain completely lacking both CupB and CupA did exhibit this upregulation under the same growth conditions. Importantly, it seems to provide a clue as to how the enzyme mechanism may work: since that having a CupB protein damaged in proton transport.

Articles (DOE-BES as sole financial support):

1. 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
2. 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
3. Miller, N.T., G. Ajlani, and R.L. Burnap ***Cyclic electron flow coupled proton pumping in Synechocystis sp. PCC6803 is dependent upon NADPH oxidation by the soluble isoform of ferredoxin:NADP-oxidoreductase*** (in preparation).

Science Objectives 2021-2022:

Now that we have a reliable system in Syn7942 and good homology model, we will focus on the CupB and NdhD4 proteins to evaluate residues that appear to coordinate the metal ion responsible for the proton-coupled carbonic anhydrase activity. This will involve construction of deletion knockouts and construction of the appropriate transformation plasmids. The truly difficult part of the project is quantifying the proteins. As the generation of antibodies to CupB have thus far failed, we are trying tagging, but can rely on the more costly (labor/money) proteomics approach that we have now successfully utilized.

Structure and Function of Rubisco Activase

Project Team
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Research Specialist -
Elif Karasu (Princeton)
Graduate Students -
Kazi Waheeda (ASU), Isabella Breen (ASU), and Hugh Wilson (Princeton)

Project Goals
We seek to gain a comprehensive understanding of the regulatory mechanism of the higher plant Rubisco activase (Rca), such as post-translational modifications, metal-ion coordination, and self-association. Experiments in mechanistic enzymology are complemented by single-molecule work and structural investigations, such as crystallographic approaches or cryogenic electron microscopy (cryo-EM), aimed at relating oligomer size and conformational states to their functional properties.

Significant Achievements (2019-2021)
Higher organization of the Rca hexamers depends on their concentrations
To understand the structural detail of the Rca hexameric packing, we froze truncated SoαRca (residue 66-359) with ATPγS for cryo-EM imaging. Surprisingly, the spatial organization of the Rca hexamers was different over a range of the Rca concentrations. Lower concentrations of the Rca proteins showed some hexamers, standing alone or packing into 1D arrays (Fig. 1A). 2D arrays were identified when the Rca concentration was 32 μM (Fig. 1B). The lattice parameters are a = 104.5 Å, b = 104.5 Å, and γ = 60° with a plane space group of $p6$ (phase residual: 35.2°) (Fig. 1B). The projection reconstruction reached at 4 Å resolution (Fig. 1B). The helical arrangement of Rca was observed in the concentration of 64 μM. The diameter and pitch of the helix are about 10 nm and 4 nm, respectively (Fig. 1C). Recent studies indicate that the Rca at concentrations of 10-100 μM without RuBP is not catalytically active, suggesting that the higher form of the Rca organization may be used to store the inactive proteins.[1]

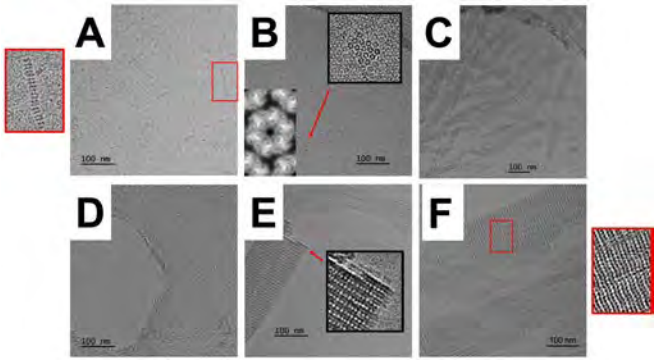

Fig. 1 | Cryo-EM images of the Rca assemblies at different concentrations.

Interaction between Rubisco and Rca is highly regulated by the N- and C-terminus of the Rca
To probe the mechanism of how Rca activates the Rubisco, we assembled the full-length SoαRca

with RuBP and ATPγS or ADP with Rubisco purified from the spinach leave tissues. We used cross-linking and negative-stain EM to analyze the binding of the two complexes. Few particles were shown in binding with a side-on orientation. However, more Rubisco-Rca complex could be observed when mixing with 10 mM ATP. The binding of the two complexes may need ATP hydrolysis in action, rather than full ADP or ATPγS.

ADP-induced subunit exchange of the Rca occurs diversely

The single-molecular assay enables the detection of the Rca assembly/disassembly events in real-time.[2,3] Fig. 2A shows a single-molecule trace at 1 µM Rca concentration with ADP. The single-molecule diffusivity, *D*, displays rich dynamics due to fast subunit exchange. We first assigned the change points in diffusivities, *D*, manually (vertically dotted red lines in the middle panel of Fig. 2A) and the regions between change points as levels of constant *D* and calculated the mean *D* of individual identified levels (Fig. 2A, red circles). The second strategy is to identify the *D* change points by a statistical approach based on log-likelihood ratio (LLR) tests (Fig. 2A, bottom). Most of the resulting levels are conserved in both methods. We further analyzed a "consensus set" of diffusivity levels and mapped out all possible oligomerization states during the ADP-induced subunit exchange. We measured hundreds of single molecules and constructed a histogram of all identified *D* levels (Fig. 2B). The result seemed to suggest that the ADP-induced subunit exchange occurs between a more diverse set of oligomers rather than a limited set that was previously determined.

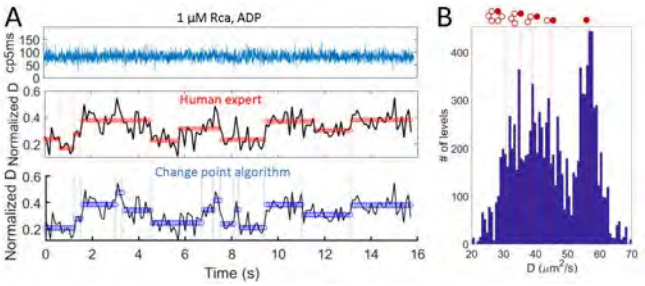

Fig. 2 | Level identification during Rca assembly/ disassembly.

Objectives for the Coming Year

To understand the versatile molecular packing of the Rca, we are pursuing high-resolution cryo-EM structural analyses. Because the Rca assemblies are sensitive to their Rca concentrations, the conventional cryo-EM sample preparation is not ideal, since the quick blotting processing dramatically changes the local concentrations of the protein samples. We plan to first characterize Rca concentrations for individual assembly states and utilize cross-linking to stabilize the molecular assemblies for high-resolution cryo-EM studies. Once the cryogenic specimens are prepared, we will use the electron crystallographic method or single-particle approach to reconstruct the density maps of the Rca assemblies.

Publications Supported by the Award

1. Serban, A. J., Breen, I. L., Bui, H. Q., Levitus, M. & Wachter, R. M. Assembly-disassembly is coupled to the ATPase cycle of tobacco Rubisco activase. *J. Biol. Chem.* **293**, 19451–19465 (2018).
2. Wilson, H. & Wang, Q. ABEL-FRET: tether-free single-molecule FRET with hydrodynamic profiling. *Nat. Methods* **18**, 816–820 (2021).
3. Wang, Q., Serban, A. J., Wachter, R. M. & Moerner, W. E. Single-molecule diffusometry reveals the nucleotide-dependent oligomerization pathways of Nicotiana tabacum Rubisco activase. *J. Chem. Phys.* **148**, 123319 (2018).

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

The *long-term goal* of our research is to determine the mechanisms controlling the acquisition, uptake, and photosynthetic reduction of CO_2 into organic compounds.

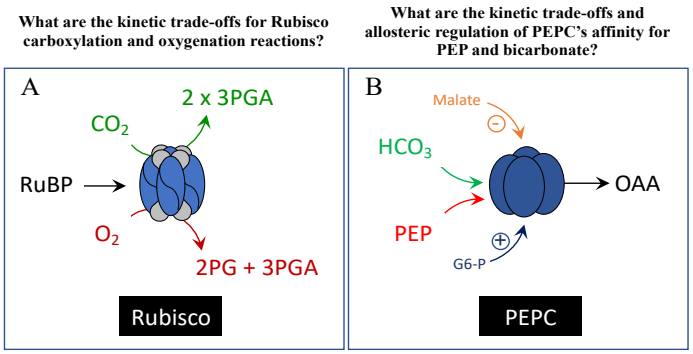

Figure 1. Rubisco and PEPC reactions. (A) Rubisco carboxylation of ribulose-1,5-bisphosphate (RuBP) with CO_2 is inhibited by O_2. Carboxylation produces 3-phosphoglycerate (3PGA), which is used to make sugar. Oxygenation generates 3PGA + 2-phosphoglycolate (2PG) but 2PG requires energy consumption by 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).

Overall objectives of this 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.

The *rationale* for this research is that understanding Rubisco and PEPC kinetic properties and their catalytic efficiencies will determine the mechanisms controlling and limiting carbon fixation.

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 over the past 2 years

We measured Rubisco kinetic parameters and isotope effect from *Rhodosprillum rubrum*, which is a model Form II type Rubisco. We characterized the PEP and HCO_3^- kinetic properties (K_{PEP} and K_{HCO3}, respectively) of diverse PEPC enzymes as well as generated chimeric PEPC isoforms to test the influence of specific regions of amino acids on the allosteric regulation (manuscripts in preparation). We also measured K_{HCO3} from a range of grass species and identified several key amino acid residues that influence PEPC kinetic properties (DiMario et al., 2021). These residues are currently being tested. We also demonstrated that carbonic anhydrase and PEPC activity can limit photosynthesis in C_4 plants at low CO_2 and high temperature (Serrano *et al*. in revision; Crawford *et al*., 2021). We also showed that leaf anatomy and biochemistry influence internal CO_2 conductance and availability for photosynthesis (Pathare et al., 2020; Pathare et al., 2021).

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 Rubisco from *R. rubrum*.
3. Initiate the characterization of temperature influence on the kinetic parameters and carbon isotope effect of diverse Rubisco isoforms.
4. Publish the survey of K_{PEP} and K_{HCO3} from diverse PEPC isoforms.
5. Generate and kinetically characterize chimeric PEPC isoforms to test amino acid regions influencing kinetic trade-offs and allosteric regulation.

Publications of the last 2 years supported by the award:

Published

1. Pathare V.S., DiMario R.J., Koteyeva N., **Cousins A.B.** (2021) C_4 grasses adapted to low mean annual precipitation exhibit leaf-level traits associated with greater mesophyll conductance and lower leaf hydraulic conductance grasses. *New Phytologist,* Under review.
2. Serrano-Romero E.A., **Cousins A.B.** (2021) The temperature response of C_4 photosynthesis with low PEPC activity. *The Plant Journal*, In revision
3. DiMario R.J., Giuliani R, Ubierna N., Slack A.D., **Cousins A.B.**, Studer A.J. (2021) Leaf carbonic anhydrase activity is needed to maintain C_4 photosynthesis in *Zea mays*. In press *Plant Cell & Environment*
4. Crawford J.D., **Cousins A.B.** (2021) Limitation of C_4 photosynthesis by low carbonic anhydrase activity increases with temperature but does not influence mesophyll CO_2 conductance. *Journal of Experimental Botany*, https://doi.org/10.1093/jxb/erab464
5. DiMario R.J., Kophs A.N., Pathare V.S., Schnable J.C., **Cousins A.B.** (2021) Kinetic variation in grass phosphoenolpyruvate carboxylases provides opportunity to enhance C_4 photosynthetic efficiency. *The Plant Journal*, 105, 1677-1688.
6. Pathare V.S., Sonawane B.V., Koteyeva N., **Cousins A.B.** (2020) C_4 grasses adapted to low mean annual precipitation exhibit leaf-level traits associated with greater mesophyll conductance and lower leaf hydraulic conductance grasses. *Plant Cell & Environment*, 43, 1897-1910.
7. Serrano-Romero E.A., **Cousins A.B.** (2020) Cold acclimation of mesophyll and bundle-sheath conductance, and leakiness in Miscanthus x giganteus. *New Phytologist* 226, 1594-1606
8. Pathare V.S., Koteyeva N., **Cousins A.B.** (2020) Increased adaxial stomatal density is associated with greater mesophyll surface area exposed to intercellular air spaces and mesophyll conductance in diverse C_4 grasses. *New Phytologist* 225, 169-182.

In prep

1. Boyd R.D., Sexton T.M., **Cousins A.B.** (2021) Temperature breakpoint and carbon isotope fractionation in Rubisco from *Oryza sativa*.

Isothermal Titration Calorimetry of Membrane Protein Interactions: FNR & the Cytochrome b_6f Complex

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Ferredoxin-NADP+ reductase (FNR) was previously inferred to bind to the cytochrome b6f complex in the electron transport chain of oxygenic photosynthesis. In the present study, this inference has been examined through analysis of the thermodynamics of the interaction between FNR and the b6f complex. Isothermal titration calorimetry (ITC) was used to characterize the physical interaction of FNR with b6f complex derived from two plant sources (Spinacia oleracea and Zea maize). ITC did not detect a significant interaction of FNR with the b6f complex in detergent solution nor with the complex reconstituted in liposomes. A previous inference of a small amplitude but defined FNR-b6f interaction is explained by FNR interaction with micelles of the UDM detergent micelles used to purify b6f. Circular dichroism (CD), employed to analyze the effect of detergent on the FNR structure, did not reveal significant changes in secondary or tertiary structures of FNR domains in the presence of UDM detergent. However, thermodynamic analysis implied a significant decrease in an interaction between the N-terminal FAD-binding and C-terminal NADP+-binding domains of FNR caused by detergent. The enthalpy, ΔHo, and the entropy, ΔSo, associated with FNR unfolding decreased 4-fold in the presence of 1 mM UDM at pH 6.5. In addition to the conclusion regarding the absence of a binding interaction of significant amplitude between FNR and the b6f complex, these studies provide a precedent for consideration of significant background protein-detergent interactions in ITC analyses involving an integral membrane protein.

Insight into O_2 Formation in Photosystem II from Substrate Water Exchange Kinetics, IR, EPR, and cryo-EM

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Overall goals of the project – To identify the two substrate water molecules that form the O-O bond, to identify the dominant substrate access and proton egress pathways that link the Mn_4Ca cluster with the thylakoid lumen, to characterize the 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 of the past 2 years – On the basis of substrate water exchange kinetics measured with the D1-D61A mutant of *Synechocystis* sp. PCC 6803 (performed in collaboration with J. Messinger and coworkers at Uppsala University), we showed that the exchange rate of the fast-exchanging substrate water (W_f) in the S_2 state is rate-limited by steric factors that limit the diffusion of bulk water through the channels that connect the Mn_4Ca cluster with the thylakoid lumen (the D1-D61A mutation decreases one of these barriers so that W_f exchange is accelerated in the S_2 state (Fig. 1)). This finding eliminates the primary argument against concluding that W3 is the fast exchanging substrate water molecule (*5*).

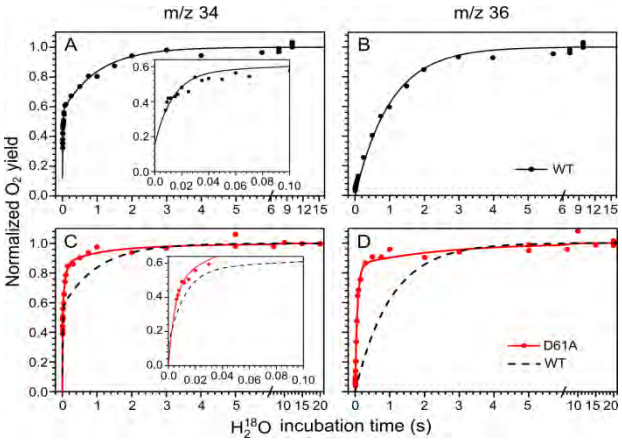

Fig. 1. Substrate water exchange in the S_2 state of WT (black) and D1-D61A (red). In D1-D61A, W_s is accelerated 15-fold and W_f is accelerated at least 3.5-fold (to the limit of our time resolution).

On the basis of an FTIR study of the D1-E189G and S mutants, we confirmed that the flexibility and H-bonding characteristics of D1-E189 are essential for this residue to facilitate the relocation of W3 from Ca to Mn1 during the S_2 to S_3 transition (*3*).

On the basis of a high-frequency pulsed EPR study of the S_3 state performed in collaboration with R. D. Britt and coworkers at UC Davis, we showed that the heterogeneity of the Mn_4Ca observed in previous EPR studies of the S_3 state is caused by a glycerol- or methanol- induced perturbation of the coordination environment of Mn4 and/or Mn3. Importantly, our data provide no evidence for a precursor S_3 state having a closed-cubane conformation with a five-coordinate Mn(IV) ion.

On the basis of EPR and optical absorption measurements performed in collaboration with G. W. Brudvig and coworkers at Yale University, we showed that the D1-S169A mutation alters the Mn_4Ca cluster in a manner that stabilizes the S_2 state and interferes with the binding of NH_3 (*1*).

On the basis of EPR and FTIR measurements performed in collaboration with R. D. Britt and coworkers at UC Davis, we showed that The D1-D170E mutation alters the equilibrium between two conformers of the Mn_4Ca cluster in the S_1 state so that a high spin form of the S_2 state is

produced during the S_1 to S_2 transition instead of the usual low-spin form that exhibits the multiline EPR signal. The mutation also alters the network of H-bonds that surrounds the Mn_4Ca cluster in much the same manner as other mutations constructed in this network and may change the coordination mode of the carboxylate at position 170 (*revised manuscript in review*).

On the basis of a cryo-EM analysis performed in collaboration with G. W. Brudvig and workers at Yale University, we determined the structure of intact wild-type PSII core complexes from *Synechocystis* sp. PCC 6803 to a resolution of 1.93 Å (*revised manuscript in review*). This is the first high-resolution structure of intact PSII from a non-thermophilic organism. This structure shows that significant differences exist between thermophilic and non-thermophilic PSII and will facilitate a reassessment of biochemical/spectroscopic data obtained with non-thermophilic organisms that have been interpreted on the basis of structures of PSII from thermophilic cyanobacteria.

Objectives for the coming year –

- Extend our studies of substrate water exchange kinetics to include mutations of residues located in the O4 (narrow) and O1 (large) H-bond networks (e.g., D1-N87A, D1-N338A, CP43-V410A, and mutations in the D1-65/D1-R334/D2-E312 triad).
- Extend our FTIR studies to include mutations of residues in the O4 and O1 pathways.
- Complete our time-resolved IR studies of proton dynamics in the D1-D61A and D1-N298A mutants and extend these studies to include mutations of residues in the D1-E65/D1-R334/D2-E312 triad, D1-N87, and D1-S169 (work performed in collaboration with H. Dau and coworkers at Freie Universität Berlin and in cooperation with R. L. Burnap and coworkers at Oklahoma State University).
- Further characterize the perturbations to the electronic structure of the Mn_4Ca cluster produced by the D1-D170E mutation and in non-O_2 evolving D1-E189 mutants.
- Obtain high-resolution cryo-EM structures of a number of site-directed mutants, starting with D1-D61A, D1-E189R, D1-D170E, and D1-S169A.

Publications of the last 2 years supported by the award –

1. Ghosh, I., Banerjee, G., Reiss, K., Kim, C. J., **Debus, R. J.**, Batista, V. and Brudvig, G. W. (2020) "D1-S169A substitution of photosystem II reveals a novel S_2-state structure," *Biochem. Biophys. Acta 1861, Article 148301* .
2. Mark, B., Coates, C. S., Sugimura, S., Baldansuren, A., Ku, J., **Debus, R. J.** and Lakshmi, K. V. (2020) "Determining the Electronic Structure of Paramagnetic Intermediates in Membrane Proteins: A high-resolution 2D ^{1}H Hyperfine Sublevel Correlation Study of the Redox-Active Tyrosines of Photosystem II," *Biochim. Biophys. Acta* 1862, Article 183422.
3. Kim, C. J. and **Debus, R. J.** (2020) "Roles of D1-Glu189 and D1-Glu329 in O_2 formation by the water-splitting Mn_4Ca cluster in Photosystem II," *Biochemistry* 59, 3902-3917.
4. Marchiori, D. A., **Debus, R. J.** and Britt, R. D. (2020) "Pulse EPR Spectroscopic Characterization of the S_3 State of the Oxygen-Evolving Complex of Photosystem II Isolated from *Synechocystis*," *Biochemistry* 59, 4864-4873.
5. de Lichtenberg, C., Kim, C. J., Chernev, P., **Debus, R. J.** and Messinger, J. (2021) "The Exchange of the Fast Substrate Water in the S_2 state of Photosystem II is Limited by Diffusion of Bulk Water through Channels – Implications for the Water Oxidation Mechanism," *Chemical Science* 12, 12763-12775.

Resolving protein-semiquinone interactions by advanced EPR spectroscopy.

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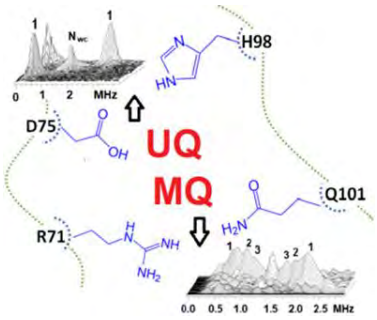
Overall research goals: Our focus is on use of modern, high-resolution EPR spectroscopy to explore the catalytic domains trapped in states with semiquinone (SQ) as an intermediate. The catalytic sites we study, - the Q_A and Q_B sites of the bacterial reaction center (RC), the Q site of the cyt bo_3 quinol oxidase, and the Q_i site of the bc_1 complex, - all operate using ubiquinone, but have different electron transfer partners, and different operating potentials. EPR probes interactions between the electron spin of SQ and local magnetic nuclei, which provide direct information about *spatial* and *electronic* structure of the SQ and the immediate protein and solvent environment. The main question to be addressed is that of how the protein environment modifies the spatial and electronic structure of the SQ in different sites to fit the physiological function.

Significant achievements 2019-2021:

The ubiquinol binding site of cyt bo_3 from E. coli accommodates menaquinone and stabilizes a functional menasemiquinone. Cyt bo_3, one of three terminal oxygen reductases in the aerobic respiratory chain of *E. coli*, has been well characterized as a ubiquinol oxidase. Its ability to catalyze the two-electron oxidation of ubiquinol-8 requires the enzyme to stabilize the one-electron oxidized ubisemiquinone (USQ) species that is a transient intermediate in the reaction. This USQ was detected and studies in detail using EPR techniques. Cyt bo_3 has been shown recently to utilize also demethylmenaquinol-8 as a substrate which, along with menaquinol-8, replaces ubiquinol-8 when *E. coli* is grown under micro-aerobic or anaerobic conditions. In the current work, we show that steady-state turnover with 1,3-dimethyl-2,4-naphthoquinone (DMN) a water-soluble menaquinol analogue, is just as efficient as with ubiquinol-1. These findings imply that the menasemiquinone (MSQ) must also be stabilized by interacting with the active site residues of cyt bo_3. The suggestion was supported by previous studies on the cyt aa_3-600 menaquinol oxidase from *Bacillus subtilis*, a close homologue of cyt bo_3 that utilizes menaquinol-7 as its native substrate and stabilizes a MSQ species. Based on the similarity of the amino acid sequences four polar residues have been implicated in binding to the quinol at the Q-site in cyt bo_3 and cyt aa_3-600: R71(R70), D75(D74), H98(H94), and Q101(E97) (*B. subtilis* numbering).



Using pulsed EPR spectroscopy and ^{15}N uniform labeling of the protein, we demonstrate that the same residues in cyt bo_3 that stabilize the USQ state also stabilize the MSQ state of menaquinone-4, with the hydrogen bond strengths and the distribution of unpaired spin density accommodated to the different substrate. Catalytic function with menaquinol is more tolerant of mutations at the active site than with ubiquinol. A mutation of one of the stabilizing residues (R71H in subunit I) that eliminates ubiquinol oxidase activity of cyt bo_3 does not abolish activity with soluble menaquinol analogues. **Ref. 2.**

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

isotropic hyperfine interaction. Their appearance is regulated by the relative values of the strain width Δa and parameter $\delta = |2a+T|-4\nu_{15N}$. Clearly resolved effects from the a-strain are only expected to appear when part of the strain broadened cross-ridge approaches or satisfies the condition $(2(a\pm\Delta a)+T) \sim 4\nu_{15N}$. The intensity and shape of the cross-features in the opposite quadrant depend on the strain distribution function and the Δa and δ values. We found similar lineshape distortions in previously published spectra from ^{15}N, ^{57}Fe ([Fe-Fe]-hydrogenase), ^{29}Si (Ti^{3+} in SiO_2–TiO_2), and ^{31}P (VO^{2+} in bones and catalytic systems) nuclei that can be explained using the approach applied for the analysis of the ^{15}N spectra of SQs. These examples clearly show that hyperfine strain produces new spectral features in 2D spectra, and ignorance of their true nature may lead to errors in spectral interpretations and resulting structural models. **Ref. 4.**

Collaborative work. The work also includes the methodological development of two-dimensional pulsed EPR spectroscopy (HYSCORE) and *E. coli* amino acid auxotrophic expression strains for the structure-function studies of recombinant proteins. and the application of these approaches to metalloclusters through informal collaborations with several laboratories of PIs supported by the Physical Biosciences program or involved in the projects funded by the BES. Our experience in pulsed EPR and available state-of-the-art equipment has attracted the attention of researches from the UIUC and other laboratories. The spectroscopic approach and isotope labeling methodology developed under this project was proven completely applicable to more complex systems as paramagnetic metals and clusters in proteins and other systems including frozen solutions and catalysts. Currently we collaborate with several laboratories of PIs supported by the Physical Biosciences program or involved in the projects funded by the BES. Most successful is the collaborative work with the laboratory of Prof. Yi Lu (UT Austin, formerly UIUC) devoted to the studies of an engineered binuclear Cu_A (Cu_2S_2) site in azurin and cytochrome *c* peroxidase with the aim to characterize peculiarities of an electronic structure. **Refs. 1,3.**

Science objectives for 2021-2022:

- We continue studying the influence of mutations on SQ_A in bacterial reaction center from *Rb. sphaeroides*. Our focus is on the M265(IS,IT,IN) mutations induced 80-120 mV negative shifts in the E_m of Q_A, with little perturbation of any other properties, including binding affinity.
- Previous studies showed that the 2-methoxy group is essential for simultaneous function of Q_A and Q_B, i.e. electron transfer from Q_A to Q_B works for 2-CH_3O-Q and does not work for 3-CH_3O-Q. We characterize the interactions of the reconstituted 2- and 3-monomethxyQs with protein in the Q_A^- state by performing 2D ESEEM and ENDOR in native and ^{15}N labeled RC, and H_2O or D_2O solvent.

References to work supported by this project 2019-2021:

1. Dikanov, S.A., Berry, S.M., Lu, Y. (2019) HYSCORE insights into the distribution of the unpaired spin density in an engineered Cu_A site in azurin and its His120Gly variant. *Inorg. Chem*. **58**, 4437–4445.
2. Ding, Z., Sun, C., Yi, S.M, Gennis, R.B., Dikanov, S.A. (2019) The ubiquinol binding site of cytochrome bo_3 from *Escherichia coli* accommodates menaquinone and stabilizes a functional menasemiquinone. *Biochemistry* **58**, 4559−4569.
3. Mirts, E.N., Dikanov, S.A., Jose, A., Solomon, E.I., Lu, Y. (2020) A binuclear Cu_A center designed in an all α-helical protein scaffold. *J. Am. Chem. Soc.* **142**, 32, 13779–13794.
4. Dikanov, S.A., Taguchi, A.T. (2020) Influence of hyperfine coupling strain on two-dimensional ESEEM spectra from I=1/2 nuclei. *Appl. Magn. Reson*. **51**, 1177-1200.
5. Iwasaki, T. Miyajima-Nakano, Y., Fukazawa, R., Lin, M.T., Matsushita, S., Hagiuda, E., Taguchi, A.T., Dikanov, S.A., Oishi, Y., Gennis, R.B. (2021) *Escherichia coli* amino acid auxotrophic expression host strains for investigating protein structure-function relationships. *J. Biochem*. **169**, 387–394. *(cover image)*
6. Crofts, A.R. (2021) The modified Q-cycle: A look back at its development and forward to a functional model. *Biochim. Biophys. Acta* **1862**, 148417.
7. Samoilova, R.I., Dikanov, S.A. Local environment of superoxide radical formed on the TiO_2 surface produced from $Ti(OiPr)_4$ exposed to H_2O_2. *Appl. Magn. Reson*. (accepted, https://doi.org/10.1007/ s00723-021-01424-0).

Understanding PSII Energy Conversion in PSIIX Crystals & *In vivo*

Principal Investigator: Charles Dismukes

Poster participants: Apostolos Zournas and Christopher Turner

Other coauthors: Kyle Mani, Colin Gates, Gennady Ananyev

Institution: Dept. Chemistry & Chemical Biology; Waksman Institute of Microbiology, Rutgers University

Project <u>Goal</u>: Understanding the molecular origin of photosynthetic inefficiencies.

- Aim 1. Cobalt substitution in the PSIIX-WOC core (Mn_4CaO_5) studied by photoassembly of the inorganic cofactors.
- Aim 2. Regulation of PSII turnover in living cells using microkinetic modelling.
-

Funding Period: 1 year + NCE, 2020-2021

Significant achievements of the past 18 months –

Aim 1. A) Selective reductants of the PSII-WOC in ultrapure crystals reveals Mn redox state & E_m^0. PSIIX crystals that are recrystallized to homogeneity have exceptional purity that allows unprecedented control of the chemical environment and is unachievable using conventional (impure) PSII complexes. We have titrated PSIIX using selective reductants that transfer either 1H, 3H or 4H (hydrogen atoms) at precise electrochemical potentials. Our results show two interesting outcomes: (a) the S_{-3} state is the lowest chemically stable redox state of the WOC that can be produced by concerted 4H reducing agents. This state releases 4 Mn^{2+}/WOC. There is no stable S_{-4} state; (b) The E_m^0 for reduction of the WOC can be estimated from this.

B) We developed a method to <u>reversibly</u> remove >99% inorganic elements from the WOC in PSIIX without losing crystallinity. The resulting apo-WOC-PSIIX can be reassembled in light using $Mn^{2+}$and Ca^{2+}, restoring >90% activity It has identical kinetics as isolated PSII w/o extrinsic subunits, indicating there is no kinetic role for these protein subunits during photoassembly. Hence, the low QY per flash during photoassembly is related to the redox instability of the oxidized intermediates, and not dependent on the extrinsic protein subunits.

C) We created the first functional "inorganic mutant" of the Manganese cofactor by photoassembly of apo-WOC-<u>PSIIX</u> with Cobalt. Co^{2+} + apo-WOC-PSIIX photoassembles 3X faster than Mn^{2+} and produces 4 X less O_2 /flash. (a) The 3X faster photoassembly kinetics suggests that proton release from the more acidic Co^{3+} assembly intermediate vs the less acidic Mn^{3+} assembly intermediate could be responsible for the acceleration. (b) The 4X lower O_2 yield of the fully reconstituted WOC using Cobalt appears to be due to faster charge recombination in the upper S states which competes with the slower O_2 evolution step: $S_4 \rightarrow S_0 + O_2$. (c) Reconstituted PSIIX(Co) activity degrades 3X faster under illumination than does PSIIX(Mn).

Photogenerated Co^{4+} may be oxidizing the PSII protein scaffold damaging it faster than Mn^{4+}. These properties can explain why cobalt is not found in any native PSII center *in vivo.*

Aim 2.
Goal: Replace the black-box Markov models of PSII turnover (phenomenological Joliot-Kok models) with a micro-kinetic model that uses actual kinetic expressions for the time dependence of individual PSII electron & proton steps. This approach will enable identification of which steps in PET are responsible for overall PSII performance.
Details: We developed a simulation package (called RODE) in Python that uses Ordinary Differential Equations to calculate fluxes in realtime for 96 reactions involving the WOC, Yz, P680, Q_A & Q_B.
Achievements: (a) *RODE* simulates the time evolution of O_2 and variable fluorescence (Fv). (b) *RODE* predicts which reaction steps influence/cause photochemical misses, double turnovers, backward transitions & photoinactivation used in Markov models (eg. Joliot-Kok and VZAD).
Ongoing work. (a) Introduce alternative ET-PT pathways in order to account for backward transitions responsible for PSII-CEF. (b) Applications of *RODE* to diverse algal & plant systems.

Objectives for the coming year (If Funded) –
Aim 1. Ground state electronic structure of cobalt reconstituted PSIIX by EPR.
Aim 2. Add a predictive model for PSII-CEF and test it experimentally. (b) Extend RODE to with additional microstates that can account for backward transitions responsible for PSII-CEF.
Aim 3 (New). Develop a fluorescence method to monitor the CO2-dependent bottlenecks in electron flow between water oxidation and CO_2 carboxylation at RuBisCO.

Publications of the last 18 months supported by the award –

1) Zhang, Y. Maliga, P., G. C. Dismukes, Engineering Photosystem II Subunit D1 in Nicotiana tabacum to increase photosynthesis at extreme light intensities. *Submitted,* in revision.
2) Gates, C., G. Ananyev, S. Roy-Chowdhury, B. Cullinane, M. Miller, P. Fromme and G.C. Dismukes, Can oxygenic photosynthesis function without manganese in vivo? Cobalt replacement in the water-oxidizing complex of PSII. *J. Phys. Chem. C, in review.*
3) Mani, K., A. Zournas and G.C. Dismukes, Bridging the gap between Kok-type and kinetic models of photosynthetic electron transport within PSII**.** ***Photosynth Res****, 2021. Aug 16.* DOI: 10.1007/s11120-021-00868-6
4) Contact us for a forthcoming release of ***RODE v1 python.***
5) ten Veldhuis, M.-C., G. Ananyev and G.C. Dismukes, Symbiosis extended: exchange of photosynthetic O2 and CO2 mutually power metabolism of lichen symbionts. ***Photosyn. Res****, 2020* 143: p. 287–299.
6) Potkay, A., M.C. Ten Veldhuis, Y. Fan, C.R.C. Mattos, G. Ananyev and G.C. Dismukes, Water and Vapor Transport in Algal-Fungal Lichen: Modeling constrained by Laboratory Experiments, an application for *Flavoparmelia caperata*. ***Plant Cell Environ****, 2020. 43(4): p. 945-964.*
7) Gates, C., G. Ananyev and G.C. Dismukes*, Realtime kinetics of the light driven steps of photosynthetic water oxidation in living organisms by "stroboscopic" fluorometry.* ***Biochim Biophys Acta Bioenerg****, 2020. 1861(8): p. 148212.*

Engineering Heterologous Metabolism in Cyanobacteria and Interrogating Impacts on Photosynthetic Performance (*subproject of Photosynthetic Energy Capture, Conversion and Storage*)

PI: Christoph Benning. Co-Investigators: Federica Brandizzi, **Daniel C. Ducat**, Gregg A. Howe, Jianping Hu, Cheryl A. Kerfeld, David M. Kramer, Beronda L. Montgomery, Thomas D. Sharkey, Josh Vermaas, Berkley Walker.
Michigan State University-DOE Plant Research Laboratory, East Lansing, Michigan 48824
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Overall goals of the project:

One theme of the collaborative activities at the MSU-DOE Plant Research Laboratory relates to integration of new modules of function into photosynthetic organisms, and design strategies that minimize unintended harmful effects from such heterologous pathways and circuits. Two representative project areas will be discussed related to this broader theme: 1) It has been recently hypothesized that cyanobacterial strains engineered to encode some heterologous metabolic pathways may exhibit improved photosynthetic performance in part due to an alteration in source/sink balances. In collaborative efforts, we have explored the interconnectivity between endogenous electron valves (*i.e.*, flavodiiron proteins and alternative oxidases), and the activation of one or more heterologous metabolic sinks - a sucrose production pathway (consuming ATP and reductant) and a reductant-only-consuming cytochrome P450. We find that expression of one or both heterologous sinks show additive impacts on photosynthesis including improved quantum yield of PSII and enhanced electron transport chain flux. Heterologous sinks may partially compensate for the loss of PSI oxidizing factors, although this compensation is incomplete. Furthermore, we find adaptations in the carbon concentrating mechanisms of cyanobacteria following engagement of such metabolic sinks. Our data suggest that Rubisco activity and organization are key outputs connected to regulatory pathways involved in metabolic balancing in cyanobacteria. 2) In order to improve pathway efficacy and control, enzymes of natural biochemical pathways are routinely subcellularly organized in space and time. By contrast, gene products for introduced metabolic pathways typically do not take advantage of the benefits of such spatio-temporal regulation. Decreasing toxic/destructive interactions of introduced pathways with endogenous metabolism so that benefits like those described above can be realized will likely require more advanced organizational approaches in engineered cells. In collaborative work, we have explored the use of bacterial microcompartment (pfam0936-domain) proteins as modules for constructing well-defined nanometer scale scaffolds *in vivo*. As part of this project, we have explored different approaches to confer new properties onto the shell proteins, including incorporation of redox-mediating cofactors, such as hemes.

Significant achievements of the past 2 years:

- We found additive enhancements on cyanobacterial photosynthetic parameters following expression of more than one heterologous sink (sucrose export and cytochrome P450)

- We observed partial rescue of PSI oxidation in sink-activated cyanobacterial strains deficient in the photoprotective proteins, Flv1/3, suggesting that diversion of excess photosynthetic capacity to productive processes can improve photosynthetic conversion efficiencies.
- In response to altered carbohydrate availability (sucrose export or import) in cyanobacteria we observe regulation centered around Rubisco activity and organization.
- We described design principles for modifying/controlling assembly of microcompartment shell proteins for use as customizable intracellular scaffolds and redox carriers.

Objectives for the coming year:

- Elaborate upon a network of two-component signaling proteins that our preliminary data suggests are involved in mediating source/sink balancing in the model cyanobacterium, *Synechococcus elongatus* PCC 7942.
- Investigate the hypothesis that dynamic carboxysome remodeling is mediated in part through the activity of factors with known roles in carboxysome positioning (*i.e.*, Maintenance of carboxysome distribution; McdAB).
- Detail post-translational modifications of Rubisco that arise in response to changes in carbohydrate export and/or import.
- Evaluate the capacity of the McdAB system to allow for positioning of synthetic scaffolds constructed from bacterial microcompartment shell proteins.

Representative Publications (last 2 years) Supported by the Award

1. Santos-Merino, M., A. Torrado, G. A. Davis, A. Rottig, T. S. Bibby, D. M. Kramer, and D. C. Ducat. 2021. Improved photosynthetic capacity and photosystem I oxidation via heterologous metabolism engineering in cyanobacteria. *PNAS* 118: https://doi.org/10.1073/pnas.2021523118.
2. Sakkos, J.K., Hernandez-Ortiz, S., Osteryoung, K.W. and Ducat, D.C., 2021. Orthogonal Degron System for Controlled Protein Degradation in Cyanobacteria. *ACS Synthetic Biology*, 10(7), pp.1667-1681. https://doi.org/10.1021/acssynbio.1c00035
3. Santos-Merino, M., Singh, A.K. and Ducat, D.C., 2021. Sink engineering in photosynthetic microbes. *Cyanobacteria Biotechnology*, pp.171-209. https://doi.org/10.1002/9783527824908.ch6
4. Huang, J., Ferlez, B.H., Young, E.J., Kerfeld, C.A., Kramer, D.M. and Ducat, D.C., 2020. Functionalization of bacterial microcompartment shell proteins with covalently attached heme. *Frontiers in bioengineering and biotechnology*, 7, p.432. https://doi.org/10.3389/fbioe.2019.00432
5. Young, E.J., Sakkos, J.K., Huang, J., Wright, J.K., Kachel, B., Fuentes-Cabrera, M., Kerfeld, C.A. and Ducat, D.C., 2019. Visualizing in vivo dynamics of designer nanoscaffolds. *Nano letters*, 20(1), pp.208-217. https://doi.org/10.1021/acs.nanolett.9b03651
6. Huang, J., Zarzycki, J., Gunner, M.R., Parson, W.W., Kern, J.F., Yano, J., Ducat, D.C. and Kramer, D.M., 2020. Mesoscopic to macroscopic electron transfer by hopping in a crystal network of cytochromes. Journal of the American Chemical Society, 142(23), pp.10459-10467. https://doi.org/10.1021/jacs.0c02729
7. Singh, A.K, Santos-Merino, M., Sakkos, J.K., Walker, B.J., Ducat D.C. 2021 Multi-layer Regulation of Rubisco in Response to Altered Carbon Status in *Synechococcus elongatus* PCC 7942. *In Revision.*

Vibronic Coupling Steers Energy Transfer within FMO in Response to Oxidative Conditions Driving Changes in Excited State Quantum Beating Signatures

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Overall goals of the project

We seek to map energy transfer pathways through photosynthetic light harvesting complexes to understand mechanisms that control and direct energy flow within and between complexes.

Significant achievements of the past 2 years

My research group seeks to dissect microscopic mechanisms and quantum dynamics of photosynthetic light harvesting. Fundamentally, we want to know how photosynthetic antenna complexes collect light and funnel it to the reaction center with high quantum efficiency and exquisite precision. Recently, we acquired data showing a complex interplay between the chlorophyll molecules and their environment within the protein. Specifically, the Fenna–Matthews–Olson complex uses redox-active cysteine residues to tune the resonance between its excitons and a pigment vibration to steer excess excitation toward a quenching site. The resonance between delocalized excited states and vibrations on individual chromophores is used to steer excitonic energy toward the reaction center. This data has forced us to reconsider the role of a structured environment in energy transfer.

These same redox conditions that moderate the vibronic coupling affect the excited state quantum coherences, or beats, observed in ultrafast optical experiments when light-matter interactions from a coherent source drive a system out of equilibrium. In our ultrafast two-dimensional electronic spectroscopy measurements on the Fenna-Matthews-Olson antenna complex, we observe that many excited state coherences present in reducing conditions, which more closely mimic the natural conditions of the complex, are absent or attenuated in oxidizing conditions. Further, the presence of these coherences is correlated with increased vibronic coupling in the system and faster, more efficient energy transfer through the complex. Our results suggest that excitonic energy transfer proceeds through a coherent mechanism in this complex and that the coherences may provide a tool to disentangle coherent relaxation from energy transfer driven by stochastic environmental fluctuations.

Objectives for the coming year

We are currently applying our spectroscopy to phycobilisomes from *Synechococcus elongatus* PCC 7942 exploring similar intrinsic photoprotection mechanisms as described above. We have found annihilation dynamics in the rods of the phycobilisome that surprisingly can be relevant under ambient lighting conditions. We are also exploring miscroscopic mechanisms of quenching of excitation in the presence of orange carotenoid protein.

Publications of the last 2 years supported by the award –

Jacob S. Higgins, Lawson T. Lloyd, Sara H. Sohail, Marco A. Allodi, John P. Otto, Rafael G. Saer, Ryan E. Wood et al. "Photosynthesis tunes quantum-mechanical mixing of electronic and vibrational states to steer exciton energy transfer." *Proceedings of the National Academy of Sciences* 118, no. 11 (2021).

Mykyta Onizhuk,* Siddhartha Sohoni,* Giulia Galli, and Gregory S. Engel. "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, no. 29 (2021): 6967-6973.

Brian S. Rolczynski, Shu-Hao Yeh, Polina Navotnaya, Lawson T. Lloyd, Alan R. Ginzburg, Haibin Zheng, Marco A. Allodi et al. "Time-Domain Line-Shape Analysis from 2D Spectroscopy to Precisely Determine Hamiltonian Parameters for a Photosynthetic Complex." *The Journal of Physical Chemistry B* 125, no. 11 (2021): 2812-2820.

Jacob S. Higgins,* Marco A. Allodi,* Lawson T. Lloyd, John P. Otto, Sara H. Sohail, Raphael G. Saer, Ryan E. Wood, Sara C. Massey, Po-Chieh Ting, Robert E. Blankenship, Gregory S. Engel, "Redox conditions correlated with vibronic coupling modulate quantum beats in photosynthetic pigment-protein complexes", PNAS (accepted) 2021

Multidisciplinary Tools for Illuminating the Details of Photosynthetic Light Harvesting Systems

Matthew B. Francis, Principal Investigator
Naomi S. Ginsberg, Phillip L. Geissler, Masakazu Iwai, and Jeffrey B. Neaton, Co-PIs
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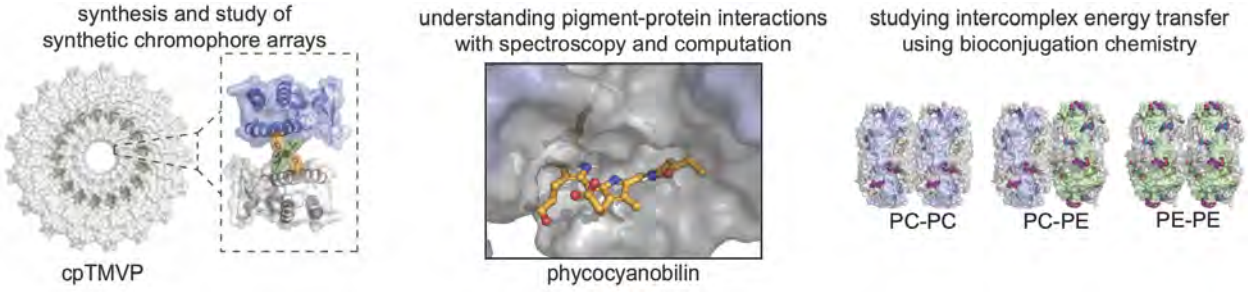
Email: mbfrancis@berkeley.edu

Overall research goals: Photosynthetic organisms rely on a series of complex light harvesting assemblies to absorb broad spectrum light and transfer the resulting excitations to the reaction centers. We are applying an integrated set of biological, chemical, spectroscopic, and computational tools to examine how the many functional components of these systems work in concert to yield higher-level function. The platforms under study include (1) engineered tobacco mosaic virus coat proteins that have been synthetically modified to produce synthetic chromophore arrays, (2) systematically altered components of phycobilisomes for the study of chromophore-protein interactions, and (3) protein complexes joined by well-defined linkages for studying energy transfer over long distances.



Significant achievements 2018-2021: Two versatile self-assembling protein scaffolds have been developed for the construction of pigment arrays with different geometries and multiplicities. These are (1) a C_2-symmetric double disk structure based on a circularly permuted tobacco mosaic virus coat protein (cpTMVP) and (2) new gene-doubled variant (dTMVP) that breaks the symmetry between the top and bottom disks to provide asymmetric protein environments for chromophores. Using these constructs:

- Ginsberg, Francis, Geissler and Neaton have introduced chromophores on both the inside and the outside surfaces of cpTMVP assemblies. Synthetically tailored linking groups have revealed that the protein environment can slow the fast relaxation processes of the chromophores—a feature believed to be crucially important for the function of natural light harvesting systems.[1]
- Time resolved fluorescence polarization spectroscopy has been used to study energy transfer in synthetic chromophore arrays. These data have been fit to simulation data to estimate the rates of energy transfer between the pigment molecules.
- Protein bioconjugation methods have been developed to couple cpTMVP-based chromophore arrays through well-defined linkages. Energy transfer has been measured between disks bearing donor and acceptor chromophores.

- Guided by MD simulations, rational design has been used to engineer cpTMVP double disks that can house arrays of porphyrin and chlorophyll molecules in a central channel. The spectroscopic, EPR, and electrochemical properties of these arrays have been determined.[2]
- Efficient enzymatic oxidative coupling reactions have been used to couple cpTMV disks bearing artificial amino acids to metal particle surfaces.[3]
- A new TMV coat protein dimer has been developed that allows the top and bottom disk surfaces to be independently addressed. This construct has been used to prepare heme and chlorophyll arrays in asymmetric ligation environments, and it has been attached to supported lipid bilayers to afford systems that mimic the fluidity of natural LH2 complexes.
- Continued advances have been made in the development of excited state electronic structure calculations that can address large chromophores and evaluate their coupling to surrounding protein environments.[4,5]

Science objectives for 2021-2022:

- A systematic series of phycocyanin proteins with minimal chromophore sets is being produced and studied spectroscopically.
- An enzymatic oxidative coupling method is being used to couple intact phycobilisome complexes through well-defined linkages. These constructs will be used to study energy transfer pathways between the assemblies.
- A heterologous expression system is being implemented to perform deep mutational scanning on phycobilisome complexes in *E. coli* hosts.
- Time-resolved ultrafast imaging approaches are being used to map energy migration through large chromophore assemblies with high spatial and temporal resolution. In particular, these methods will be applied to light harvesting complexes embedded in liposomes.
- Computational efforts are continuing to simulate the bath fluctuations that occur in both natural and synthetic light harvesting systems and relate them to energy transfer performance.
- Combinations of the MD and DFT methods developed in this program are being used to study the complex photophysical behavior of phycobilin pigments in different protein environments.

References to work supported by this project 2018-2021:

1. Delor, M.; Dai, J.; Roberts, T. D.; Rogers, J. R.; Hamed, S. M.; **Neaton, J. B.**; **Geissler, P. L.**; **Francis, M. B.**; **Ginsberg, N. S.** "Exploiting Chromophore–Protein Interactions through Linker Engineering To Tune Photoinduced Dynamics in a Biomimetic Light-Harvesting Platform" *J. Am. Chem. Soc.* **2018**, *140*, 6278-6287.
2. 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.
3. 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.
4. 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.
5. 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.

Towards Structure Determination of the of the Photosynthetic Supercomplex to understand the Regulation of sustained Cyclic Electron Flow (CEF) in the Antarctic photopsychrophile Chlamydomonas sp. UWO241

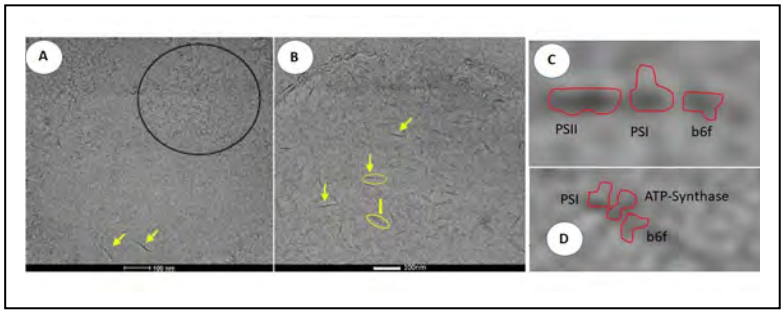
Principal Investigator name: Petra Fromme
Other Investigators: Jay-How Yang and Jackson Carrion
Address: Biodesign Center for Applied Structural Discovery& School of Molecular Sciences, Arizona State University, 1001 S McAllister Arizona State University, 85287-7401Tempe, AZ
Contact information for Principal Investigator: E-mail: pfromme@asu.edu, phone: 480 965 9028 7840 (office) and 480 326 7840 (cell)

Overall goals of the project: The overall goal of this project (led by the main PI Rachael Morgan-Kiss) is to describe the function of sustained CEF and assembly of the PSI supercomplex in the Antarctic psychrophile Chlamydomonas sp. UWO241. This part of the project at ASU led by Petra Fromme aims to determine the structure of the UWO241 PSI supercomplex through cryo-EM studies. Outcomes of this project will support research focused on meeting future energy and food needs by advancing our understanding of how extremophilic phototrophs use sustained CEF and rewired carbon metabolism to survive long-term exposure to environmental stressors. We aim to gain detailed information on the protein composition and the interaction of the proteins and cofactors in the supercomplex at molecular resolution. The goal is to identify the key amino acids in the formation and stabilization of the supercomplex including details on the cofactor interactions that will reveal the electron transfer pathways that drive the cyclic electron transfer in the supercomplex.

Significant achievements of the past 2 years

The major effort during the last year has focused on the stabilization of the supercomplex in preparation for the cryo-EM studies. In our initial studies the supercomplex was stabilized by extraction in the mild detergent alpha-dodecylmaltoside. The complex was stable in sucrose and could be isolated by sucrose density gradient centrifugation, for functional studies and biochemical characterization. However, EM studies cannot be performed in the presence of sucrose. However, the removal of sucrose was a huge challenge, as the supercomplex disintegrated upon removal of sucrose even using very fast methods like use of desalting centrifugation columns, where the time from sucrose removal to deposition on the cryo-EM grid was < 2 min. We investigated different approaches that included stabilization of the supercomplex by divalent cations, where we found that Mg2+ stabilized the complex but led to aggregation. We also investigated different crosslinking strategies but they also led to protein aggregation. We have used negative stain EM with our screening microscope at ASU to visualize the supercomplexes isolated under different conditions with the goal to optimize the isolation and grid preparation conditions towards conditions suitable for high resolution cryo-EM studies. The most promising conditions were achieved by removing the sucrose directly on the grid from samples isolated at ASU freshly from frozen cells. The samples were imaged by cryo-EM using our Titan Krios instrument with a K2 detector and show that the supercomplex forms elongated particles 300-500A in size (Figure 1).

Figure 1: Cryo-EM of the Supercomplexes *(A) Supercomplexes isolated from cells shipped at 4C to ASU showed supercomplexes (elongated particles of 400-500A length) and aggregates (black circle)* ***B) Supercomplexes*** *freshly prepared at ASU from frozen cells. High concentration of supercomplexes were observed suitable for single particle image analysis, which is ongoing. Selected particles enlarged from (B) in C and D provide indication that multiple different supercomplexes can be isolated, which will be sorted in different classes by image analysis.*



Most recently we have also investigated the further stabilization of the supercomplexes by decrease of the detergent concentration and stabilization by lipids with very promising results (Figure 2).

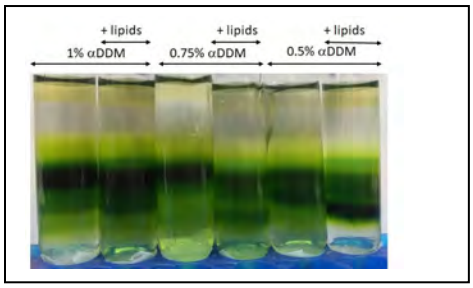


Figure 2: The supercomplex stability is dramatically improved by reduction of the detergent concentration and the addition of lipids during the isolation procedure. *Reduction of the detergent concentration (1%, 0.75% and 0.5%) during the solubilization at 0.45mM Chl and addition of lipids (1mg/ml asolection) during the purification strongly stabilized the supercomplexes*

Objectives for the coming year *Functional studies with ultrafast spectroscopy*

The objective for the coming year will be to extend our functional studies to characterize the function of the supercomplexes by ultrafast spectroscopy. Here we plan to use the streak camera techniques that have been successfully used in the past to characterize the efficiency of the large Photosystem I-IsiA complex (Chauhan et al Biochemistry 2011) to study the light capturing and electron transfer events in the supercomplex from the arctic green algae Chlamydomonas sp. UWO24.

Stabilization of the supercomplex by detergent-free extraction in native nanodiscs

Our most recent studies showed that we can stabilize the supercomplex further by its isolation in the presence of lipids. The next objective is to further stabilize the supercomplex by native nanodiscs (DIBMA). We plan to use this alternate approach to achieve detergent-free extraction of the supercomplex from the membrane and maintain it in the native lipids environment for the structural studies by Cryo-EM. The goal will be to determine its structure in its native lipid environment.

Publications of the last 2 years supported by the award

Yang, J.H., Carrion, J., Morgan-Kiss, R. and Fromme, P.
"Isolation, characterization, stabilization and characterization of the Photosystem I supercomplex from the Antarctic photopsychrophile green algae Chlamydomonas sp. UWO241 for cryo-electron microscopy studies." **J of Protein Expression and Purification in preparation**

This publication will be followed by the publication on the first structure of the supercomplex by Cryo-EM.

Function of the Low Molecular Mass Dicluster Ferredoxins in *Heliobacterium modesticaldum*

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[1]Department of Biochemistry and Molecular Biology, [2]Department of Chemistry, The Pennsylvania State University, University Park, PA 16802

Overall goals of the project – The most studied Type 1 RC, Photosystem I (PS I), is a heterodimer of the PsaA and PsaB core polypeptides, but it also contains an 8.9 kDa polypeptide known as PsaC that ligates two [4Fe-4S] clusters F_A and F_B. PsaC is tightly bound to the PS I core and can only be displaced using chaotropic agents such as 6.8 M urea. In contrast, while the Type 1 reaction center from *Heliobacterium modesticaldum* is a homodimer of the PshA core polypeptide, it does *not* contain a tightly bound dicluster ferredoxin. Instead, the dicluster ferredoxins PshBI and PshBII are associated with the reaction center at low ionic strengths but they are easily removed with 0.5 M NaCl. Annotation of the *H. modesticaldum* genome shows the presence of six low molecular mass proteins denoted PshB1(HM1_1462), PshB2 (HM1_1461), HM1_2505, HM1_0357, HM1_2767 and FdxB (HM1_0860) that may function as diffusible electron acceptors from F_X (**Figure 1**). The goal of this project is to determine the roles of these low molecular mass ferredoxins, specifically, which of them have the ability to accept electrons from F_X.

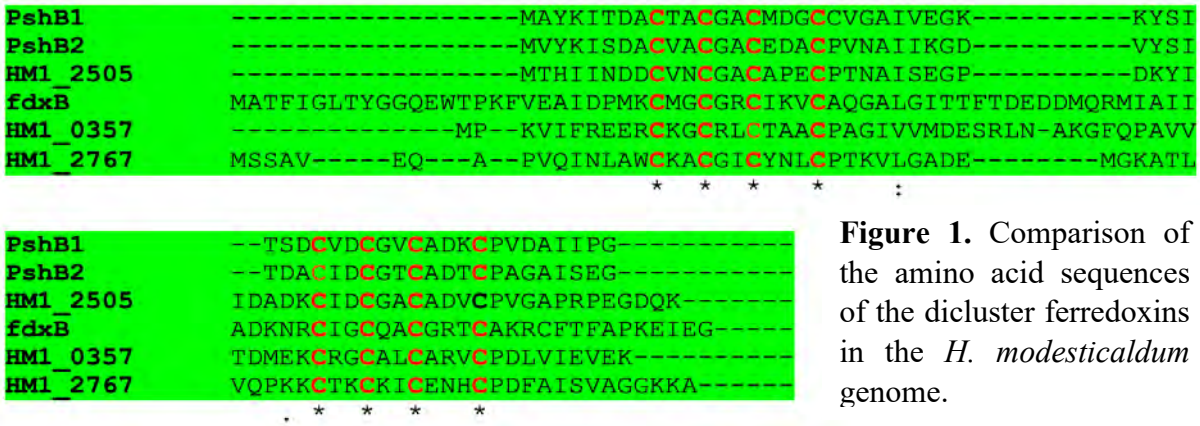

Figure 1. Comparison of the amino acid sequences of the dicluster ferredoxins in the *H. modesticaldum* genome.

Significant achievements of the past 2 years – The low molecular weight ferredoxins PshB1, PshBII, HM2505, HM1_2505, HM1_0357 and FdxB were cloned and expressed in *Escherichia coli.* Each was characterized by optical and EPR spectroscopy, and redox potentiometry. Using analytical techniques such as proteomics, pull down assays, and time resolved optical spectroscopy, we found that **PshB1**, **PshB2** and **HM1_2505** accept electrons directly from F_X; these three ferredoxins are acidic and have pI values less than 4.5 (**Figure 2**). In contrast, **HM1_0357, HM1_2767,** and **FdxB** do not accept electrons from F_X; these three ferredoxins are basic and have pI values greater than 7.4. The conclusion is that electrostatic interactions govern whether these ferredoxins are capable of interacting with the HbRC. Proteomic analysis of *H. modesticaldum* grown under nitrogen replete and deplete conditions shows significant expression of the *fdxB* gene only in the latter, along with the *nif* genes involved in nitrogen fixation. Therefore, there is a high probability that FdxB plays a role in supplying electrons required for nitrogen

fixation. It has been assumed that FdxB accept electrons from the HbRC, which would then reduce *nifH* and finally the MoFe protein of nitrogenase. However, based on our studies, **FdxB** does not obtain its electrons directly from F_X.

Ferredoxin	M.W (Da)	E_m	pI
PshB1	5440	-480 ± 11 mV[5]	4.37
		-524 ± 13 mV[5]	
PshB2	5379	-453 ± 6 mV[5]	3.76
		-527 ± 6 mV[5]	
HM1_2505	6225	-457 ± 5 mV[5]	4.11
		-533 ± 8 mV[5]	
FdxB	9838	-419 ± 2 mV [un]	7.45
		-473 ± 2 mV [un]	
HM1_0357	7459	-	8.15
HM1_2767	7515	-	8.68

Figure 2. Comparison of the molecular masses, midpoint potentials (where measured) and pI values of the low molecular mass ferredoxins in the *H. modesticaldum* genome.

[5]Redox titrations from: B. Ferlez, J. Cowgill, W. Dong, C. Gisriel, S. Lin, M. Flores, K. Walters, D. Cetnar, K.E. Redding, J.H. Golbeck, *Biochemistry*, 55 (2016) 2358-2370.

Objectives for the coming year – Deletion mutant strains ΔPshB1, ΔPshB2, ΔPshB1/ΔPshB2 and ΔHM1_2505 were supplied by Kevin Redding's group and the cells were grown to stationary phase. The cell lysates were analyzed by mass spectroscopy, and the results show the expected absence of the targeted ferredoxin(s). Objectives for the coming year include (i) measuring growth curves for the ferredoxin deletion strains in the presence and absence of added fixed nitrogen; (ii) quantitative mass spectrometry studies to determine whether and by how much the non-affected ferredoxins are up- or down-regulated; and (iii) which metabolic pathways are affected by their absence. String analysis from uniport.org hints that PshB1 may be associated with pyruvate ferredoxin oxidoreductase (PFOR), 2-oxoacid ferredoxin oxidoreductase and arginosuccinate lyase; PshB2 with NADH-quinone oxidoreductase, acyl carrier proteins and NADH-dehydrogenase; and HM1_2505 with aminotransferase and ferrous iron uptake protein. These potential protein partners will be studied in future work using pulldown assays of the *H. modesticaldum* cell lysate. Of particular interest is the role of FdxB in nitrogen fixation, and some of next year's work will be devoted to determining from which metabolic pool it receives its electrons and how this influences the energy balance of the cell.

Publications of the last 2 years supported by the award –

A. Agostini, M. Bortolus, B. Ferlez, K. Walters, J.H. Golbeck, A. van der Est, D. Carbonera, Differential sensitivity to oxygen among the bacteriochlorophylls *g* in the Type-I reaction center of *Heliobacterium modesticaldum*, *Photochem Photobiol Sci*, 20 (2021) 747-759.

P. Charles, V. Kalendra, Z. He, M.H. Khatami, J.H. Golbeck, A. van der Est, K.V. Lakshmi, D.A. Bryant, Two-dimensional ^{67}Zn HYSCORE spectroscopy reveals that a Zn-bacteriochlorophyll *aP*' dimer is the primary donor (P840) in the Type-1 reaction centers of *Chloracidobacterium thermophilum*, *Phys Chem Chem Phys*, 22 (2020) 6457-6467.

Z. He, B. Ferlez, V. Kurashov, M. Tank, J.H. Golbeck, D.A. Bryant, Reaction centers of the thermophilic microaerophile, *Chloracidobacterium thermophilum* (Acidobacteria) I: Biochemical and biophysical characterization, *Photosynth Res,* 142 (2019) 87-103.

Involvement of Pyrenoid Putative Methyltransferase in Photosystem I Biogenesis in *Chlamydomonas reinhardtii*

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While oxygen is produced by photosynthetic processes and is critical for most life on earth, it can also react with molecules in the cell and cause oxidative damage. To identify mechanisms that allow cells to cope with high environmental oxygen levels (produced by photosynthesis) and identify processes associated with oxygen sensitivity, we performed a high-throughput oxygen sensitivity screen on a genome-wide insertional, mapped mutant library of the unicellular alga *Chlamydomonas reinhardtii*. This screen identified several genes that, when disrupted, caused oxygen sensitivity. One of these genes encodes a protein with homology to a 'Rubisco' methyltransferase (RMT2). The *rmt2* mutant is defective for photosynthetic electron transport, which is primarily caused by a decrease in the level of PSI (by ~85%). RMT2 appears to be required for efficient PSI biogenesis; the low level of PSI in the mutant explains its oxygen sensitivity. RMT2 localizes to the pyrenoid, an organelle in many algae that houses the CO_2-fixing enzyme Rubisco. While we show that many proteins of the photosynthetic electron transport chain, including those of PSI, appear to be methylated, we have not observed a difference in the methylation pattern in the *rmt2* mutant and wild type cells. We have recently used proximity tagging, which indicates that RMT2 interacts with various proteins associated with PSI and its biogenesis. While we still cannot be sure if RMT2 has methyltransferase activity, the relationship between RMT2, PSI biogenesis and the pyrenoid is intriguing and raises the possibility that this compartment participates in the biogenesis of photosynthetic complexes.

Studies of Photosynthetic Reaction Centers and Biomimetic Systems

Principal Investigator name Marilyn Gunner
Other participant names as appropriate **Victor S. Batista,** Gary W. Brudvig, William Armstrong, Richard J. Debus, K.V. Lakshmi, Jimin Wang, Ke R. Yang, Krystle Reiss, Divya Matta.
Name and address of awardee institution: City College of New York
Contact information for Principal Investigator: mgunner@ccny.cuny.edu

Overall goals of the project –
Photosystem II (PSII) – a protein complex found in thylakoid membranes of chloroplasts of higher plants and algae, and in internal membranes of cyanobacteria – is able to oxidize water into oxygen during the light period of photosynthesis. The project uses computational methods including quantum mechanics/molecular mechanics (QM/MM), DFT, Molecular Dynamics, Monte Carlo sampling and Network analysis to study the reactions in PSII and type II bacterial reaction centers (RCs). In PSII water oxidation is catalyzed by the Oxygen Evolving Complex (OEC) deeply buried in the D1 subunit, on the lumen (positive) side of the membrane embedded protein. The OEC, a Mn-Ca-oxo cluster carries out four proton-coupled electron transfers cycling through the five S-states of the Kok-cycle. Quinones buried on the acceptor side remove protons from the stromal (negative) side of the membrane. Our goals are to use computer simulations and comparison with experiment to characterize the positions of waters and protons in and around the OEC in the later states in the Kok cycle that prepare the OEC for O-O bond formation; to investigate the water channels around the OEC that bring in substrate H_2O and remove protons to the lumen as well as the pathways that bring protons to the quinones; determine how the protein selects the correct cofactor to bind in each pocket and modulates the E_m and pK_a of cofactors once bound.

Significant achievements of the past 2 years –
We have studied the OEC catalytic mechanism by characterizing the protonation states of the OEC, the role of proton release in the lag phases Kok cycle. and the functional roles of surrounding water channels. (1) There is no proton release when the OEC is oxidized from S_1 to S_2. We have shown the proton is trapped by quantum delocalization at a low-barrier hydrogen bond between W1 and D1-D61. This is revealed by QM/MM calculations of isotropic proton hyperfine coupling constants, and verified by direct comparisons to experimental data from two-dimensional hyperfine sublevel correlation (HYSCORE) spectroscopy and extended X-ray absorption fine structure (EXAFS). (2) Analysis of the D1-S169A substitution near the OEC reveals a novel S_2-state structure that is more stable than found in the wild-type PSII. (4) Re-examination of the crystallographic XFEL data finds that there is no crystallographic evidence for an additional water molecule in the OEC exposed to two flashes of light. (5) There are three proton exit paths leading away from the OEC. We have found that near the OEC proton transfer pathways are highly interconnected. However, exit of protons through the broad channel is preferred.

Objectives for the coming year –

Work at Yale will focus on studies of O-O bond formation as influenced by V185N and N181A mutants, including calculations of ^{18}O kinetic isotope effects to allow direct comparisons to experimental measurements. This will address the mechanistic hypothesis suggested by our recent DFT-QM/MM calculations, showing that both of those mutants affect the structure of hydron bonding network next to the OEC and modulates the pK_a of D1-D61, the putative residue responsible for deprotonation of substrate water molecules for translocation to the lumen. Work at CCNY will analyze the proton transfer pathways from the OEC and uptake to the quinones in wild-type and mutated PSII and purple non-sulfur bacterial RCs. The relative affinity of Chl a, d and f in the binding sites of PSII optimized for white light and far red light will be determined to find the aspects of the structure responsible for selectivity.

Publications of the last 2 years supported by the award –

1. Biochim. Biophys. Acta Bioenerg. 1862(8): 148446 (2021) Proton exit pathways surrounding the oxygen evolving complex of photosystem II, by Divya Kaur, Yingying Zhang, Krystle M. Reiss, Manoj Mandal, Gary W. Brudvig, Victor S. Batista, and M. R. Gunner
2. Front Chem. 9: 660954 (2021) Protein Motifs for Proton Transfers That Build the Transmembrane Proton Gradient, by Divya Kaur, Umesh Khaniya, Yingying Zhang, and M.R. Gunner,
3. BBA Advances 1: 100019 (2021) Quantitative assessment of chlorophyll types in cryo-EM maps of photosystem I adapted to far-red light, by Christopher J. Gisriel, Hao-Li Huang, Krystle Reiss, David A. Flesher, Victor S. Batista, Donald A. Bryant, Gary W. Brudvig, and Jimin Wang
4, Proc. Natl. Acad. Sci. USA 118(24): e2023982118 (2021) Do crystallographic XFEL data support binding of a water molecule to the oxygen-evolving complex of photosystem II exposed to two flashes of light?, by Jimin Wang, William H. Armstrong, and Victor S. Batista
5. J. Am. Chem. Soc. 142: 8324-8332 (2021) Is Deprotonation of the Oxygen-Evolving Complex of Photosystem II during the S1 → S2 Transition Suppressed by Proton Quantum Delocalization?, by Ke R. Yang, K. V. Lakshmi, Gary W. Brudvig, and Victor S. Batista
6. Biochim. Biophys. Acta Bioenerg. 1861(12) 148301 (2020) D1-S169A substitution of photosystem II reveals a novel S2-state structure, by Ipsita Ghosh, Gourab Banerjee, Krystle Reiss, Christopher J. Kim, Richard J. Debus, Victor S. Batista, and Gary W. Brudvig
7. Biochemistry 59(30): 2823-2831 (2020) Identification of a Na^+-Binding Site Near the Oxygen-Evolving Complex of Spinach Photosystem II, by Jimin Wang, Joshua M. Perez-Cruet, Hao-Li Huang, Krystle Reiss, Christopher J. Gisriel, Gourab Banerjee, Divya Kaur, Ipsita Ghosh, Alisha Dziarski, M. R. Gunner, Victor S. Batista, and Gary W. Brudvig

Two disulfide reducing pathways are required for plastid cytochrome *c* assembly

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Overall research goals: The long-term goal of our investigation is to detail the redox landscape in the thylakoid, a membrane enclosed compartment required for photosynthesis. The molecular identity of the catalysts controlling thiol-disulfide exchanges, their relevant targets of action and the importance of thiol-based chemistry for photosynthesis in general are the long-term experimental questions we are currently pursuing. Through previous work, we documented a disulfide bond forming enzyme (LTO1) require for the biogenesis Photosystem II (PSII) (**Fig. 1**). Here we are further dissecting thiol-dependent redox chemistry in the lumen with an emphasis on disulfide reduction in the context of cytochrome b_6f assembly and a specific focus on CCS5 a thioredoxin-like protein and CCS4, a protein of unknow function.

Significant achievements: Cytochrome b_6f assembly requires its structural subunit cytochrome *f* (a metalloprotein defined as a cytochrome of the *c* type) to be converted to its holoform by covalent attachment of heme to the thiol groups of the C*XX*CH motif. The current view is that the heme-linking cysteines of apocytochrome *f* are oxidized following translocation in the lumen by an unknown oxidant (LTO1, another disulfide bond forming enzyme or O_2) and subsequently reduced (providing free -SH) for the heme attachment reaction (**Fig. 1**). The delivery of reductants relies on a trans-thylakoid redox pathway where thiol-disulfide oxido-reductases CCDA and CCS5 are proposed to transfer electrons, sequentially from stroma to the thylakoid lumen via a cascade of thiol-disulfide exchanges, to reduce disulfide-bonded apocytochrome *f* in the lumen (**Fig. 1**). The source of reductants in the stroma is still unknown but thioredoxin *m* (Trx-*m*) is suspected to be the electron donor (**Fig. 1**). CCS4 is a thylakoid-bound protein with a stroma-facing C-terminal domain whose biochemical activity is obscure as the protein lacks any known motifs suggestive of function. CCS4 and CCS5 were first uncovered in *Chlamydomonas reinhardtii,* and a *ccs4*-null or *ccs5*-null mutant is partially deficient for photosynthetic growth and accumulates decreased levels of holocytochrome *f*. We established that CCS4, initially thought to be restricted to a subgroup of algae is conserved throughout the green lineage. CCS4-like proteins display very little sequence similarity but are all characterized by a predicted membrane anchor and a hydrophilic domain whose total number of charged residues appears to be conserved. We showed that loss of CCS4 function (but not loss of CCS5) results in a down-accumulation of CCDA suggesting that one of the functions of CCS4 is to stabilize CCDA. Expression of *CCDA* in the *ccs4*-null mutant partially suppresses the photosynthetic deficient phenotype and restores the level of CCDA. Prior work in *Arabidopsis* suggested the existence of an unknown 'second' reducing pathway that is CCDA-independent and our genetic studies in *Chlamydomonas* indicated that CCS4 is a component of this second reducing pathway. Indeed, the *ccs4 ccs5* double mutant exhibits a synthetic photosynthetic defect characterized by a complete block in holocytochrome *f* assembly, which can be rescued by application of exogenous thiols. This indicates that CCS5 and CCS4 are operating in independent pathways controlling the reduction of disulfide-bonded

apocytochrome *f* in the lumen. Gain-of-function mutations in the *CCS4* gene can suppress a *ccs5*-null mutant, suggesting that the activity of this pathway can be enhanced in the absence of the CCDA-dependent pathway. We postulate that CCS4, in addition to its function in stabilizing CCDA, also operates in an unknown trans- thylakoid pathway distinct from the known CCDA/CCS5 disulfide – reducing pathway (**Fig. 1**). The source of reducing power and the mechanism by which it is transduced across the thylakoid membrane via the CCDA-independent route remain currently unknown. We recently discovered that a lumen-facing Glutathione-transferase like (GST-L) might be the component of this CCDA-independent reducing pathway based on the following findings: a) a *gstl* mutant is partially deficient for cytochrome *f* accumulation and b) a recombinant form of the molecule can reduce a disulfide in apocytochrome *f* in a GSH-dependent manner.

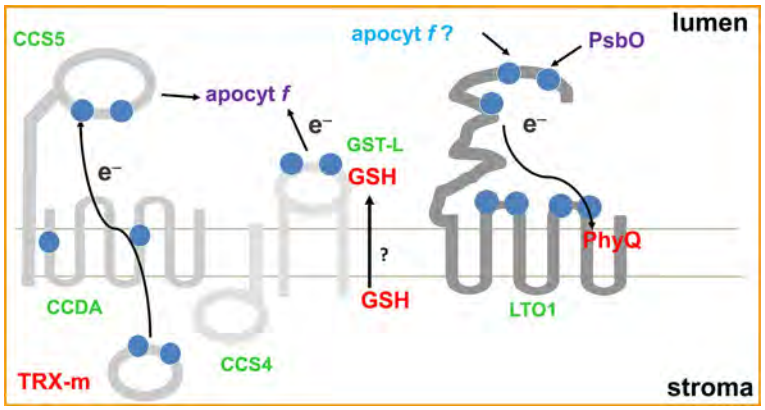

Fig.1. Trans-thylakoid thiol-metabolizing pathways. *Disulfide reducing (TRX-m, CCDA, CCS5 in light gray and CCS4, GST-L in pale gray) and forming pathways (LTO1, in dark gray) and some of their known (purple) or suspected targets (light blue) are represented. Conserved cysteines are in blue. Electrons (e^-) routes are indicated by arrows. The final electron acceptor of the LTO1-dependent pathway is presumed to be a phylloquinone (PhyQ). GSH is the reductant used by GST-L and must be transported from the stroma to the lumen (?)*

Objective of the coming year:

A possible role for CCS4 in the CCDA-dependent and independent pathway is to recruit reducing power to the thylakoid membrane. While the source of reductant in the CCDA-independent pathway remains unknown, Trx-*m* is postulated to provide electrons to the the CCDA-dependent pathway (**Fig. 1**). One possibility is that the soluble domain of CCS4 interacts with Trx-*m* to favor the distribution of electrons to CCDA. The physical interaction between CCS4 and Trx-*m* will be assessed a) in vitro via gel filtration using a recombinant form of the soluble domain of CCS4 and Trx-*m* and b) in vivo by testing if Trx-*m* can be pulled down along CCS4 using a *ccs4*-null strain expressing a *CCS4-HA* gene

References to work supported by this project:

(1) Das, A. and Hamel, P. (2021) Cytochrome *c* assembly in "Encyclopedia of Biological Chemistry, 3rd edition (Ed. Joseph M. Jez, Publisher: Elsevier Inc.)

Characterizing Rubisco by Phylogeny-Informed Mutagenesis

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Overall goals of the project

a) Generation and analyses of tobacco Rubisco with mutated large subunit (LSU)
b) Improvement of *E. coli* expression of tobacco Rubisco enzymes
c) Reconstruction of ancestral Rubisco from phylogeny of Solanaceae
d) Utilization of the *E. coli* expression system for mutated tobacco Rubisco.
e) Characterize the kinetic properties of ancestral Rubiscos
f) Introduce Rubisco with superior properties in *E. coli* into transgenic tobacco

Significant achievements of the past 2 years

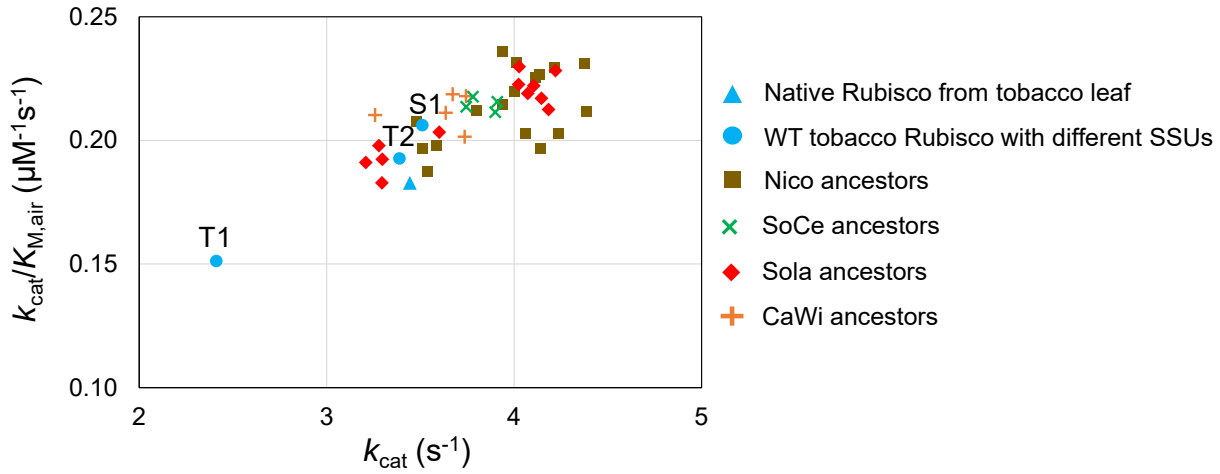
a) Tobacco plants with LSU mutations. Ten transgenic plants were produced using a new method we developed for introduction of point mutations into the chloroplast genome (1). Six mutations were chosen as characteristic of the C_3 to C_4 transition, and others were at the interface of the two subunits. All plants grew well, but none had improved enzyme kinetics.

b) *E. coli* expression system for tobacco. The three-plasmid system for expression of Arabidopsis Rubisco developed by the Hayer-Hartl lab was converted into a two-plasmid system that expressed chaperones needed for improved expression of tobacco Rubisco under auto-induction conditions (2). Each tobacco LSU/small subunit (SSU) pair was expressed separately in an *E. coli* strain and enzyme kinetic properties were determined. Several LSU/SSU pairs exhibited k_{cat} similar to the native enzyme. The small subunit from trichome-expressed Rubisco exhibited higher K_c and $k_{cat.}$

c) Acquisition of tobacco LSU and small subunit (SSU) sequences. We developed an efficient computational workflow that utilizes multiple programs to assemble *de novo* any transcript of interest from RNA-Seq data. With this new workflow, we obtained over 112 SSU sequences from 17 genera and 44 LSU sequences from 15 genera of Solanaceae in addition to those already available publicly. The python scripts are available at https://github.com/myattlin/de-novo-assembly.

d) Construction of phylogenetic tree for Rubisco in Solanaceae. We applied Bayesian inference and maximum likelihood approaches independently to perform phylogenetic analyses of the Solanaceae Rubisco subunits and predict ancestral sequences for both LSU and SSU at four levels of internal nodes named Nico, SoCe, Sola and CaWi. We chose 98 most probable ancestral Rubisco enzymes at these nodes for further analyses below.

e) Expression and characterization of predicted ancestral Rubisco enzymes. We produced each of the 98 predicted ancestral Rubisco enzymes of Solanaceae in *E. coli* and measured their kinetic properties. At least 22 of those predicted ancestors displayed characteristics of a typical C_4 Rubisco with higher RuBP carboxylation rates (k_{cat}) and/or efficiencies ($k_{cat}/K_{M,air}$) at 25°C compared to the native Rubisco from tobacco leaves as well as wild-type tobacco Rubisco expressed in *E. coli* with individual SSUs (figure below). Since these ancestral nodes coincide with periods that gave rise to C_4 photosynthesis, our findings indicate that the kinetics of ancestral C_3 Rubisco enzymes prior to the evolution of C_4 photosynthesis are more similar to that of a C_4 Rubisco. We also found that these predicted ancestors maintained their superior catalytic performance at a higher temperature (30°C) and possessed a similar ability to distinguish CO_2 and O_2 (specificity factor) as can a typical C_3 Rubisco.



Objectives for the coming year

1) We will introduce genes encoding particular ancestral Rubisco enzymes into the chloroplast genome of SeLS tobacco plants carrying CRISPR deletions of native SSU genes. The cyanobacteria Rubisco genes in the chloroplast genome of SeLS plants will be replaced during the process.

2) Once we obtain the plants expressing the ancestral Rubisco enzymes, we will evaluate growth, Rubisco accumulation and enzyme properties in those transformed plants.

Publications of the last 2 years supported by the award

(1) 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

(2) Lin MT, Stone WD, Chaudhari V, Hanson MR. Small subunits can determine enzyme kinetics of tobacco Rubisco expressed in *Escherichia coli*. Nat Plants. 2020 Oct;6(10):1289-1299. doi: 10.1038/s41477-020-00761-5. PMID: 32929197

(3) Hanson, MR and PL Conklin. 2020 Stromules, functional extensions of plastids within the plant cell. Curr Opin Plant Biology 58-25-32. PMID: 33137706. (review article)

Time Resolved Infrared Difference Spectroscopy for the Study of the Cofactors Involved in Electron Transfer in Photosystem I

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Overall goals of this project –

The goal of this project is to gain a molecular-level understanding of the factors that modulate the functional properties of pigments involved in electron transfer (ET) in photosystem I (PSI). To address this goal, we use time-resolved (from femtoseconds to seconds) visible and infrared difference spectroscopy to study PSI complexes with modified cofactor composition.

To drive spectral band interpretation and assignment a second goal is to develop and use computational methods to simulate the experimental spectra. We focus on ONIOM-type (QM:MM) quantum chemical methods for this purpose. A second computational objective is to develop methods for modeling the myriad of ET processes that occur in native and modified PSI, at both 298 and 77 K.

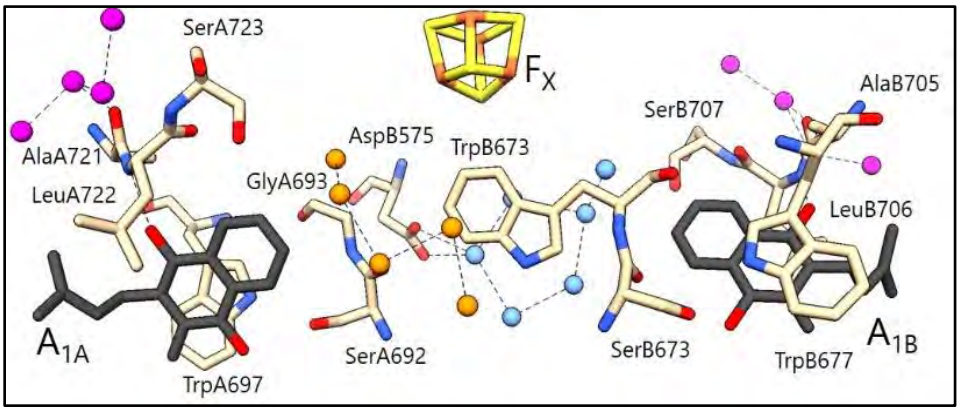

Fig. 1 Structural organization of the quinone and F_X cofactors in PSI. Select amino acids and water molecules are also shown. Possible H-bonding interactions are depicted as dotted lines. *Dark grey*: carbon atoms of ET cofactors, *light khaki*: all other carbon atoms, *red*: oxygen, *blue*: nitrogen, *yellow*: sulfur. *Magenta/orange/light blue*: oxygen atoms of water molecules.

Significant achievements of the past 2 years –

1. Studied unlabeled and fully ^{13}C labeled PSI with several different naphthoquinones (NQ's) incorporated into the A_1 binding site. This allowed unambiguous identification (for the first time) of bands associated with the neutral state of the incorporated quinones.[6]
2. Time resolved FTIR difference spectra for PSI with different di-substituted NQ's incorporated were produced. FTIR spectra for the same quinones in solution were also produced along with associated quantum chemical vibrational frequency calculations. This latter work led to a new approach to identify neutral state quinone bands in FTIR difference spectra.[2]
3. Undertook QM/MM vibrational frequency calculations to model FTIR spectra obtained using purple bacterial reaction centers with phylloquinone in the Q_A binding site.[7] This allowed a comparison of calculations for the same quinone in different binding sites.
4. Studied PSI containing a TrpB673Phe site-directed mutation (Fig. 1). In this mutant we showed that forward ET from A_1 to F_X can be reversibly inhibited and reactivated.[5]

5. Studied PSI containing a MA664H site-directed mutation. In this mutant we showed that a second hydrogen bond to the A_{1A} pigment is introduced.
6. Wrote a proposal and received a beamtime allocation on the infrared light source at LBNL. Proposal was to undertake polarized FTIR difference spectroscopy experiments on PSI microcrystals. This work is in collaboration with Jan Kern. First experiments on microcrystals on the IR beamline were recently undertaken.
7. In collaboration with Holger Dau/Dennis Nurnberg, performed nanosecond time-resolved IR spectroscopy experiments on native PSI under physiological conditions (room temperature).[1]
8. Extended our studies to consider hydrogen bonding to (quinone-like) flavins.[3]

Objectives for the coming year –

1) Study PSI with different BQ's incorporated. Our previous work suggests altered bioenergetics compared to NQ's. Assess mechanism underlying the altered bioenergetics.
2) Use static and time-resolved FTIR difference spectroscopy to study PSI where several site-directed mutations have been introduced to modify the P700, A_{-1}, A_0 and A_1 pigments.
3) Further develop QM/MM computational methods to simulate the experimental spectra, especially for bound neutral quinones.
4) Use femtosecond time-resolved spectroscopy in the visible, at both 298 and 77 K (in collaboration), to study forward electron transfer from A_0^- to A_1 in PSI with non-native quinones incorporated.
5) Use quantum cascade laser-based nanosecond time-resolved infrared spectroscopy to study native and modified PSI samples under physiological conditions.
6) Develop and use the IR light source at LBNL to undertake FTIR difference spectroscopy of PSI microcrystals. Beamtime has been awarded and can be extended.
7. Develop and use a QCL based IR light source to undertake FTIR difference micro-spectroscopy on PSI microcrystals.

Publications in 2020-2021 supported by the award –

1. Mausle, S., Simon, P., Makita, H., Agarwala, N., Nurnberg, D., Dau, H. and Hastings, G. (2021) Nanosecond Time-Resolved Infrared Spectroscopy for the Study of Photosystem I Photosynthetic Reaction Centers. *Manuscript to be submitted.*
2. Agarwala, N., Rohani, L. and Hastings, G. (2021) Experimental and Calculated Infrared Spectra of Disubstituted Naphthoquinones. *Spectrochimica Acta* A: *Molecular and Biomolecular Spectroscopy*, Accepted.
3. Kabir, M., Orozco-Gonzalez, Y., Hastings, G. and Samer Gozem (2021) The effect of hydrogen-bonding interactions on the infrared vibrational spectrum of flavin. *Spectrochimica Acta* A: *Molecular and Biomolecular Spectroscopy*, **262**, 5:120110.
4. Rohani, L. and Hastings, G. (2021) Assessment of the orientation and conformation of pigments in protein binding sites from infrared difference spectra. *Biochim. et Biophys. Acta, Bioenergetics.* **1862**, 4, 148366.
5. Agarwala, A., Makita, H., Luo, L., Xu, W. and Hastings, G. (2020) Reversible inhibition and reactivation of electron transfer in photosystem I. *Photosynth. Res.* 145, 97–109.
6. Makita, H. and Hastings, G. (2020) Time-resolved FTIR difference spectroscopy for the study of quinones in the A_1 binding site in photosystem I: Identification of neutral state quinone bands. *Biochim. et Biophys. Acta, Bioenergetics.* **1861**, 5-6, 148173.
7. Rohani, L. and Hastings, G. (2021) Vibrational Properties of Phyllosemiquinone in the Q_A Binding Site of Purple Bacterial Reaction Centers. *Manuscript in preparation.*

Protein Targeting to the Chloroplast Thylakoid Membrane: Structure and Function of a Targeting Complex

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Overall goals of the project –

The overall goal of this project is to gain a fundamental molecular understanding of the key events underlying the vectorial targeting of light harvesting complex proteins (LHCs) to the thylakoid membrane. LHCs are imported into the chloroplast and then bound by cpSRP to form a soluble transit complex. cpSRP is a heterodimer composed of a conserved GTPase, cpSRP54, and a subunit unique to chloroplasts, cpSRP43. While cpSRP43 binds LHC targeting substrates, cpSRP54 functions to direct the targeting complex to an SRP receptor (cpFtsY) at the thylakoid membrane. Once at the membrane, cpSRP43 binds a protein insertase (Alb3) via a stroma-exposed disordered C-term, which stimulates release of LHC from cpSRP and insertion into the thylakoid.

We hypothesize that the sequence of targeting events is regulated by changes in the structural dynamics of each targeting component to impact their binding affinities and support a stepwise order of protein interactions that define the vectorial LHC targeting/insertion pathway. This hypothesis is supported by our studies (Gao et al., 2015) showing that cpSRP43 is a highly dynamic protein that reduces its dynamics upon binding cpSPR54, an event producing a ~3-fold increase in binding affinity of cpSRP43 for LHC targeting substate. We further showed (Henderson et al., 2016) that cpSRP54 *increases* its dynamics upon binding cpSRP43 and, through MD simulations, identified residues on cpSRP54 and cpSRP43 that are critical for efficient transit complex formation and/or LHC integration, which were then experimentally verified.

With a goal to understand how vectoral LHC targeting and thylakoid insertion relies on interaction-induced changes to structural dynamics of the targeting components, we are employing a combination of experimental and computational approaches including small angle X-ray scattering (SAXS), single molecule fluorescence resonance energy transfer (smFRET), site-directed mutagenesis and all-atom molecular dynamics simulations. Together, with assays that reconstitute each step of the LHC integration pathway, we are beginning to uncover an atomic-level model of targeting protein conformations responsible for vectoral LHC targeting events.

Significant achievements of the past 2 years

1. Structural dynamics of the spSRP heterodimer is affected by binding LHC substrate. SAXS examination of cpSRP identified changes in its global structure and predicted a wide smFRET distribution for dye placement in both cpSRP43 CD1-domain and cpSRP54 M-domain. This prediction was confirmed and used to demonstrate a more compact “locked” structure upon LHC peptide binding (Figure 1). The structures observed allowed us to predict regions of cpSRP43 and cpSR54 that interact to lock this conformation, which was confirmed in functional assays. These same studies demonstrated a critical interaction between LHC and cpSRP54 M-domain for efficient transit complex formation and demonstrated that the N-terminus of cpSRP43 functions at a step following transit complex formation that is critical for LHC insertion into thylakoids. We hypothesize that the

cpSRP43 N-domain is used to properly orient the cpSRP/LHC transit complex for binding to cpFtsY at the membrane.

2. Structural dynamics of Alb3 C-terminus respond to binding by cpSRP43. The disordered C-terminus of Alb3 (Alb3-Cterm) is known to interact with cpSRP43 as a pre-requisite for LHC transfer from cpSRP and thylakoid insertion by Alb3. However, details of this interaction remain controversial. Using our all-atom enhanced sampling MD simulations, we found that there are specific regions in Alb3-Cterm with a moderate propensity to form α-helical structures. CD spectroscopy and smFRET verified the presence of transient structure in Motifs I and II near the N-terminus of Alb3-Cterm, which are sensitive to chemical denaturant or helix breaking point mutations (Baucom et al 2021). We have recently found that Alb3-Cterm becomes more compact upon binding cpSPR43 (a higher FRET state is observed, figure 2). However, when the transient secondary structure is disrupted by a helix breaking mutation, this compaction is no longer observed, suggesting that this transient secondary structure plays a major role in binding to cpSRP43.

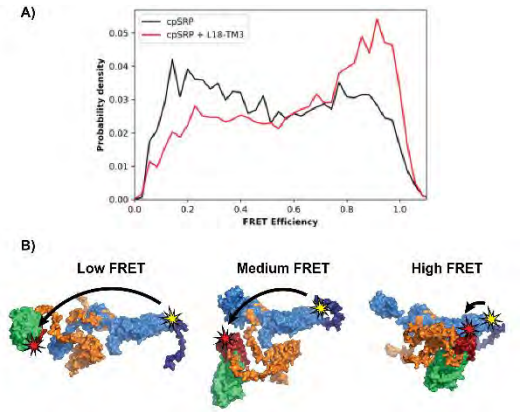

Figure 1 – (A) smFRET histograms of cpSRP before and after binding the substrate (L18-TM3). (B) Structures from SAXS data that allowed us to design the smFRET assay to show that the compact high FRET structure is "locked in" after binding to the substrate

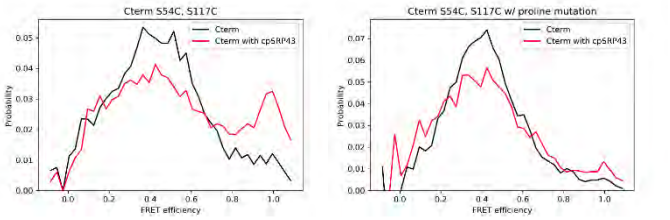

Figure 2 – smFRET histograms of Alb3-Cterm before and after binding cpSRP43. Left panel shows the C-term without the helix breaking mutant and the right panel shows the C-term when a helix breaking mutation (proline) is introduced

Objectives for the coming year –

1. The transient secondary structure of Alb3-Cterm and how it affects the interaction with cpSRP43 was performed in solution. To investigate the role of the membrane environment on the interaction between cpSRP43 and Alb3-Cterm, we will anchor the Alb3-Cterm into liposomes using a Tha4-Alb3-Cterm construct and perform CD and smFRET experiments to determine the role of the membrane in modulating the transient secondary structure and interaction. This may shed some light onto the somewhat conflicting literature on the cpSRP43/Alb3-Cterm interaction.
2. We will also investigate other regions of the Alb3-Cterm for transient secondary structure and interaction with cpSRP43, and how they are affected by helix breaking mutations. This will allow us to pinpoint more accurately which regions of Alb3-Cterm are the most critical for interaction with cpSRP43 and subsequent LHC integration.

Publications of the last 2 years supported by the award –

Dustin R. Baucom, Mercede Furr, Vivek Govind Kumar, Patience Okoto, James L. Losey, Ralph L. Henry, Mahmoud Moradi, Thallapuranam Krishnaswamy Suresh Kumar and Colin D. Heyes. Transient Local Secondary Structure in the Intrinsically Disordered C-Term of the Albino3 Insertase. Biophys. J. (2021) In Press (Online Oct 16) https://doi.org/10.1016/j.bpj.2021.10.013

Elucidating the Mechanism of OCP-mediated Antenna Quenching

DE-FG02-91ER20021

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Overall goals of the project – The Orange Carotenoid Protein (OCP) of cyanobacteria is a relatively new member of the family of photoactive proteins; intense light causes this water soluble carotenoprotein to convert from a dark stable orange form, OCP^O, to an active red form, OCP^R. The active OCP^R form is able to bind to the cyanobacterial light-harvesting antenna complex, the phycobilisome (PBS), where it enables efficient "quenching" of bilin pigments, allowing excess absorbed light energy to be dissipated as heat instead of being transferred to the PSII or PSI reaction centers. We have also discovered that the OCP evolved from the fusion of two carotenoid binding proteins. Our goal is to elucidate the structural and mechanistic details of PBS quenching by the OCP and determine if this mechanism is also active in its ancestral domain homologs

Significant achievements of the past 2 years – We have made progress in determining the structure of the complex between the OCP and the PBS, we have determined the structure of the C-terminal domain homolog carotenoid protein (CCP) and have probed the function of the beta2 surface of the OCP in photoactivation and quenching.

Objectives for the coming year – Complete structural studies and use to rationalize the observed role of the beta2 surface in quenching. Further explore the potential for quenching by the OCP domain homologs.

Publications of the last 2 years supported by the award –

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, in press.

Ralston, C.Y. and Kerfeld, C.A. Integrated Structural Studies for Elucidating Carotenoid-Protein Interactions. Advances in Experimental Medicine and Biology May 2021. doi: 10.1007/5584_2020_615.

Dominguez-Martin, M.A., Hammel, M., Gupta, S., Lechno-Yossef, S., Sutter, Rosenberg, D.J., Chen, Y., Petzold, C.J., Ralston, C.Y., Polívka, T. and Kerfeld, C.A. Structural analysis of a new carotenoid-binding protein: The C-terminal domain homolog of the OCP. Scientific Reports 10:15564, 2020.

Kuznetsova, V., Dominguez-Martin, M.A., Bao, H., Gupta, S., Sutter, M., Kloz, M., Chen, Y., Petzold, C.J., Ralston, C.Y., Kerfeld, C.A. and Polívka, T. Ultrafast spectroscopy and structural analysis explain differences between the photoactivation mechanism of OCP1 and OCP2. Biochimica Biophysica Acta 1861: 148120, 2019.

Structural Dynamics in Photosynthetic Reaction Centers

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Overall goals of the project

One of the fundamental questions regarding light driven charge transfer in natural photosynthetic system is how charge-separated states are stabilized and 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) will be used to structurally probe the dynamics of photosynthetic systems in the sub-picosecond to microseconds time scale. These studies will 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. 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 of the past 2 years

The first step to study the details of the cofactor-protein interaction during charge separation in PS I was to establish the protocols for measurements of damage free diffraction data of PS I under near physiological conditions. We developed a protocol that allows generation of uniformly sized 10 µm crystals, that are compatible with established sample delivery methods for serial femtosecond crystallography and small enough for uniform illumination in optical pump X-ray probe studies. In the next step, we utilized our Drop-On-Tape sample delivery system to collect room temperature (RT) diffraction data from these crystals in the dark-adapted state using an XFEL, that resulted in a RT structure at 2.75 Å resolution. The most prominent feature of the structure is a clear expansion of the entire trimeric complex in the membrane plane compared to the cryogenic data. Distinct differences were seen in the environment of the phylloquinones in both the A and

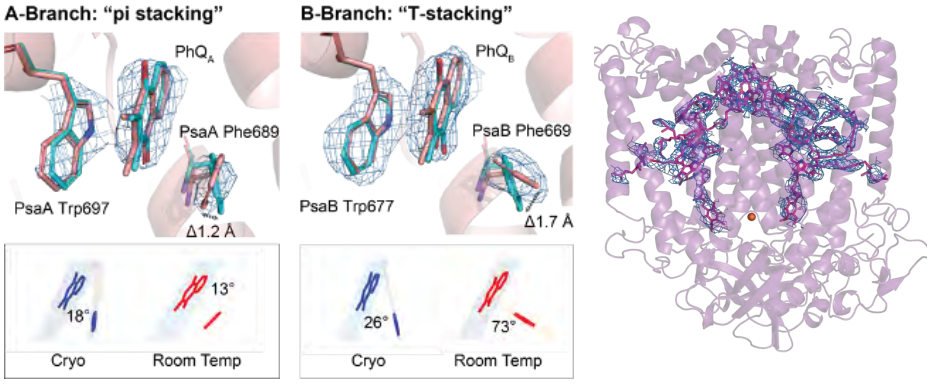

Fig. 1: Left and Middle: Asymmetry around the two phylloquinone binding pockets in PS I observed at room temperature. Right: 3.6 Å room temperature structure of *R. sphaeroides* RC with electron density for cofactors shown as blue mesh.

the B branches (Fig. 1 left and middle), that may have implications for the directionality of electron transfer. Residues PsaA-Phe689 and PsaB-Phe669, which are directly next to the phylloquinones PhQ_A and PhQ_B, respectively, are oriented very differently with respect to the phylloquinones, and may contribute to the differences in the rates of electron transfer between the two branches. The details are described in a recent publication (Keable et al 2021).

To extend our studies to a type II reaction center, we started efforts to crystallize the photosynthetic bacterial reaction center protein from *Rhodobacter sphaeroides* in collaboration with the groups of Drs. Phillip Laible and Christine Kirmaier. Crystals of ~100 μm in size were screened at the Advanced Light Source at room temperature, and diffraction was observed out to 2.3 Å. Based on these initial conditions, we improved the crystallization protocol and obtained crystals of 10-20 μm size and these were used for collection of SFX data at the XFEL at RT and a merged dataset to 3.6 Å was obtained, showing clear electron density of the electron transfer co-factors (Fig. 1, right).

Objectives for the coming year

We plan to collect time resolved fs X-ray diffraction data from illuminated PS I in the ps to ns delay time range. We also started working with the group of Prof. Wu Xu on specific point mutants in PS I from *Synechocystis* PCC6803 with the long term goal to perform time resolved structural studies on these. In addition, we will pursue the work on the reaction center from *R. sphaeroides* in collaboration with Laible/Kirmaier and will establish conditions for crystallization of specific point mutants that selectively target the environment of individual cofactors in the electron transfer chain.

Publications of the last 2 years supported by the award

Reviews:

1. U. Bergmann, J. Kern, R. Schoenlein, P. Wernet, V. K. Yachandra, J. Yano, Using X-ray free-electron lasers for spectroscopy of molecular catalysts and metalloenzymes. *Nature Review Physics* **3** (2021) 264-282.
2. 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).

Original Publications:

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** (2021) 21787.
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** (2021) 36-48.
3. N. K. Sauter, J. Kern, J. Yano, J. M. Holton, Towards the spatial resolution of metalloprotein charge states by detailed modeling of XFEL crystallographic diffraction, *Acta Cryst. D* **76** (2020) 176-192.
4. E. S. Burgie, et al., Photoreversible interconversion of a phytochrome photosensory module in the crystalline state, *Proc. Natl. Acad. Sci. U S A* **117** (2020) 300-307.

CONTROL OF THE THYLAKOID ULTRASTRUCTURE BY K^+ AND CL^- TRANSPORTER/CHANELS

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Overall goals of the project –

1. Establishing and developing electron microscopic methods for ultrastructural thylakoid dynamics in intact leaf tissues.
2. Examination of light-induced ultrastructural dynamics of thylakoid membranes in plants (like swelling of the thylakoid lumen or changes in thylakoid membrane thickness).
3. Determine the role of thylakoid membrane ion transporter and channels for ultrastructural thylakoid dynamics.
4. Determine light-induced lateral redistributions of cytochrome b6f complexes between stacked and unstacked thylakoid domains.
5. Examine the consequences of lateral cytochrome b6f complex distribution dynamics for linear and cyclic electron transport.
6. Unraveling the role of small regulatory proteins for lateral cyt b6f redistribution dynamics.

Significant achievements of the past 2 years –

1. We established a methodical pipeline for ultrastructural analysis of thylakoid membranes by electron microscopy.
2. We characterized in detail light induced dynamics of the thylakoid membrane network.
3. We generated and characterized single and higher order mutants of thylakoid ion transporter/channel mutants.
4. We developed and optimized a quantitative mathematical model for photosynthetic light and dark reactions that was verified by a set of experimental data.
5. We characterized light-induced lateral redistributions of cytochrome b6f complexes for wildtype and mutant plants of small regulatory proteins (kinases, phosphatases, PGR5, PGRL1).
6. We developed immunogold-labeling of thylakoid membrane protein complexes combined with TEM with leaf discs.

Objectives for the coming year –

1. Finalizing the ultrastructural and functional analysis of thylakoid membrane ion transporter/channel mutants.
2. Finalize the lateral redistribution analysis combined with ultrastructural thylakoid membrane analysis and functional measurements for wildtype plants.
3. Extend the approach in 2. to mutants affected in low abundant regulatory proteins.
4. Optimize immuno-gold labeling for key photosynthetic protein complexes in thylakoid membranes.

Publications of the last 2 years supported by the award –

1. H.M.O. Oung, R. Mukhopadhyay, V. Svoboda, D. Charuvi, Z. Reich, **H. Kirchhoff** (2021) Differential response of the photosynthetic machinery to dehydration in older and younger resurrection plants. *J. Exp. Bot*. accepted
   The electron microscopic work was supported by a US Department of Energy grant (BES #DE -SC0017160).
2. B.H. Kang, C.T. Anderson, S. Arimura, E. Bayer, M. Bezanilla, M. A. Botella, F. Brandizzi, T. M. Burch-Smith, K. D. Chapman, K. D nser, Y. Gu, Y. Jaillais, H. Kirchhoff, M. S. Otegui, A. Rosado, Y. Tang, J. Kleine-Vehn, P. Wang, B. K. Zolman (2021) A glossary of plant cell structures: Current insights and future questions. *The Plant Cell*, accepted
   Acknowledged support from US Department of Energy (DESC0017160).
3. **H. Kirchhoff** (2021) Proteoliposomes for Studying Lipid-protein Interactions in Membranes *in vitro. Bio-protocol, in press*
   Acknowledged support from US Department of Energy (DE-SC0017160).
4. M. Li, V. Svoboda, G. Davis, D. Kramer, H.H. Kunz, **H. Kirchhoff** (2021) Impact of ion fluxes across thylakoid membranes on photosynthetic electron transport and photoprotection. *Nature Plants* 7, 979-988
   Acknowledged support from US Department of Energy grant (DE-SC0017160).
5. **F. Müh**, B. van Oort, S. Puthiyaveetil, **H. Kirchhoff** (2021) Thylakoid membrane stacking to grana in plants: Physicochemical forces at work. *Nature Plants* 7, 279-281
   Acknowledged support from US Department of Energy DE- SC0017160.
6. 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. Highlighted as article of the week in ASPB Signal Dec 10, 2020
   Acknowledged support from Department of Energy DE- SC0017160.
7. R. Höhner, M. Pribil, M. Herbstová, L.S. Lopez, H.-H. Kunz, M. Li, M. Wood, V. Svoboda, S. Puthiyaveetil, D. Leister, **H. Kirchhoff** (2020) Plastocyanin is the long-range electron carrier between photosystem II and photosystem I in plants. *Proc. Natl. Acad. Sci. USA* 117, 15354-15362
   The EM work was supported by a Department of Energy (DOE-DE-SC0017160.
8. S Tietz, M Leuenberger, R Höhner, AH Olson, GR Fleming, **H Kirchhoff** (2020) A proteoliposome-based system reveals how lipids control photosynthetic light harvesting. *J. Biol. Chem*. 295, 1857-1866 (recommended to read by JBC editor; Covered as Editor's choice in Science journal, *Science* 367).
   Acknowledged support from US Department of Energy (DE-SC 0017160).
9. D. Schneider, L.S. Lopez, M. Li, J.D. Crawford, H. Kirchhoff, **H.H. Kunz** (2019) Fluctuating light experiments and semi-automated plant phenotyping enabled by self-built growth racks and simple upgrades to the IMAGING-PAM. *Plant Methods* 15, 156.
   Acknowledged support from US DOE-BES, (#DE-SC0017160).
10. D.A. Gacek, C.-P. Holleboom, S. Tietz, **H. Kirchhoff**, **P.J. Walla** (2019) The role of the PsbS protein for carotenoid-chlorophyll coupling in grana thylakoids. *FEBS Letters* 593, 3190-3197.
    Acknowledged support from US Department of Energy (DE-SC 0017160).
11. **H. Kirchhoff** (2019) Chloroplast ultrastructure in plants. Invited review article for *New Phytologist* 223, 565-574.
    Acknowledged support from US Department of Energy (DOE-DE-SC0017160).
12. H. Koochak. S. Puthiyaveetil, D. Mullendore, M. Li, **H. Kirchhoff** (2019) The structural and functional domains of plant thylakoid membranes. *The Plant Journal* 97, 412-429. (Reseach highlight and cover)
    Acknowledged support from DOE-BES (DE-SC0017160).

The Dynamic Energy Budget of Photosynthesis

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Overall goals of the project - Photosynthesis must balance how much energy is stored and in what forms to precisely meet the ratio required for biochemical demands. Failure of these balancing mechanisms can lead to photodamage and loss of energy, particularly under rapidly changing environmental conditions. These processes are critical for efforts to improve the efficiency of photosynthesis, e.g., by introducing CO_2 concentrating mechanisms, altering metabolism or biosynthetic pathways to shunt energy to alternative products. These balancing processes must also be rapid and robust to contend with the rapid and unpredictable fluctuations in environmental conditions and metabolic demands that occur in nature. The goal of the project is to understand at biochemical/biophysical levels how the formation, storage, responses to the thylakoid protonmotive force (*pmf*) impact these balancing mechanisms. Part of the effort is focused on discovering and understanding new processes that appear under non-laboratory conditions and in non-model systems.

Significant achievements of the past 2 years-

What limits the ability of light reactions to use rapidly increased light under read world conditions? We found that found that ability of plants to use rapidly increased light was limited mainly by two processes related to the thylakoid *pmf*: 1) the rapid buildup of NPQ leading to net oxidation of Q_A and P_{700}; and 2) photosynthetic control (PCON), i.e., slowing of plastoquinol oxidation at the cytochrome b_6f complex, leading to reduction of Q_A and oxidation of P_{700}. Interestingly, "over-reduction" of photosystem I only occurred at very short times after light fluctuations, suggesting that the light reactions are tuned to avoid this situation.

Discovery of new kinetic limitations to the use or dissipation of rapidly increased intensities of light. These responses were highly dependent on leaf temperature and initial light intensity, with a transition from NPQ to PCON limitations at surprisingly moderate temperatures (<20°C), identifying a large (real world) impactor on photoprotection responses. We were able to reproduce these effects in the laboratory and show that the transition is not related to the formation of ΔpH or to the xanthophyll cycle, but appear to reflect a newly identified rate limiting step, most likely involving antenna conformational changes. At the other extreme of high temperatures, we observed that fast, *pmf*-related NPQ changes become less important and slow NPQ changes, likely related to PSII damage, prevail.

Diversity in the proton circuit of photosynthesis. Using a combination of detailed, high throughput phenotyping of diversity panels of cowpea and Arabidopsis, we showed nature has tuned photosynthetic responses to changing temperature by tweaking the network of regulatory processes involving the formation and sensing of the thylakoid *pmf*, including cyclic electron flow, ATP synthase activity and the responses of qE to lumen pH.

Evidence that "excess NPQ" is not excessive. We showed that photosynthetic electron transfer is often strongly limited by NPQ in excess of that needed to maintain Q_A redox state. However, this occurs in a wide range of species and conditions, suggesting that this phenomenon is not "wasteful" but likely to be critical for preventing photodamage, particularly at high light.

Science objectives for the coming year -

The role of rapid light-induced CEF. We have demonstrated that rapid photosynthetic responses are accompanied by activation of CEF, which contributes substantially to the buildup of *pmf* and that this rapid activation of CEF can be explained by feedback inhibition of plastoquinone reductases by ATP levels. We are currently asking whether this CEF is required to build up of ΔpH for regulation, or to supply ATP to meet altered metabolic demands.

The importance of the Δ*ψ* component of thylakoid *pmf*. In our past work, we presented evidence that the partitioning of thylakoid *pmf* into Δ*ψ* and ΔpH components is important for regulation of photosynthesis. Much of the evidence is based on deconvoluting the electrochromic shift (ECS) absorbance signal. We have developed a new method of measuring the ECS using signals in the near infrared. Using this independent method, we verified our previous observations over a wide range of conditions, supporting a key role for *pmf* partitioning in regulation of photosynthesis. Moreover, the method is applicable to both laboratory and field studies, allowing us to assess importance of this effect under real world conditions.

Mechanisms of limitation to NPQ activation and electron flow. Determine the specific NPQ-induction processes that are "frozen" out at lower leaf temperatures, determine if these are related to lipid-protein interactions, and to what extent natural variations impact the effects. We will also assess the extent to which the observed shifts between modes of limitations/regulation of the light reactions impact efforts to improve photosynthetic efficiency.

Selected Publications of the last 2 years supported by the award -

Fisher, N., T. Bricker, and •. D. M. Kramer. 2019. "Regulation of Cyclic Electron Flow Pathways by Adenylate Status in Higher Plant Chloroplasts." Biochimica et Biophysica Acta 1860 (11): 148081.

Fisher, Nicholas, and David M. Kramer. 2021. "Photosynthesis | Mechanisms and Regulation of Photosynthetic Cyclic Electron Flow Around Photosystem I." In *Encyclopedia of Biological Chemistry III (Third Edition)*, edited by Joseph Jez, 391–98. Oxford: Elsevier.

Hoh, D., Osei-Bonsu, Chattopadhyay, A., Kanazawa, A., Fisher, N., Tessmer, OL, Cruz, JA, Roberts, P.A., Huynh, B-L., Hall, D. and Kramer, D.M. (2021) Genetic Variation in Photosynthetic Responses to Chilling Modulates Proton Motive Force, Cyclic Electron Flow and Photosystem II Photoinhibition. Submitted

Kanazawa, A, Chattopadhyay, A., Kuhlgert, S., Tuitupou, H., Maiti, T. and Kramer, D.M. (2021) Light potentials of photosynthetic energy storage in the field: what limits the ability to use or dissipate rapidly increased light energy?" Roy Soc Open Sci, Accepted (see bioRxiv. https://doi.org/10.1101/2021.08.26.457798)

Kanazawa, A., Neofotis, P., Davis, G.S., Fisher, N. and Kramer, D.M. (2020) "Diversity in photoprotection and energy balancing in terrestrial and aquatic phototrophs." In Photosynthesis in Algae: Biochemical and Physiological Mechanisms, edited by Anthony W. D. Larkum, Arthur R. Grossman, and John A. Raven, 299–327. Springer International Publishing

Walker, B. J., Kramer, D. M., Fisher, N. and Fu, Xinyu (2020) Flexibility in the energy balancing network of photosynthesis enables safe operation under changing environmental conditions. Plants 9: 301.

Diversification and function of bilin chromophores in oxygenic photosynthesis

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Nathan C. Rockwell, Co-I
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Overall goals of the project. Previous work under this project has focused on cyanobacterial photoreceptors in the phytochrome superfamily. These phytochromes and cyanobacteriochromes use the linear tetrapyrrole (bilin) chromophores also used by light-harvesting phycobiliproteins. In studying these photoreceptors, we have successfully combined evolutionary analyses with a robust pipeline for expressing recombinant candidate proteins in *Escherichia coli* strains engineered to produce bilins. This work provided a comprehensive chemical and biophysical understanding of spectral tuning from the near UV to the near IR by this family of photosensors. In recent months, the objectives of our project have shifted to examine the role of bilins in chlorophyll biosynthesis, a question more closely allied with the core mission of Photosynthetic Systems.

Basic research into biological photosynthesis provides a paradigm for dynamic, self-assembling, self-regulating systems that harvest solar energy. Such work holds promise for providing sustainable food sources for the expanding human population, for development of improved biofuels, and for new approaches for biosynthesis of diverse compounds with reduced energy needs. Photosynthesis relies on the biosynthesis of chlorophyll. Chlorophyll is synthesized via a common trunk pathway that also synthesizes heme and bilins, and the enzyme magnesium chelatase (MgCh) carries out the committed step for chlorophyll synthesis. Photosynthesis emerged in an anaerobic world and is sensitive to oxygen due to the inherent photosensitization of porphyrins and magnesium tetrapyrroles. This project focuses on the role of bilin-binding proteins in synthesis and maintenance of the photosynthetic apparatus in an oxygen atmosphere.

Previously, we have shown that the bilin biosynthesis pathway is ubiquitous in photosynthetic eukaryotes, even though many of these organisms lack known bilin-based light-harvesting or light-sensing proteins. We have also shown that one such organism, the green alga *Chlamydomonas reinhartdii*, requires this pathway for phototrophic growth. In a collaboration with researchers in Australia and China, we have revealed that bilins stimulate MgCh activity in this alga by binding to GUN4, a protein cofactor of MgCh found in all oxygenic species except for the early-diverging cyanobacterial genus *Gloeobacter*. Our results show that GUN4:bilin complexes allosterically activate MgCh activity and allow accumulation of the protoporphyrin-binding CHLH subunit of MgCh. Our ongoing studies address three questions.

1. ***How do bilins stimulate chlorophyll biosynthesis in C. reinhardtii?***
2. ***Do different bilins regulate chlorophyll biosynthesis in other photosynthetic organisms?***
3. ***How did photosynthetic organisms acquire bilin biosynthesis?***

Significant achievements in the past 2 years. Work on the current goals began in late 2020. Our most significance discovery in this time has been documentation of the mechanistic importance of bilins for MgCh activity by binding to GUN4 (Zhang et al, 2021). GUN4 also stabilizes the bilin-dependent accumulation of the protoporphyrin IX-binding CrCHLH1 subunit of MgCh in light-grown *C. reinhardtii* cells. Exogenous application of biliverdin IXα reverses the loss of CrCHLH1 in a bilin-deficient heme oxygenase (*hmox1*) mutant but not in a *gun4* mutant. We propose that retention of bilin biosynthesis in all photosynthetic eukaryotes arises from the need for GUN4:bilin complexes for sustained chlorophyll biosynthesis. In unpublished work, we have completed a phylogenetic analysis demonstrating that GUN4 has co-evolved in some algae with ferredoxin-dependent bilin reductases, the last step in bilin biosynthesis, rather than with MgCh subunits.

Objectives for the coming year. Work in the coming year will address all three goals. We will examine the effects of bilin on Chlamydomonas MgCh using fluorescence and using mutations in the bilin/porphyrin-binding pocket that are currently being evaluated. We will begin to explore the roles of bilins from diverse algae using PCYA enzymes and GUN4 proteins from *Gloeochaete wittrockiana* (glaucophyte), *Cyanidioschyzon merolae* (rhodophyte), *Chroomonas mesostigmatica* (cryptophyte), and *Karenia mikimotoi* (dinoflagellate). We will examine the origins of bilin biosynthesis by characterizing "pre-PcyA" enzymes we have identified via phylogenetic analysis and by examining the atypical phycobiliproteins associated with those proteins and also found in some cyanobacteria. We are also examining bilin binding in a candidate GUN4 paralog found in one isolate of *Gloeobacter* but not in others. Work on algae and on *Gloeobacter* can then be extended to GUN4•CHLH interactions using a plate assay we are currently developing in *E. coli*, reconstituting and/or rescuing phototoxicity with a minimal number of components.

Publications of the last 2 years supported by the award. Work under our current goals has resulted in one publication. Work under the previous goals is also listed in an abbreviated format.

Publication under current goals:

Zhang, W., Willows, R.D., Deng, R., Li, Z., Li, M., Wang, Y., Guo, Y., Shi, W., Fan, Q., Martin, S.S., Rockwell, N.C., Lagarias, J.C. and Duanmu, D. (2021) Bilin-dependent regulation of chlorophyll biosynthesis by GUN4. Proc. Nat'l Acad. Sci. (USA) 118: e2104443118.

Publications under previous goals:

Song et al. (2020) J. Biol. Chem. 295: 6754-6766.

Fushimi et al. (2020) Proc. Nat'l Acad. Sci. (USA) 117: 15573-15580.

Jenkinset al. (2020) Biochemistry 59: 4015-4028.

Moreno et al. (2020) Proc. Nat'l Acad. Sci. (USA) 117: 27962-27970.

Kirpich et al. (2021) Biochemistry 60: 274-288.

Bandara et al. (2021) Proc. Nat'l Acad. Sci. (USA) 118: e2025094118.

Blain-Hartung et al. (2021) Plant Physiol., 187: 632-645.

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 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 dissimilarities in time constants for forward ET and charge recombination on the two pathways (Fig. 1B).

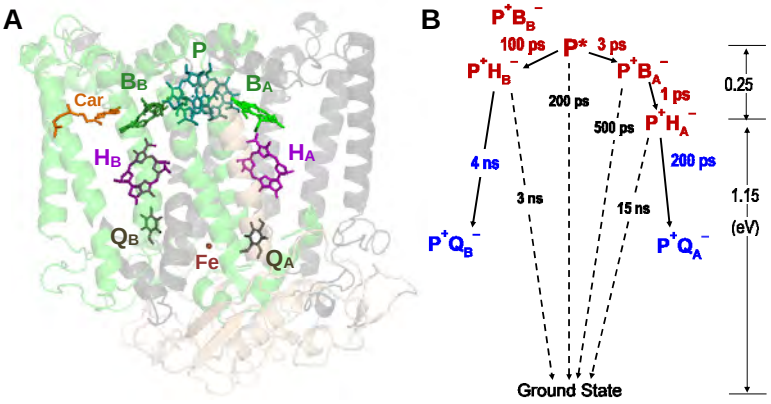

Fig. 1. *(A)* R. sphaeroides *RC (pdb:1PCR). (B) Model free energy diagram and time constants for primary (red) and secondary (blue) processes: forward ET (solid) and charge recombination (dashed).*

The goal of this project is to engineer an RC that performs transmembrane charge separation efficiently and exclusively via the B-side cofactors. Such an RC – and, equally, the steps toward it – will provide a fundamental understanding of how ET between the A-side cofactors is optimized and ET between the B-side cofactors is inhibited in the native RC. Protein-cofactor interactions in RCs from the purple photosynthetic bacteria are manipulated using a semi-directed, molecular-evolution approach. In this way, productive substitutions can be found that would not have been chosen using the more standard site-directed approach (which is also utilized as needed). The photochemistry is analyzed using time-resolved spectroscopic studies spanning <1 ps to >10 s. Combinations of mutations are employed to minimize ET from the excited primary electron donor (P*) to the A-side cofactors and maximize ET from P* to the B-side cofactors (Fig. 1B). Another goal is to slow competing $P^+H_B^-$ charge recombination (Fig. 1B). Selective pressure for photosynthetic growth is employed to identify variants that are photocompetent using solely B-side ET. The principles realized will afford deep insights into RC function and design of biomimetic systems for solar energy conversion.

Significant achievements 9/2019 – 9/2021: Studies have focused on enhancing both the primary and secondary ET processes on the B side as well as further detuning A-side ET.

(1) We engineered *R. sphaeroides* mutant RCs that give astonishing ~90% yields of $P^+H_B^-$. This breakthrough achievement was found for RCs where Arg replaces LeuL185 near H_B in two multiply mutated parent RCs. The 90% yield of ET to H_B occurs via two P* populations, one in which P* decays in ~4 ps via a two-step process involving $P^+B_B^-$ and a second in which P* → $P^+H_B^-$ conversion takes place in ~20 ps by superexchange. These results address the long-standing dichotomy of A- versus B-side initial charge separation and have implications for the

mechanism(s) and timescale of initial ET that are required to achieve a near-quantitative yield of unidirectional charge separation. [Ref. 1 below.]

(2) Mutant RCs with Glu at L185 contain an unusual magnesium chlorin in place of H_B. This chlorin *does* participate in photo-induced ET albeit in low yield. A bacterial RC that incorporates a chlorin naturally (i.e., not by chemical treatment of purified RCs) is remarkable and points to new strategies for tailoring the functional characteristics of RC cofactors or mimetic assemblies. [Ref. 2 below.]

(3) The RCs described above derive from two sets of *R. sphaeroides* mutants wherein all amino acids were substituted for LeuL185. A number of substitutions led to the retention of H_B and several to incorporation of a bacteriochlorophyll. Several variants afford a ~70–90% yield of ET to H_B and some show a notable slowing of $P^+H_B^-$ charge recombination. Both traits support B-side transmembrane charge separation. [Ref. 2 below.]

(4) $P^+H_B^- \rightarrow P^+Q_B^-$ ET is a bottleneck on the B side relative to $P^+H_A^- \rightarrow P^+Q_A^-$ ET on the A side (Fig. 1B, blue). The yield of $P^+Q_B^-$ formed by B-side ET is improved markedly by equipping the channel between H_B and Q_B with up to two non-native tryptophan residues (Fig. 2) in concert with a threonine residue to optimize Q_B binding and (possibly) electronic coupling. In two mutants having a $P^+H_B^-$ yield of ~70%, the measured overall yield of $P^+Q_B^-$ is ~60%. This means that the yield of the $P^+H_B^- \rightarrow P^+Q_B^-$ secondary ET step is ~90%! A paper on this work is being readied for submission.

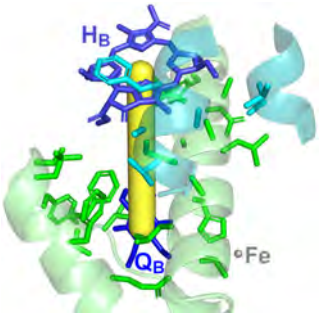

Fig. 2. Sites of Trp substitutions (green) between H_B and Q_B studied for enhanced yields of secondary ET.

(5) Collaborated with Dr. Jennifer Ogilvie on 2D electronic spectroscopy measurements, furnishing mutants and insights into experimental designs and data analysis. [Ref. 3 below.]

(6) Coordinated RC crystal generation with team led by Jan Kern at LBNL for analysis of structure-function relationships using neutron and time-resolved x-ray diffraction.

(7) Leveraged cryo-electron microscopy advances by Karen Davies at the Diamond Light Source to explore the structural basis of directional ET in *R. capsulatus* complexes.

Main science objectives for FY2022:

- Engineer mutants in which ET from P* to H_B occurs mainly via a fast two-step process.
- Develop mutants that simultaneously give ~90% yields of both primary and secondary ET.
- Continue collaborative studies with Drs. Jennifer Ogilvie, Jan Kern and Karen Davies.
- Seek phenotypic revertant strains that are photocompetent via ET to B-side cofactors only.

References to work supported by this project 9/2019 – 9/2021:

1. *Switching Sides – Re-Engineered Primary Charge Separation in the Bacterial Photosynthetic Reaction Center,* P. D. Laible, D. K. Hanson, J. C. Buhrmaster, G. A. Tira, K. M. Faries, D. Holten, and C. Kirmaier, Proceedings of the National Academy of Sciences U.S.A. **2020**, *117*, 865–871.
2. *In Situ, Protein-Mediated Generation of a Photochemically Active Chlorophyll Analog in a Mutant Bacterial Photosynthetic Reaction Center*, 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, Biochemistry **2021**, *60*, 1260–1275.
3. *Hidden Vibronic and Excitonic Structure and Coherence Transfer in the Bacterial Reaction Center*, V. Policht, A. Niedringhaus, C. Spitzfaden, P. Laible, D. Bocian, C. Kirmaier, D. Holten, T. Mančal, and J. Ogilvie, Science Advances **2021** (submitted).

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

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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 an understanding of charge separation by studying the transfer of quantum states from the primary donor to the acceptors of PSI.

1. **Significant Achievements 2019-21. (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 X-ray crystal structures provide a model for the Mn_4Ca-oxo cluster. However, the structure of the OEC and the mechanism of delivery and binding of substrate have been elusive. 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)** In this study, we used ammonia binding to provide valuable insights on substrate delivery in the OEC. We performed two-dimensional (2D) hyperfine sublevel correlation (HYSCORE) spectroscopy that provided direct 'snapshots' of the binding of NH_3 and water molecules in the S_2 state of the Mn_4Ca-oxo cluster.[1] These results, for the first time, unambiguously assign the binding sites of ammonia and water ligands in the S_2 state. **(2)** The serine-169 residue (D1-S169) in the OEC is hydrogen (H)-bonded to the waters, W_1 and W_X, that 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.[2] 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. **(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 studies of water-derived ligands in the presence of an extensive network of H-bonds in the S_2 state.[3] We identified a quantum-delocalized proton between the water-derived ligand, W_1, and D1-D61 residue. The quantum delocalization of the proton results in a much lower zero-point energy, which precludes its release in the $S_1 \rightarrow S_2$ transition of the OEC. **(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 understand the functional differences. **(1)** We used 2D ^{1}H HYSCORE to determine the electronic structure of $Y_Z^{\bullet}$ and $Y_D^{\bullet}$. We find that the key differences include (i) increased electronic asymmetry, (ii) difference in the relative orientation of the side chain and (iii) stronger H-bonding in $Y_Z^{\bullet}$, which likely renders it kinetically competent for water oxidation.[4] **(2)** A detailed understanding of PCET at Y_Z has been complicated as it is kinetically competent in water oxidation. We recently utilized a combination of 2D ^{14}N HYSCORE and DFT to investigate the electronic structure of an artificial reaction center, benzimidazole-phenol porphyrin ($BiP–PF_{10}$), that mimics the PCET reactions at Y_Z. The results demonstrate the significance of charge delocalization and inclusion of explicit water molecules

on the tuning of PCET in BiP–PF_{10} suggesting that these features must be considered in predicting of electronic properties of Y_Z.[5] **(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 calculations to explore the nature the $A_0^{\cdot-}$ state. Analysis of the hyperfine coupling constants revealed 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.[6] Interestingly, this dimerization occurs independently of the axial ligand, as seen when the Met that ligates Chl_3 is changed to a His, with the only noticeable change being a shift in the asymmetry.[7] The dimerization of the primary acceptor likely serves to ensure charge separation is energetically downhill, and that subsequent recombination is slowed. **(E) Zn-Bacteriochlorophyll a_P' dimer is the primary donor (P_{840}) in the *Chloracidobacterium thermophilum RC***: *Chloracidobacterium* (*C.*) *thermophilum* is a microaerophilic, chlorophototrophic species in the phylum *Acidobacteria* that uses a homodimeric Type I RC with (bacterio)chlorophyll ((B)Chl) cofactors. Pigment analyses have shown that the RCs contain BChl a_P, Chl a_{PD}, and Zn^{2+}-BChl a_P' in the approximate ratio 7.1:5.4:1. However, the functional roles of the three different Chl species are not yet fully understood. It was recently demonstrated that Chl a_{PD} is the primary electron acceptor. Because Zn^{2+}-(B)Chl a_P' is present at low abundance, it was suggested that the primary electron donor might be a dimer of Zn^{2+}-BChl a_P' molecules. We utilized isotopic enrichment and high-resolution two-dimensional (2D) ^{14}N and ^{67}Zn HYSCORE spectroscopy to demonstrate that the primary donor cation, P_{840}^+, in the *C. thermophilum* RC is indeed a Zn^{2+}-BChl a_P' dimer.[8] To our knowledge this is the only example of a photochemical RC in which the Chl molecules of the primary donor are metallated differently than those of the antenna.

Science Objectives for 2021-22. (i) We determined the electronic structure of water-derived ligands in the S_2 state of the OEC. We are extending these studies to include the determination of the binding and protonation states of the substrate waters in the high-spin S_2 and S_3-state intermediates of the OEC. (ii) We are investigating the mechanism of light-induced PCET in genetic variants of the tyrosines of PSII and (iii) we are developing a system to investigate the transmission of quantum electron spin states in PSI.

References to Work Supported by this Project 2019-21.

1. Kalendra *et al*. "Mechanism of Binding of Ammonia as a Substrate Analog in the Oxygen-evolving Complex of Photosystem II" (2021) Manuscript in revision.
2. Kalendra *et al*. "Binding of Substrate Water in the S_2 to S_3 State Transition of the D1-S169A Variant of Photosystem II" (2021) Manuscript in revision.
3. 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, 8324-8332.
4. Mark *et al*. "Determining the Electronic Structure Paramagnetic Intermediates in Membrane Proteins: A High-resolution 2D ^1H Hyperfine Sublevel Correlation Study of the Redox-active Tyrosines of Photosystem II" (2020) *Biochim. Biophys. Acta- Biomemb*., 1862, 183422.
5. 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.
6. Gorka *et al*. "A Dimeric Chlorophyll Electron Acceptor Differentiates Type I from Type II Photosynthetic Reaction Centers" (2021) *iScience* (Cell Press), 24, 102719.
7. 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.
8. Charles *et al*. "The Role of Zinc-Bacteriochlorophyll a' in the Primary Photochemistry of Chloroacidobacterium thermophilum" (2020) *Phys. Chem. Chem. Phys*., 22, 6457.
9. Gorka *et al*. "Shedding Light on Primary Donors in Photosynthetic Reaction Centers" (2021) *Front. Microbiol*., 12, 735666.
10. Chai *et al*. "The Role of Hydrogen Bonds in PCET at the Redox-Active Tyrosine Residues in Photosystem II" (2019) *Phys. Chem. Chem. Phys*., 21, 8721.

Probing Molecular Mechanism of Action of the Cyanobacterial Orange Carotenoid Protein by Using Structural Mass Spectrometry

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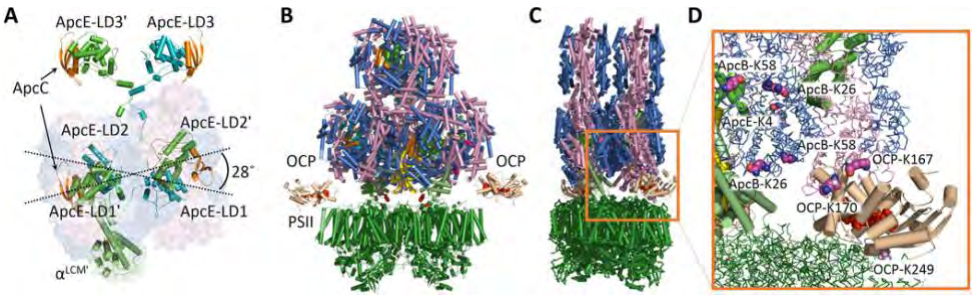
Overall goals of the project - Develop structural mass spectrometry techniques to probe the molecular mechanism of orange carotenoid protein/FRP, ligand binding stoichiometry/affinity, structural location of OCP on the thylakoid membrane and their structural reorientation upon binding/quenching of excitation energy of phycobilisome.

Significant achievements of the past 2 years – Have used structural mass spectrometry and generated a near atomic level of cyanobacterial phycobilisome core structure to facilitate the OCP binding/quenching mechanism research in the field. We firstly identified an acute X-shape of the two basal cylinders of cyanobacterial PBS (Fig. A). The extra space between PBS and the planar stromal side of Photosystem II allows access of regulatory molecules, such as OCP or others (Fig. B,C,D). The result was published on ***Science Advance*** Vol. 7. No.2 DOI: 10.1126/sciadv.aba5743



News Release: https://source.wustl.edu/2021/01/orange-is-the-new-block/

Objectives for the coming year – Develop new mass spectrometry associated chemistry to elucidate the structural sequestration/mobilization of both OCP and FRP on the thylakoid membrane in their photoprotection cycles.

Publications of the last 2 years supported by the award –

Johnson, V.M., Biswas, S., Pakrasi, H.B. and Liu, H. (2021) Psb27, a Photosystem II assembly protein, enables quenching of excess light energy during its participation in the PSII life cycle, ***Photosynthesis Research*** (submitted)

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

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

Sonani, R.R., Roszak, A.W., Liu, H., Gross, M.L., Blankenship, R.E., Madamwar, D., and Cogdell, R.J. (2020) Revisiting high-resolution crystal structure of *Phomidium rubidum* phycocyanin. ***Photosynthesis Res*** 144, 349-360

*Lou, W., *Niedzwiedzki, M.D, Blankenship, R.E., and Liu, H. (2020) Binding of red form of Orange Carotenoid Protein (OCP) to phycobilisome is not sufficient for quenching. ***Biochim Biophys Acta*** (Bioenergetics). 1861(3):148155 * these authors contribute equally

Photoprotective Structural Change Led by Intramolecular Vibrational Switching

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Picosecond protein structural dynamics enable efficient conformational transitions during biological function. We examine the switching of these long range collective structural vibrations of the photoprotective protein orange carotenoid protein (OCP) using anisotropic terahertz microspectroscopy, which provides dynamical fingerprinting for biomacromolecules by anisotropic absorption of protein crystals (1, 2). OCP controls efficiency of the phycobilisome (PBS), the light harvesting antenna in cyanobacteria, to prevent oxidative damage. In low light, carotenoid bound OCP appears orange (OCP^o). Absorption of blue-green light results in a 12 translocation of the carotenoid into the N-terminal domain (NTD) and conversion to an active red state (OCP^R) which interacts with the light harvesting antenna, the phycobilisome (PBS), to induce fluorescence quenching(3). We examine the changes in the collective vibrations with photoexcitation under two conditions. Under excitation similar to the solution phase switching we detect reversible photoswitching of the THz vibrational bands through double difference analysis. However, switching of the OCP crystal optical absorbance is not observed. Using conditions to achieve optical absorbance switching in the crystal, we confirm that the intramolecular vibrational changes arise from the transition to the photoprotective state. We compare the measured spectral changes to those calculated using normal mode ensemble analysis (NMEA) for apo and holo OCP. NMEA accounts for population sampling of the rugged energy landscape (4). The calculated spectral difference in the 38-55 cm^{-1} range is strikingly similar to the observed changes. To assign the spectral features to structural motions, we examine the correlations in backbone motions for the 38-41 cm^{-1} spectral peak. Incredibly, long-range correlations persist with the averaging over the energy landscape. The changes in the correlated motions span the CTD and NTD, and may provide the dynamical bias towards the reorganization of the NTD for the translocation of the carotenoid and the separation of the NTD from the CTD necessary for the interaction with the phycobilisome.

This work is supported by DOE grant DE-SC0016317

1. Deng, Y. T.; McKinney, J. A.; George, D. K.; Niessen, K. A.; Sharma, A.; Markelz, A. G., Near-Field Stationary Sample Terahertz Spectroscopic Polarimetry for Biomolecular Structural Dynamics Determination. *Acs Photonics* **2021,** *8* (2), 658-668.
2. Niessen, K. A.; Xu, M. Y.; George, D. K.; Chen, M. C.; Ferre-D'Amare, A. R.; Snell, E. H.; Cody, V.; Pace, J.; Schmidt, M.; Markelz, A. G., Protein and RNA dynamical fingerprinting. *Nat. Comm.* **2019,** *10*.
3. Leverenz, R. L.; Sutter, M.; Wilson, A.; Gupta, S.; Thurotte, A.; de Carbon, C. B.; Petzold, C. J.; Ralston, C.; Perreau, F.; Kirilovsky, D., et al., A 12 angstrom carotenoid translocation in a photoswitch associated with cyanobacterial photoprotection. *Science* **2015,** *348* (6242), 1463-1466.
4. Romo, T. D.; Grossfield, A.; Markelz, A. G. Persistent Protein Motions in a Rugged Energy Landscape Revealed by Normal Mode Ensemble Analysis *J. Chem. Inf. Model.* [Online], 2020. https://doi.org/10.1021/acs.jcim.0c00879 (accessed 2020/10/26).

Iron economy in photosynthesis

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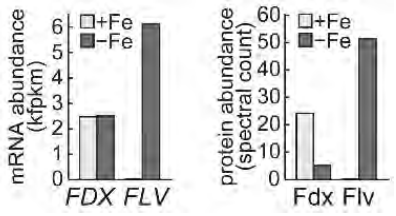
Overall goals of the project – Background: Iron (Fe) is an essential nutrient for life because of its broad function as a catalyst. Despite its abundance, Fe has limited bioavailability because of its insolubility in the Fe(III) state, which is the prevalent species in aerobic environments. About 30% of the oceans, responsible for about half of global primary productivity, are Fe-limited, and a similar fraction of the world's croplands have poor iron availability. Therefore, primary metabolism in photosynthetic organisms often occurs in situations of Fe-deficiency stress in nature. The bioenergetic organelles house much of the metabolic capacity of eukaryotic cells and accordingly are sinks for Fe utilization. About 80-90% of leaf Fe is in the chloroplast with half of that amount associated with thylakoid membranes. In Chlamydomonas, a suitable microbial reference organism for dissecting iron-limitation stress responses, we previously described changes in antenna protein abundance and physical / functional connection to reaction centers as being critical acclimation steps for regulation of excitation energy distribution. Some green algae, including *Dunaliella* spp. and *Chromochloris zofingiensis* have the option of reducing the Fe quota with a flavodoxin to replace ferredoxin, which in addition to PSI, is a major Fe sink in photosynthetic organisms. These algae also induce alternate chlorophyll binding putative antenna proteins.



Flv is dramatically induced in *Dunaliella* spp. under low Fe nutrition. Transcript abundances for ferredoxin and flavodoxin are from RNA-Seq experiments and protein abundances are from quantitative proteomics at EMSL, validated by immunoblotting. Fdx = ferredoxin, Flv = flavodoxin. Dunaliella cells are photo-autotrophic with a reduced Fe quota because they can replace ferredoxin by flavodoxin.

Objectives: During the project period of the present award, 1) we will compile a portfolio of Chlamydomonas Fe proteins and use quantitative proteomics to estimate the chloroplast luxury vs. economy Fe quota in photoheterotrophic vs. photoautotrophic cells and 2) estimate the changes in the absolute abundances of PSI and LHCI polypeptides in Fe economy mode, especially in the context of trophic transitions (CO_2 to acetate); 2) we will exploit stable isotope pulse-chase experiments to track Fe movement from ferredoxin to ferritin during acclimation of Chlamydomonas to low Fe, and 3) we will use single particle electron microscopy under cryogenic conditions (collaboration with Iwai, Niyogi and Nogales) to capture structural modifications to the PSI-LHCI super-complexes in Fe-limited 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 of the past 2 years – 1) We employed XFM in *Chlamydomonas reinhardtii* to determine single-cell and organelle trace metal quotas within algal cells in situations of trace metal overaccumulation (Fe and Cu). Quantitation of the Fe and Cu contents of individual cells and compartments via XFM, over a range of cellular metal quotas created by nutritional and genetic perturbations, indicated excellent correlation with bulk data from corresponding cell cultures, establishing a framework to distinguish the nutritional status of single cells. 2) We applied single-cell RNA sequencing (scRNA-seq) to probe the heterogeneity of Chlamydomonas cell populations. scRNA-seq successfully separated single cells into nonoverlapping cell clusters enabling us to readily distinguish iron from nitrogen deficiency despite a shared tendency to arrest photosynthesis and cell division to economize resources. A substantial source of variation between cells originated from their endogenous diurnal phase, although cultures were grown in constant light. We exploited this result to show that circadian iron responses may be conserved from algae to land plants.

Objectives for the coming year – 1) What is the inventory of Chlamydomonas chloroplast Fe proteins, and how does that inventory change in response to Fe nutrition and trophic growth style? We propose to use comparative proteomics of Fe-replete vs. Fe-deficient and Fe-limited cells to construct inventories of Fe proteins that contribute to the chloroplast Fe quota. 2) How is PSI-LHCI organization in Chlamydomonas affected by poor Fe nutrition? We will use the MS/MS technique of selected reaction monitoring (SRM) in a targeted proteomics approach to quantitatively evaluate changes to the PSI-LHCI super-complex. 3) How do changes in the processing / abundance of Psa and Lhca subunits in cells experiencing poor Fe nutrition impact the organization of the PSI-LHCI complex? What is the organization and role of novel chlorophyll proteins that replace Lhca in Fe-limited Dunaliella? We will address this question by single-particle cryo-EM analysis of PSI-LHCI complexes from Fe-limited Chlamydomonas and Dunaliella cells.

Publications of the last 2 years supported by the award – (previous award, peer-reviewed)

1) Tsednee, M., Castruita, M., Salome, P.A., Sharma, A., Lewis, B.E., Schmollinger, S.R., Holbrook, K., Otegui, M., Khatua, K., Das, S., Datta, A., Chen, S., Ramon, C., Ralle, M., Weber, P.K., Stemmler, T.L., Pett-Ridge, J., Hoffman, B.M., Merchant, S.S. (2019) Manganese co-localizes with calcium and phosphorus in Chlamydomonas acidocalcisomes and is mobilized in manganese-deficient conditions. **J. Biol. Chem**. 294, 17626-17641. https://doi.org/10.1074/jbc.RA119.009130

2) Schmollinger, S., Chen, S., Strenkert, D., Hui, C., Ralle, M., Merchant, S.S. (2021) Single-cell visualization and quantification of trace metals in Chlamydomonas lysosome related organelles, **Proc. Natl. Acad. Sci. USA** 118:e2026811118 https://doi.org/10.1073/pnas.2026811118

3) Ma, F., Salome, P.A., Pellegrini, M. Merchant, S.S. Single-Cell RNA Sequencing of Batch Chlamydomonas Cultures Reveals Heterogeneity in their Diurnal Cycle Phase (2021) **Plant Cell**, 31:1042-1057. doi: https://doi.org/10.1101/2020.09.15.298844

Cyclic electron flow and thylakoid ultrastructure during long term stress in the photopsychrophile, *Chlamydomonas* sp. UWO241

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Overall goals of the project: The goal of this project is to describe the function of sustained CEF and assembly a PSI supercomplex (SC) in the Antarctic photopsychrophile *Chlamydomonas* sp. UWO241 (UWO241). Major objectives are: **1)** determine the functional role(s) of sustained CEF and its impacts on downstream carbon metabolism in UWO241; **2)** solve the structure of the UWO241 SC through proteomic and structural studies; **3)** determine whether *C. reinhardtii* also assembles a SC to support sustained CEF under high salinity. Outcomes of this project will support research focused on meeting future energy and food needs by advancing our understanding of how extremophilic phototrophs use sustained CEF to survive long-term environmental stress.

Significant achievements of the past 2 years (graduate and undergraduate* students)**

[1] Long-term acclimation in UWO241 (S. Stahl-Rommel**, S. D'Silva*, D. Popson**). We have described that UWO241 utilizes high CEF as a universal mechanism to support robust growth and photosynthesis under high salinity, high light, or low temperatures [1]. High CEF was correlated with a large capacity for NPQ and very low ROS (superoxide, H_2O_2) production. UWO241 also exhibits constitutively high activity for two key enzymes in the ascorbate pathway and maintains a large ascorbate pool, potentially resulting in highly efficient ROS detoxification. We propose that tight control over photostasis and ROS are critical for UWO241 to maintain a functional photosynthetic apparatus during long-term exposure to environmental stress (Fig. 1).

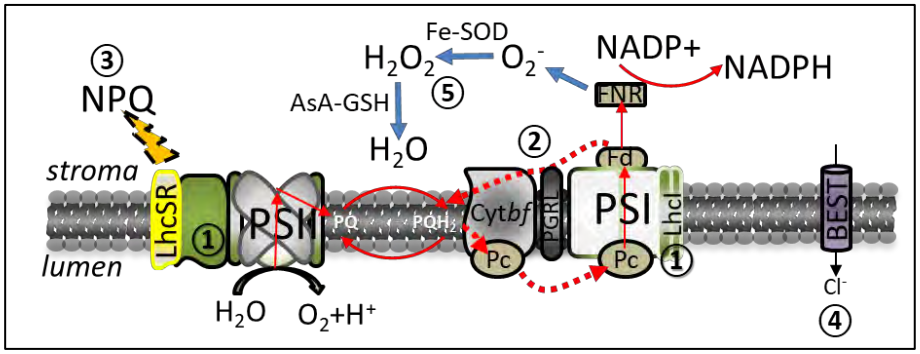

Figure 1. An updated model of the photosynthetic apparatus of UWO241 (Stahl-Rommel et al. In press).

[2] Impact of high salinity on state transitions and CEF (I. Kalra**). Past research has shown that UWO241 has constitutive CEF, representing a natural state transition mutant. We tested whether either phenotype is associated with long-term exposure to low temperatures or high salinity by comparing state transition capacity and CEF rates in UWO241 (psychrophilic & halotolerant), *Chlamydomonas* sp. ICE-MDV (psychrophilic only), and *C. reinhardtii* (neither). High salinity activated CEF suppresses state transition capacity in all three strains. These phenotypes were most pronounced in UWO241 which is a natural state transition mutant and exhibits constitutive levels of high CEF under low temperature or high salinity (manuscript in prep).

[3] High salt evolved *C. reinhardtii* strain (I. Kalra**, I Jacques*). We developed a high salt-evolved strain (Ev) of *C. reinhardtii* which exhibits high growth rates and maintains high photosynthetic activity in autotrophic medium at NaCl levels (100 mM) which are non-permissive for the Wt

strain. Relative to the Wt strain, the Ev strain possesses higher rates of CEF under either low or high salt conditions. Whole cell proteomics showed that the Ev strain exhibited downregulation of ROS-scavenging enzymes as well as key enzymes of photorespiration (manuscript in prep).

[4] High light acclimation in UWO241 (D. Popson**, S. D'Silva*). While UWO241 is adapted to extreme shade, high rates of CEF have been associated with improved resistance to photoinhibition. We have shown that UWO241 possesses remarkable ability to avoid PSII photoinhibition which is enhanced when cells are pre-acclimated to either high light or low temperatures. Conversely, high salt-acclimated cells exhibited enhanced sensitivity to photoinhibition (manuscript in prep).

[5] Genomics, transcriptomics. In collaboration with David Smith (Western Ontario) and Marina Cvetkovska (University of Ottawa), we published the draft genome of UWO241 [4] and published a popular science article in a Frontiers journal for young scientists [5]. We also annotated a low temperature high salt transcriptome of UWO241 [6]. Both datasets are deposited in NCBI (Bioprojects PRJNA547753 and PRJNA575885).

[6] Cryo-ET pilot study. We began a new collaboration with the SLAC National Accelerator Laboratory to determine the feasibility of capturing thylakoid membrane architecture within vitreously-frozen cells of UWO241. Fresh cultures of low and high-salt acclimated cultures were sent to SLAC, and 3D cryo-electron tomograms were successfully generated from the high salt cultures. The SLAC investigators are currently working on sub-tomogram averaging of the thylakoids to localize the PSI supercomplexes. We will also send purified supercomplexes for single particle analysis.

Objectives for the coming year

With the exception of structural studies in Objective 2, we are near-completion on our project goals and are focused on writing and submitting manuscripts. **Objective 1**. We are writing two more manuscripts, one focusing on UWO241 response to short-term and long-term exposure to high light, and a second comparing CEF, SC formation, and attenuation of state transitions across three algal species with varying temperature and salinity tolerance. **Objective 2**. We will continue to work with our new collaborators at SLAC on development of Cryo-EM and Cryo-ET methods to visualize the UWO241 SC. **Objective 3**. We are writing a manuscript comparing the physiology and proteomes between Wt and Ev strains of *C. reinhardtii*.

Publications of the last 2 years supported by the award (Co-PIs and PI; **graduate students, *undergraduate students, on this project)

[1] 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. UWO 241 (renamed *Chlamydomonas priscuii*) *Photosynthesis Research*. *In press*.

[2] Huner N.P.A., Smith D.R., Cvetkovska M., Zhang X., Ivanov A., Szyska-Mroz B., **Morgan-Kiss R**. (2021) Photosynthetic adaptation to polar life: photopsychrophily and psychrotolerance as emergent properties. Journal of Plant Physiology, Humbolt Review, *In Press*.

[3] Smith D.R., Leung A., Zhang X., Cvetkovska M., **Morgan-Kiss R.M.**, Huner N. (2021) The cold-loving Antarctic alga UWO241: it keeps a licking and keeps on ticking. Frontiers in Young Minds – Biodiversity. *In Press*.

[4] Zhang, X., Cvetkovska, M., **Morgan-Kiss, R**., Hüner, N.P.A., and Smith, D.R. (2021) Draft genome sequence of the Antarctic green alga *Chlamydomonas* sp. UWO241. *iScience* 102084-102084

[5] 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

[6] Raymond, J.A., **Morgan-Kiss, R.,** 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

Photosynthetic Energy Transduction Core Program: Dissecting the Electron-Transfer Mechanism of Photosynthetic Flavodiirons

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Overall goals of the project: The overall goal of the Photosynthetic Energy Transduction core program at NREL is to understand the fundamental mechanisms for coupling reaction center photochemistry to electron transport and reduction-oxidation reactions of enzymes. The process of electron transformation is fundamental to solar energy conversion and is exquisitely controlled in photosynthesis from light capture to chemical conversion. This entails coordinating reaction phenomena across networks that occur over vast time (nanoseconds-to-seconds) and length (subatomic to molecular) scales with reaction intermediates that can be elusive to define due to dynamic and shifting energy landscapes. To address these challenges, the research objectives will determine the physical interactions and functional properties of reaction center photochemistry, the biophysical properties and reactivity of ferredoxins in coupling electron-transport to redox enzymes, and how redox enzymes and cofactors coordinate fluxes to achieve extraordinary turnover and substrate selectivity. In doing so, the program will evolve toward a fundamental understanding on the diverse array of photosynthetic components and interactions that are essential for successfully managing electron-transport under highly variable conditions for the efficient conversion of sunlight to chemicals and fuels.

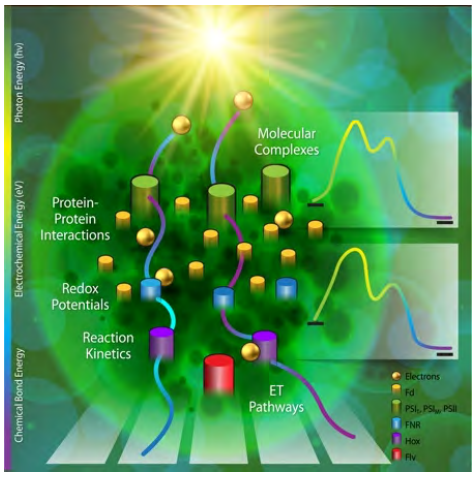

Figure 1. Electron transformation reactions in photosynthesis consist of highly coordinated protein interactions that form thermodynamic landscapes for directing electrons to selective catalytic reactions.

Significant achievements of the past 2 years: As part of these efforts, one area of focus has been to understand how photosynthetic electron-flux is synchronized to catalytic reactions. Using model enzymes such as Hox [NiFe]-hydrogenase, [FeFe]-hydrogenase, and flavodiirons (Flv), we have been probing mechanisms of electron-transport via metal sites and organic cofactors, how active-sites couple flux to productive proton-coupled electron-transfer (PCET) chemistry, and how enzymes carry out PCET reactions with certain reactivity. Specific achievements are presented here on dissecting the electron-transfer mechanism of photosynthetic Flvs, which catalytic the selective 4-electron reduction of O_2 to H_2O by coupling to NAD(P)H oxidation (Figure 2). Utilizing thermodynamic and kinetic measurements on purified Flv3 from

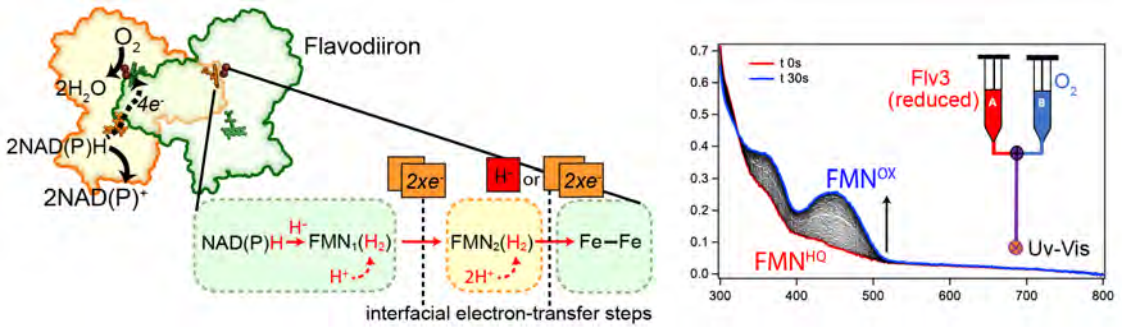

Figure 2. Left: Schematic for coupling electron-transfer steps across the homodimeric Flv to the O_2 reduction reaction. Right: Stopped-flow measurements of reduced Flv3 mixed with O_2 showing 2-electron oxidation of FMN_{HQ} to FMN_{Ox}.

Synechocystis sp. PCC 6803, our findings indicate a reaction mechanism designed to minimize formation of reactive, 1-electron flavin semiquinone species that could lead to the formation of damaging reactive oxygen species. Corresponding in-vivo studies utilizing *flv1* or *flv3* knock-out strains show changes in PSI oligomeric state and altered photochemical properties in the absence of Flv1 or Flv3, solidifying a functional and more expansive role of Flv in alternative electron-flow pathways for operating under dynamic environmental conditions.

Objectives for the coming year –

- Elucidate the functional role(s) of Photosystem I (PSI) supercomplexes employed under altered cellular redox conditions.
- Determine the reduction potentials, biophysical properties, and reaction profiles of ferredoxins to understand their function in mediating electron transport between PSI and redox enzymes.
- Understand how redox enzymes such as Hox [NiFe]-hydrogenase, [FeFe]-hydrogenase, and Flv mediate electron transformation reactions with catalysis and how redox cofactors are tuned to impart exquisite control over enzyme reactivity.

Publications of the last 2 years supported by the award –

1. 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. *Sustain. Energy Fuels*. **3**: 3191-3200.
2. Lubner, C. E. 2019. Bacteria 'read' light to gain a competitive advantage. *J. Bacteriol*., 201:e0082-19.
3. Brown, K.A., King, P.W. 2020. Coupling biology to synthetic nanomaterials for semi-artificial photosynthesis. *Photosyn. Res.* **143**:193-203.
4. Artz, J.H., Tokmina-Lukaszewska, M., Mulder, D.W., Lubner, C.E., Gutekunst, K., Appel, J., Bothner, B., Boehm, M., and King, P.W. 2020. Defining the structure and reactivity of the HoxEFU complex from *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* **295**:9445-9454.

Exploring structure-function relationships governing transport by the major cyanobacterial bicarbonate transporters SbtA and BicA

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Overall goals of the project:

Cyanobacteria play a large and essential role in the global carbon cycle, while also representing promising microbial platforms for the direct bioproduction of fuels and chemicals from solar energy and carbon dioxide (CO_2). In both cases, a critical step in the overall photosynthetic process is the transport of inorganic carbon (C_i) across the cell membrane, which occurs via different mechanisms and associated transporter proteins that target either CO_2 or bicarbonate (HCO_3^-) In the latter case, BicA (a constitutively-expressed, high-flux/low-affinity Na^+/HCO_3^- symporter) and SbtA (a low-flux/high-affinity Na^+/HCO_3^- symporter, expressed only under low CO_2 conditions) have been identified as the two main cyanobacterial HCO_3^- transporters. Using a combination of detailed functional and high-resolution structural studies, this project seeks to help fill current knowledge gaps regarding our collective understanding of the key mechanisms of HCO_3^- transport employed by BicA and SbtA.

Significant achievements of the past 2 years:

To facilitate functional screening of SbtA and BicA, we engineered a Δ4 (Δ*ndhD3* Δ*ndhD4* Δ*sbtA* Δ*bicA*) mutant of *Synechococcus* sp. PCC 7002 which can grow only elevated CO_2 (e.g., 5%) but not under air (Fig. 1). Low CO_2 (air or 0.5% CO_2) growth can be restored, however, following the introduction of a functional copy of SbtA, BicA, or derived mutants. This strain can serve as a facile screen for preliminary testing of the function of SbtA and BicA mutants of interest, which will be developed to test current and future hypotheses.

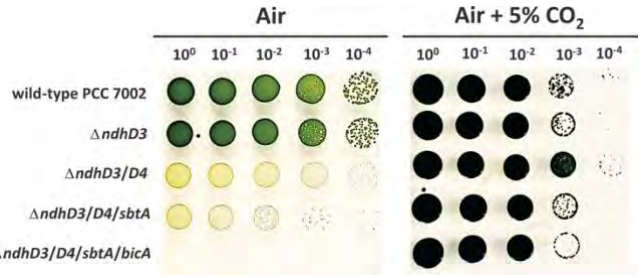

Figure 1. Construction of a PCC 7002 Δ4 for functional screening of SbtA and BicA mutants.

The Δ4 strain was first used to evaluate mutants of BicA, which is a member of the broader SulP/SLC26 family of anion transporters whose function has been extensively studied as result of their human health relevance (e.g., SLC26A2, SLC26A4). Using this information, a subset of six mutations known to alter the transport function of other SulP/SLC26 transporters (both positively and negatively) was mapped to and introduced at their corresponding residues in BicA. The resulting BicA* mutants were then screened via complementation studies using the above PCC 7002 Δ4 mutant. Among these, one mutation – F232A – has shown particular promise with respect to enhancing the function of BicA, as determined based on improved growth under 0.5% CO_2. Specifically, expression of this mutant resulted in a 38% reduction in doubling time relative to wild-type BicA (Fig. 2). F232A is located along a periplasmic loop between transmembrane segments 7 and 8, which we postulate could provide some structural control over the transport action of BicA (Fig. 3). Accordingly, we are currently further interrogating this residue via site

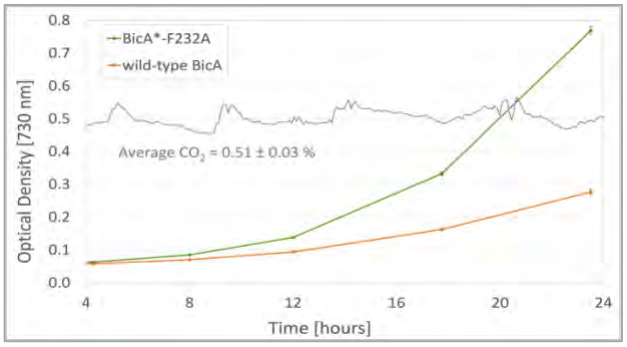

Figure 2. Comparing differences in exponential growth of the Δ4 expressing F232A BicA vs. wild-type BicA.*

saturation mutagenesis as well as the entire loop via an alanine substitution method. We are also currently mapping non-conserved residues across BicA homologs with known activities differences to further diversify our screening approach. A similar approach is also being applied with respect to functionally diverse SbtA homologs, which will similarly be initially screened using the Δ4 strain.

Partial structures of BicA were recently reported for both the transmembrane and STAS domains. Furthermore, these structures were obtained using sample produced by and purified from *Escherichia coli*. Since BicA is not known to be functional in *E. coli*, the important objective of obtaining the full-length structure of functional BicA still remains, and we have been pursuing this by focusing on cyanobacterial expression hosts. To this end, we are leveraging a recently developed T7 RNA polymerase expression system with the potential to boost BicA yields while still ensuring that the functional protein is obtained. Once optimized, the same approach will be used to also improve yields of all BicA* mutants of interest (including F232A). These samples will be purified and used for structural studies in order to better understand the influence of such mutations of observed activities differences, as well as improve our understanding of how BicA functions.

Objectives for the coming year:

We will continue to screen mutations of interest on BicA, first using growth-based assays then then via O_2 evolution experiments. We will also begin ramping up studies on SbtA using analogous approaches and, in particular, by mapping residues of interest between different homologs shown to display vastly different activities. In addition, random mutant libraries will be generated and screened for both BicA and SbtA in order to increase diversity. Optimization of our cyanobacterial expression platform will continue with a focus on first determining achievable yields of BicA and SbtA as well as factors influencing those yields. High yields of SbtA and especially full-length BicA will then facilitate structure determination efforts for wild-type and superior mutants.

Publications of the last 2 years supported by the award:

Conference Presentations:

'Protein Engineering to Improve the Function of BicA: A Constitutively Expressed, High-Flux Bicarbonate Transporter in *Synechococcus* sp. PCC 7002', Parrish, S., Bu, G., Jones, C., Nielsen, D.R., Nannenga, B.L. American Institute of Chemical Engineers (AIChE) Annual Meeting, Boston, MA (2021)

Regulation of Photosynthesis

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Thien Crisanto, Lam Lam, Tsung-Yen Lee, Dhruv Patel, Audrey Short, Collin Steen, Graduate Students
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Overall goals of the project – 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 genetic and biochemical techniques 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 of the past 2 years – To unravel the complex relationship of the known actors in NPQ, we performed chlorophyll fluorescence lifetime snapshot measurements during regular and irregular repeated light/dark cycles. By comparing the data in *Arabidopsis* with a mathematical model that describes both fast and slow quenching responses, we found that the rapidly reversible quenching response depends on the state of the slower response and that PsbS and Zea have distinct roles in both quenching and recovery. In *Chlamydomonas reinhardtii*, we observed a striking increase in quenching in the dark in the *npq4 lhcsr1* mutant, which lacks both LHCSR3 and LHCSR1, the sites of qE in this green alga. We showed via a mutant lacking STT7 kinase that this "out of phase" behavior most likely arises from state transition. The response to periodic light/dark sequences is quite different for the first and subsequent light periods. In the photosynthetic stramenopile *Nannochloropsis oceanica*, overall induction of NPQ appears logarithmic. This led us with help from David Limmer (UCB/LBL) to write a simple mathematical model for the *N. oceanica* NPQ response where the product of [Zea] (or more precisely de-epoxidized xanthophylls) and the protonated LHCX1 is the main generator of the light response. Using a forward genetics approach, we identified ROQH1 as a stromal protein that is involved in turning off slowly reversible qH in *Arabidopsis*, possibly through interaction with LHCII, a site of qH. In *N. oceanica*, we found that a violaxanthin de-epoxidase-like enzyme, VDL, catalyzes the conversion of violaxanthin to all-trans neoxanthin, the first step in the synthesis of allenic carotenoids. Homologs of this enzyme in other marine algae are involved in synthesis of fucoxanthin and peridinin, the most abundant carotenoids in marine algae. We also isolated and characterized *hlr1*, the first mutant affecting a member of the LHCR family of light-harvesting proteins, which are found in algae that contain a red algal plastid. By sequencing the genomes of 660 *Chlamydomonas* insertional mutants that are defective in photosynthesis, we identified 253

higher-confidence photosynthesis genes, two-thirds of which have not been previously characterized. Eight of these mutants affect CPSFL1, a conserved Sec14 domain protein in the chloroplast that affects carotenoid biosynthesis and the accumulation of carotenoid-rich chloroplast structures such as plastoglobules and the eyespot.

Objectives for the coming year –

- Perform structure-function analysis of LHCX1 in *N. oceanica* using knock-in mutations and/or episomal expression of mutant proteins, and investigate functions of LHCX2 and LHCX3.
- Use snapshot TA spectroscopy to measure timescales of CT and EET quenching in *N. oceanica* mutants that lack qE or qZ.
- Use snapshot TA spectroscopy to measure timescales of CT and EET quenching in thylakoids from *Nicotiana benthamiana* mutants affecting PsbS and xanthophylls.
- Measure the exciton diffusion length by exciton-exciton annihilation.
- Determine the location of PsbS in photosystem II using single-particle cryo-EM analysis.
- Reconstitute qE in proteoliposomes containing thylakoid lipids, oriented LHCII, zeaxanthin, and PsbS.
- Generate fluorescently tagged LHCII to visualize its dynamics in vivo.

Publications of the last 2 years supported by the award –

1. Oldemeyer S, Haddad AZ, Fleming GR (2020). Interconnection of the antenna pigment 8-HDF and flavin facilitates red light reception in a bifunctional animal-like cryptochrome. *Biochemistry* 59: 594-604.
2. Amstutz CL, Fristedt R, Schultink A, Merchant SS, Niyogi KK, Malnoë A (2020). ROQH1 is a negative regulator of lipocalin-dependent non-photochemical quenching in *Arabidopsis thaliana*. *Nature Plants* 6: 154-166.
3. Tietz S, Leuenberger M, Höhner R, Olson AH, Fleming GR, Kirchhoff H (2020). A proteoliposome-based system reveals how lipids control photosynthetic light harvesting. *J Biol Chem* 295: 1857-1866.
4. Dautermann O, Lyska D, Andersen-Ranberg J, Becker M, Gartmann H, Krämer LC, Mayr K, Pieper D, Rij LM, Niyogi KK, Lohr M (2020). An algal enzyme required for biosynthesis of the most abundant marine carotenoids. *Sci Adv* 6: eaaw9183.
5. Onoa B, Fukuda S, Iwai M, Bustamante C, Niyogi KK (2020). High-speed atomic force microscopy visualizes mobility of photosynthetic proteins in grana thylakoid membranes. *Biophys J* 118: 1876-1886.
6. Hertle AP, García-Cerdán JG, Armbruster U, Shih R, Lee JJ, Wong W, Niyogi KK (2020). A Sec14 domain protein is required for photoautotrophic growth and chloroplast vesicle formation in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 117: 9101-9111.
7. García-Cerdán JG, Schmid EM, Takeuchi T, McRae I, McDonald K, Yordduangjun N, Hassan AM, Grob P, Xu CS, Hess HF, Fletcher DA, Nogales E, Niyogi KK (2020). Chloroplast Sec14-like 1 (CPSFL1) is essential for normal chloroplast development and promotes carotenoid accumulation in *Chlamydomonas*. *Proc Natl Acad Sci USA* 117: 12452–12463.
8. Steen CJ, Morris JM, Short AH, Niyogi KK, Fleming GR (2020). Complex roles of PsbS and xanthophylls in the regulation of nonphotochemical quenching in *Arabidopsis thaliana* under fluctuating light. *J Phys Chem B* 124: 10311-10325.
9. Lu Y, Gan Q, Iwai M, Alboresi A, Burlacot A, Dautermann O, Takahashi H, Crisanto T, Peltier G, Morosinotto T, Melis A, Niyogi KK (2021). Role of an ancient light-harvesting protein of PSI in light absorption and photoprotection. *Nat Commun* 12: 679.
10. Wakao S, Shih PM, Guan K, Schackwitz W, Ye J, Patel D, Shih RM, Dent RM, Chovatia M, Sharma A, Martin J, Wei C-L, Niyogi KK (2021). Discovery of photosynthesis genes through whole-genome sequencing of acetate-requiring mutants of *Chlamydomonas reinhardtii*. *PLoS Genet* 17: e1009725.
11. Wakao S, Niyogi KK (2021). *Chlamydomonas* as a model for reactive oxygen species signaling and thiol redox regulation in the green lineage. *Plant Physiol* 187: 687-698.

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

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Overall goals of the project:

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

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

Significant achievements of the past 2 years

To meet goals 1) and 3) we have performed two-dimensional electronic spectroscopy (2DES) experiments on the BRC from purple bacteria, revealing hidden excitonic and vibronic structure. Through analysis of the coherent dynamics of the BRC we have resolved specific coherent signatures that allow us to definitively assign the upper exciton energy of the "special pair." This assignment has been controversial in the past due to the low oscillator strength of the transition. We show that the coherent signatures in the 2DES data clearly reveal the presence of this state due to vibronic coupling within the BRC and rapid energy transfer to the lower exciton state. We support our assignment by simulations of coherent dynamics of a reduced excitonic model of the BRC. The simulations also identify nonsecular vibronic coherence transfer processes that are generally neglected in standard models of photosynthetic energy transfer and charge separation. In addition, our analysis of the coherent dynamics reveals multiple quasi-resonances between key intramolecular pigment vibrations and excited state energy gaps in the BRC. The functional significance of such electronic-vibrational resonances and the identified nonsecular coherence transfer processes for photosynthetic energy conversion remains an open question. This work was recently accepted at Science Advances.[1]

Towards meeting goals 1) and 2), we are performing kinetic analysis of our broadband 2D data from the BRC and the PSII RC to both test excitonic models, as well as charge separation mechanisms. In addition, we have collaborated with Kevin Redding to study both the excitonic structure and the charge separation in the heliobacterial reaction center (HbRC). The HbRC has

been proposed to most closely resemble the common ancestor of photosynthetic reaction centers, motivating a detailed understanding of its structure-function relationship to gain insight into the divergence of charge separation mechanisms in Type I and Type II RCs. The recent elucidation of the HbRC crystal structure motivates advanced spectroscopic studies of its excitonic structure and charge separation mechanism. We performed multispectral 2DES of the HbRC and corresponding numerical simulations, resolving the electronic structure and testing and refining recent excitonic models. Through extensive examination of the kinetic data by lifetime density analysis and global target analysis, we reveal that charge separation proceeds via a single pathway in which the distinct A_0 chlorophyll *a* pigment is the primary electron acceptor. Our findings have general implications for the understanding of photosynthetic charge separation mechanisms, and how they might be tuned to achieve different functional goals. This work was published in Nature Communications[2].

Towards meeting goals 1) and 2) we have worked with theory collaborator Darius Abramavicius to develop a theoretical framework for modeling 2D electronic Stark spectroscopy (2DESS)[3]. We previously developed 2DESS, which combines two-dimensional electronic spectroscopy (2DES) and Stark spectroscopy as a sensitive new approach that could detect charge transfer (CT) states. In order to understand how the presence of CT states manifest in 2DESS, we performed computational modeling and calculations of 2DESS as well as 2DES and Stark spectra, studying a photosynthetic dimer inspired by the PSII RC. We identified specific cases where qualitatively different sets of system parameters produce similar Stark and 2DES spectra but significantly different 2DESS spectra, showing the potential for 2DESS to aid in identifying CT states and their coupling to excitonic states.

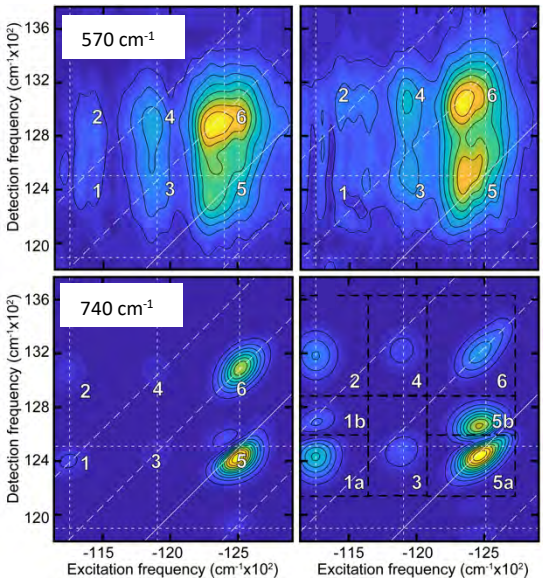

Fig. 1: Experimental (top row) and simulated (bottom row) coherence maps derived from 2DES studies of the BRC for ω_2 = 570 cm^{-1} and = 740 cm^{-1} featuring the prominent signatures (labeled 1-6) of vibronic coherence.

Objectives for the coming year

- 2DEV experiments of the PSII RC
- 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

Publications of the last 2 years supported by the award[1-5]

[1] V. R. Policht *et al.*, Science Advances **accepted** (2021)

[2] Y. Song *et al.*, Nature Communications **12** (2021) 2801.

[3] H. H. Nguyen *et al.*, The Journal of Chemical Physics **153** (2020) 144203.

[4] M. R. Wasielewski *et al.*, Nature Reviews Chemistry (2020)

[5] J. S. Cao *et al.*, Science Advances **6**, eaaz4888 (2020)

CRISPR-based Manipulation of Photosystems in Cyanobacteria

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Overall goals of the project –

The overall goal of this project is to understand the assembly process of Photosystem (PSII) and the function of cysteine-mediated quenching in antenna proteins. We are developing synthetic biology tools for use in cyanobacteria in the lab, specifically CRISPR and CRISPRi, to study these processes.

Significant achievements of the past 2 years –

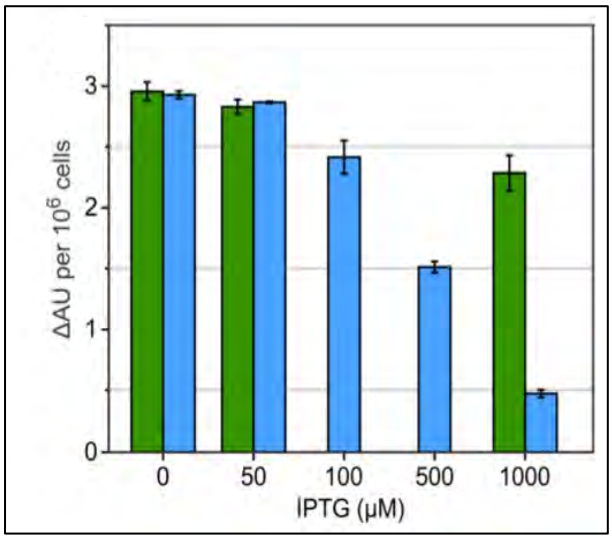

Figure 1. Quantitation of PSI content using a Joliot-type spectrometer for the control strain (green) and 2973::*btpA*i (blue).

During 2019-2020, we developed two CRISPRi systems to target photosynthetic complexes. An IPTG inducible CRISPRi system was designed to target the PSI biogenesis protein BtpA in the cyanobacterium *Synechococcus elongatus* UTEX 2973 (UTEX 2973). This system, when induced, was able to reduce PSI titer by 87% under photoautotrophic conditions (Figure 1). The system was tightly regulated and no change in PSI content was observed in the absence of inducer.

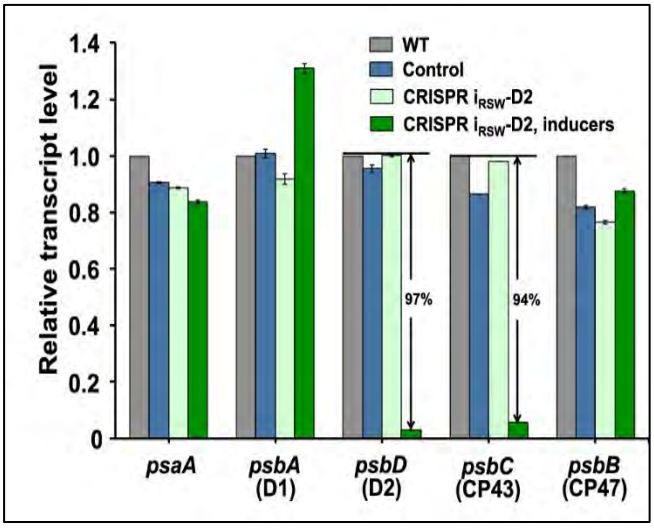

Figure 2. qPCR on PSII genes targeted by CRISPRi-D2 strain.

We also wanted to use *Synechocystis* 6803 (S6803), as it can grow heterotrophically on glucose, but the inducible IPTG promoter system is not functional in S6803. We developed a chimeric promoter, $P_{rhaBAD\text{-}RSW}$, which exhibits tight control and a broad, titratable inducible range in S6803. We used this promoter to control a CRISPRi system targeting the D2 protein of PSII, which eliminated *psbD* (D2 protein) and *psbC* (CP43 protein) expression in the induced system (Figure 2), and as a result abolished PSII activity. This work

provides a new platform to study PSII biogenesis, whereby protein complex intermediates can be tracked dynamically following de-repression.

CRISPRi was used to target the CtpA protease involved in D1 protein processing. However, we are not yet successful in controlling the expression of this enzyme which is functional even at low expression levels. New expression constructs are currently investigation to overcome this limitation.

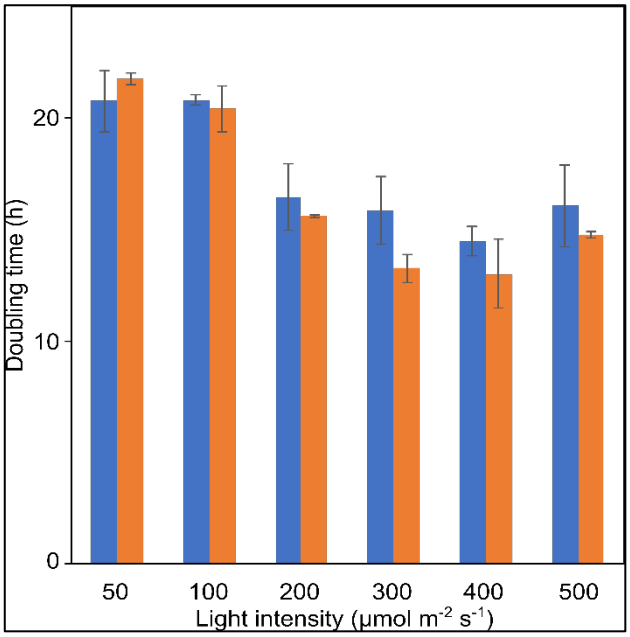

Figure 3. Doubling time of CP47His (blue), CP47His-CP43:V277C (orange) strains under different light intensities.

We identified a cysteine-mediated mechanism of energy dissipation in the PSI-free-IsiA complex in S6803. We introduced this quenching mechanism into the PSII core antenna CP43 protein, a structural homolog of IsiA. This mutation to cysteine in the CP43:V277C strain conferred resilience to high light exposure (Figure 3). We found that the mutation has a minimal impact on photosynthetic electron transfer but protects PSII under photodamaging conditions.

Objectives for the coming year

During the coming year, we plan to target additional PSII proteins. Down regulation of such genes will allow a closer examination of the understudied steps in the PSII life cycle.

Publications during the last 2 years supported by the award –

Weisz, D. A., et. al., (2019) A novel chlorophyll protein complex in the repair cycle of Photosystem II. Proc. Natl. Acad. Sci. USA, 116: 21907

Knoot, C.J. et al., (2020), Tunable repression of key photosynthetic processes using Cas12a CRISPR Interference in the fast-growing cyanobacterium *Synechococcus* sp. UTEX 2973, ACS Synth. Biol., 9(1).

Liu, D. et al., (2020), A Reversibly Induced CRISPRi System Targeting Photosystem II in the Cyanobacterium *Synechocystis* sp. PCC 6803, ACS Synth. Biol., 9(6).

Chen, H.-Y. et al., (2021), A novel mode of photoprotection mediated by a cysteine residue in the chlorophyll protein IsiA, mBio, 12(1).

Liu, H., et al., (2021) Structure of Cyanobacterial Phycobilisome Core Revealed by Structural Modeling and Chemical Cross-linking. Sci. Adv., 7: eaba5743

Biswas, S. et al., (under review), Engineering high-light tolerance in *Synechocystis* sp. PCC 6803 by modifying the CP43 antenna protein.

Johnson, V. M., et al., (under review) Psb27, a Photosystem II assembly protein, enables quenching of excess light energy during its participation in the PSII lifecycle.

Dissecting Mechanisms of Reductant Flow from the Chloroplast

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Overall Research Goals

We are using the model photosynthetic alga *Chlamydomonas reinhardtii* (Chlamydomonas throughout) to develop an informed understanding of key cellular components that participate in the flow of reduced metabolites from the chloroplast and to determine how metabolite exchange between subcellular compartments is used to manage high-light stress and maintain whole-cell redox and energy balance. Our recent studies on a subset of Chlamydomonas triose-phosphate and malate/oxaloacetate transporters show that null mutants for some of these transporters are unable to survive in high light (>1000 μmol photons m^{-2} s^{-1}), conditions where wild type cells continue to thrive. These results are consistent with the participation of these transporters in metabolite export from the chloroplast (and exchange of metabolites with other organelles), redox balancing, and net photosynthetic performance. Our primary hypotheses are: *i*) oxaloacetate, malate, 3-phosphoglycerate (3-PGA), and glyceraldehyde phosphate/dihydroxyacetone phosphate (GAP/DHAP) form the core of redox exchange between cellular compartments; *ii*) triose-phosphate and oxaloacetate/malate transporters (TPT and OMT, respectively) are critical components of the machinery that exports reduced metabolites (malate, 3-PGA and GAP) from Chlamydomonas chloroplasts; and *iii*) redox exchange via metabolite shuttles is critical for Chlamydomonas stress management (and viability of cells experiencing stress) and the integration of the cell's metabolic networks in both the light and dark.

Significant achievements 2019-2021

We have successfully developed robust CRISPR-Cas9 protocols for the reliable and facile disruption of targeted genes in Chlamydomonas. Using this powerful genome-

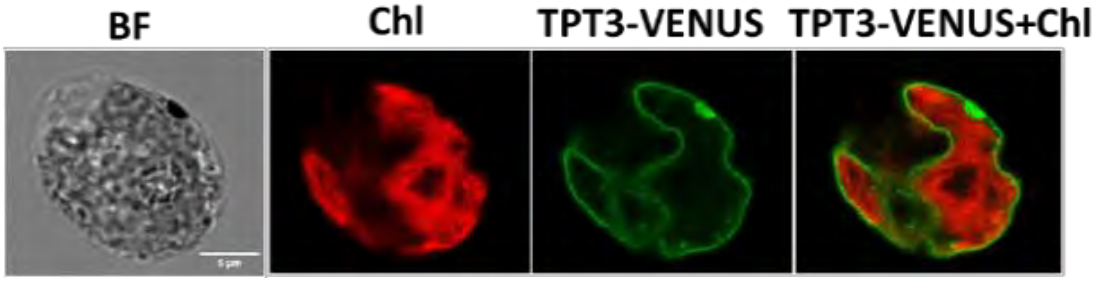

Figure 1. Cellular localization of TPT3 in Chlamydomonas. Bright field (BF), chlorophyll autofluorescence (Chl) in red; Venus fluorescence (TPT3-VENUS) in green; and merged chlorophyll and Venus signals (TPT3-VENUS+Chl).

editing technique, we have disrupted genes encoding both TPT2 and TPT3. Two distinct *tpt3* disruption strains were generated (unique Cas9 cut sites in each). Both of these mutants are light sensitive with respect to the parental control, and this phenotype is rescued (complemented) by a wild type *TPT3* gene expressed ectopically in the mutant cells. TPT3 is on the chloroplast envelope based on localization using a TPT3-VENUS fusion protein (**Fig. 1**). The *tpt3* mutants are unable to grow photoautotrophically when placed in even moderate light (~300-350 μmol photons m^{-2} s^{-1}) (**Fig. 2**), whereas they grow well in low light (<30 μmol photons m^{-2} s^{-1}). TPT2 and TPT3 were purified and incorporated into liposomes *in vitro*. Phosphate exchange

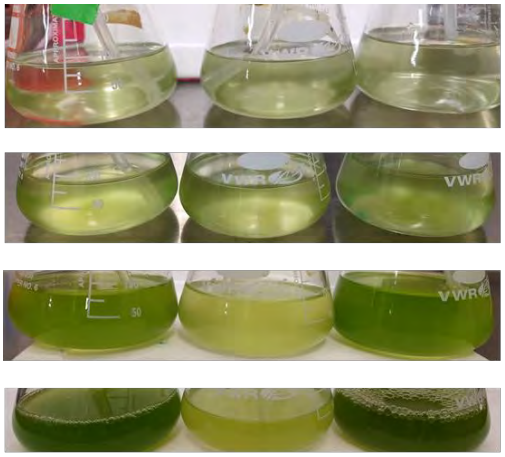

Figure 2. Chlamydomonas cells shifted from low light to high light as indicated. (L to R) parental control, *tpt3* mutant, complemented strain.

assays showed that TPT2 and TPT3 were most active for the transport of 3-PGA, G3P and DHAP, but could also transport phosphoenolpyruvate, with TPT3 demonstrating a much higher rate of transport than TPT2 (collaboration with A. Plett and N. Linka, Heinrich-Heine Universität Düsseldorf, Germany). Photosynthetic performance diminishes after *tpt3* mutants are shifted from low-light to higher light intensities and initial characterizations indicate that increased levels of reactive oxygen species are generated by the *tpt3* mutants. Lastly, higher levels of starch are observed in the *tpt3* mutants, potentially due to their inability to export 3-PGA from the chloroplast.

Science objectives for 2021-2022

- Conduct comprehensive intracellular (and extracellular) metabolite analysis to determine differential metabolite export as a consequence of a lack of TPT3 activity, and determine whether metabolites are being secreted into the medium in *tpt3* mutants.
- Conduct membrane inlet mass spectrometry (MIMS) experiments to determine if O_2 reduction is impacted in mutants unable to export chloroplast metabolites to mitochondrial respiratory complexes.
- Investigate the distinct metabolic role(s) for each of the Chlamydomonas MDH enzymes. These enzymes are likely critical for NADPH oxidation under highly reducing conditions in the chloroplast, TCA cycle function for sustaining respiratory activity, supplying electrons to alternative oxidases, photorespiration, and the assimilation of acetate.
- Define the roles of additional *tpt*, *omt* and *mdh* mutants in chloroplast metabolite exchange.

References to work supported by this project 2019-2021

1. Saroussi, S., Karns, D., Thomas, D., Posewitz, M., Grossman, A.R. (2019) Alternative outlets for sustaining photosynthetic electron transport during dark to light transitions. *Proc Natl Acad Sci USA*. 201903185 DOI: 10.1073/pnas.1903185116.
2. Posewitz, M.C., Atteia, A., Hemschemeier, A., Happe, T., Grossman, A.R. (2021) Metabolic networks during anoxia. *Chamydomonas Sourcebook*. In revision.
3. Hemschemeier, A., Posewitz, M.C., Happe, T. (2021) Hydrogenases and hydrogen production. *Chamydomonas Sourcebook*. In revision.
4. Kaye, Y., Huang, W., Saroussi, S., Idoine, A., Clowez, S., Sanz-Luque, E., Grossman, A.R. (2019) *Chlamydomonas reinhardtii* Mitochondrial Alternative Oxidases Allows Survival in High Light. *J Biol Chem* 294(4):1380-1395. doi: 10.1074/jbc.RA118.004667.

Molecular mechanisms of photosystem II disassembly

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Overall goals of the project

Photosystem II (PSII) catalyzes the photolysis of water and thus derives electrons, protons, and molecular oxygen necessary for metabolism. The remarkable water-oxidation and the associated electron transport reactions of PSII, however, expose it to reactive oxygen species, which irreversibly damages its key D1 reaction center protein. The photodamaged D1 is degraded and replaced by a newly synthesized copy through an intricate repair process known as the PSII repair cycle. In plants PSII occurs as a large 1.3 MDa supercomplex in tightly-stacked granal regions, which introduces many constraints for repair. The overall goals of this project are:

1. To identify the molecular mechanisms that drive the orderly disassembly of PSII for repair.
2. To identify the mechanisms that ensure the reassembly of repaired cores with peripheral antenna.

Significant achievements of the past 2 years

1. Core proteins of PSII are known to become phosphorylated in light. Based on a phosphorylation map of the C2S2M2 supercomplex of PSII, we have predicted the existence of two types of core phosphorylation sites: those involved in the peripheral antenna dissociation and those driving the monomerization of dimeric cores. Analysis of PSII assembly forms in a core kinase mutant and an overexpressor plants supports our hypothesis. The core kinase (STN8) overexpressor plants show a larger proportion of smaller disassembled PSII supercomplexes and especially a higher abundance of dimeric reaction center cores, compared to wild type.
2. Based on phosphoproteomic analysis of thylakoid proteins, we identify PsbL as a novel PSII core phosphoprotein. A superimposition of the PsbL phosphosite on PSII structure shows a putative function for PsbL phosphorylation in core monomerization together with the two PsbH phosphosites.
3. Using ectopic plastid expression of the plastid gene *psbD* on an *rrn16* promoter, the Co-PI has generated a phosphosite mutant of the PSII D2 protein in tobacco. The functional and biogenetic assembly of PSII in this mutant will be examined at the PI laboratory.

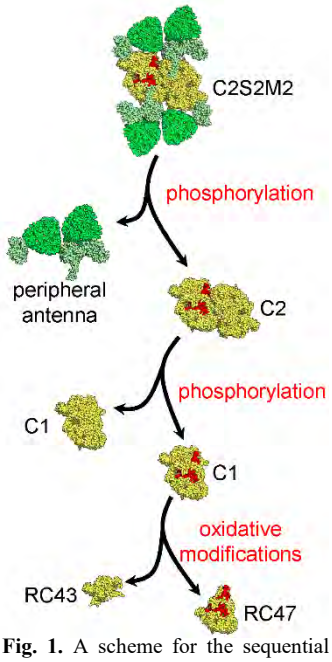

Fig. 1. A scheme for the sequential disassembly of PSII showing unique roles for phosphorylation and oxidative modifications. The damaged D1 subunit is colored red.

4. We have demonstrated that a treatment of *Arabidopsis* thylakoids with H_2O_2 results in an increased conversion of the monomeric cores into two subcomplexes: RC47 and RC43 (Fig. 1). Using LC-MS-MS analysis we show that the oxidative modifications (damage) correlate with

the degree of PSII disassembly, with the fully disassembled RC47 complex harboring more frequent and unique oxidative modifications. Some of these oxidative modifications occur at the D1-CP43 interface, suggesting a mechanism for the disassembly of the monomeric cores into RC47 and RC43. This novel observation reveals a hitherto unexpected role for damage in PSII disassembly and further points to an economical disassembly in which only the damaged monomeric cores disassemble for repair while leaving the undamaged cores for photochemistry.

Objectives for the coming year

1. A key objective will be to build on our observations on the role of phosphorylation in PSII disassembly. We will further analyze PSII functional and biogenetic assembly in core kinase mutant and overexpressor plants under high light. The phenotype of these mutant plants should be more apparent in high light conditions, which exacerbate photodamage. The functional assembly of PSII will be analyzed by chlorophyll fluorescence induction measurements and the biogenetic assembly during the repair cycle, by blue native gel electrophoresis. Emphasis will be given to the elucidation of the PSII disassembly mechanism in the core kinase mutant, which altogether lacks core phosphorylation. We will also build on our observation of PsbL phosphorylation by identifying the kinase that phosphorylates it and by unraveling its functional consequence. For identifying the PsbL kinase, we will employ a phosphoproteomics approach, and for the analysis of functional consequence, we will incubate isolated dimeric cores with recombinant *Arabidopsis* STN8. Our hypothesis is that PsbL phosphorylation works alongside PsbH phosphorylation to monomerize the dimeric cores. The D2 phosphosite mutant will be analyzed to test the existence of predicted peripheral antenna dissociation phosphosites. Specifically, we expect that this mutant is unable to dissociate the M-trimer efficiently.
2. Another objective for the coming year will be to fully elucidate the role of oxidative modifications in PSII disassembly. By mass spectrometry, we will analyze whether the innate modification found under the growth light are the same as those incurred during H_2O_2 treatment. We further plan to analyze the damage-mediated disassembly of isolated monomeric PSII cores in vitro. We will first isolate the monomeric core by sucrose density gradient centrifugation and then incubate it with H_2O_2. The status of PSII assembly will be analyzed by blue native gel electrophoresis, and the oxidative modifications mapped by LC-MS-MS.

Publications of the last 2 years supported by the award

1. McKenzie SD, Puthiyaveetil S (2022) Oxidative modifications as molecular drivers of plant photosystem II disassembly (in preparation).
2. Müh F, van Oort B, Puthiyaveetil S, Kirchhoff H (2021) Reply to: Is the debate over grana stacking formation finally solved? *Nature Plants* 7, 279-281.
3. Puthiyaveetil S, Kayanja G, McKenzie SD, Ibrahim IM (2021) Transcription initiation as a control point in plastid gene expression. *BBA - Gene Regulatory Mechanisms* 1864(3): 194689.

Genetic & Biophysical Dissection of Electron Transport in Heliobacteria Driven by the Heliobacterial Reaction Center (HbRC)

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Overall goals of the project (Redding, Fromme, Golbeck groups)

(1) Genetically test the model of light-driven cyclic electron flow pathways in heliobacteria by deleting genes for proteins in the pathways and assessing the activities of the mutants
(2) Measure the *in vivo* abundance of each ferredoxin by mass spectrometry and the affinity of each for the HbRC by isothermal calorimetry.
(3) Investigate key structural features of the electron transfer chain within the HbRC by a combination of site-directed mutagenesis and X-ray crystallography.
(4) Explore the reduction of quinone to quinol in a proteoliposome system using biochemical techniques and probe reduction of bound quinone to a semiquinone in a mutant lacking F_X.
(5) Elucidate the electronic structure of the primary electron donor and acceptor using advanced pulsed EPR techniques and quantum mechanical computational chemistry, as well as determine the mechanism of primary charge separation using a combination of site-directed mutagenesis and ultra-fast pump-probe spectroscopy.
(6) Determine requirements for HbRC assembling by co-expressing heliobacterial proteins in *R. sphaeroides* cells that synthesize BChl *g* and the HbRC subunits.

Significant achievements of the past 2 years:

- Created a system to express a His-tagged version of the *pshA* gene in the *. pshA* mutant [1].
- Created 3 site-directed mutants targeting the A_0 chlorophyll (Chl) *a* cofactor, the primary acceptor in the HbRC [2], based on our 2.2-Å resolution crystal structure. Mutants targeting the axial ligand (PshA-S545A) or the H-bond donor to the 13^1-keto oxygen (PshA-S553A) resulted in a ~50% loss in quantum yield of charge separation.
- Constructed a deletion mutant of the gene encoding PshX, the minor subunit of the HbRC [3]. The only phenotypes seen in the *ΔpshX* mutant were a slight lowering (~15%) of the HbRC level *in vivo* and the loss of one of the lowest energy pigments.
- Created a proteoliposome system incorporating purified HbRC into liposomes composed of phospholipids mimicking the heliobacterial membrane [4]. We found that (1) the HbRC was preferentially inserted with the donor side facing outside and (2) inclusion of a Ni(II)-NTA-lipid allowed decoration of the membrane with a His_6-tagged cytochrome (cyt) c_{553} and restored rapid reduction of oxidized primary donor as in the heliobacterial membrane.
- Collaborated with the group of Jennifer Ogilvie to study energy transfer and charge separation (CS) in the HbRC using their multi-dimensional electronic spectroscopy methodology [5]. They found that (1) direct excitation of the A_0 Chl *a* resulted in a significant fraction of HbRCs undergoing rapid CS (0.8 ps); (2) excitation of bulk BChl *g* resulted in

much slower CS due to equilibration with low-energy pigments; (3) in both cases, CS resulted in a radical pair assigned to $(P_{800}Acc)^{+}A_0^{-}$, followed by a slower (19 ps) transition to the stable $P_{800}^{+}A_0^{-}$ state, the state typically observed as the initial product of CS. The excited state leading to trapping is likely composed primarily of the P_{800} and Acc BChl *g* pigments.

- Deleted the genes for the core subunits of the cyt *bc* complex [6]. The *ΔpetCBDA* mutant lacks the genes encoding the cyt b_6/SuIV, Rieske, and di-heme cyt *c* subunits. As expected, this mutant is non-phototrophic, although it still possesses active HbRC. It has no cyt *bc* activity, as detected by re-reduction of cyt c_{553} in living cells after a laser-flash. The rate of this reaction slows ~100-fold in the absence of the cyt *bc* complex, the same as seen when saturating amounts of azoxystrobin (cyt *bc* inhibitor) are added to wild-type cultures.Moreover, the fraction of oxidized primary donor (P_{800}^{+}) of the HbRC in the presence of actinic light is much higher in the *ΔpetCBDA* mutant.
- Deleted the genes for the 3 small, acidic, di-cluster ferredoxins shown by the Golbeck group to interact with the HbRC: PshB1, PshB2, HM1_2505. We have also constructed the Δ*pshB1B2* double mutant and we are attempting to make the triple mutant.

Objectives for the coming year:

- Construct and study mutants in the HbRC designed to target P_{800} and Acc cofactors.
- Construct mutants in the HbRC designed to target the menaquinone (MQ) binding site. Construct a system in which cyt c_{553} is attached to the HbRC in detergent micelles.
- Create mutants in the F_X cluster that shift its potential. Create mutants unable to bind the acidic ferredoxins (well).

Publications of the last 2 years supported by the award:

1. Orf, G. S.; Redding, K. E., Expression and purification of affinity-tagged variants of the photochemical reaction center from Heliobacterium modesticaldum. *Photosynth Res* **2019,** *142* (3), 335-348. https://www.ncbi.nlm.nih.gov/pubmed/31542861

2. Orf, G. S.; Redding, K. E., Perturbation of the primary acceptor chlorophyll site in the heliobacterial reaction center by coordinating amino acid substitution. *Biochim Biophys Acta Bioenerg* **2021,** *1862* (1), 148324. https://www.ncbi.nlm.nih.gov/pubmed/33039349

3. Orf, G. S.; Gisriel, C. J.; Granstrom, J.; Baker, P. L.; Redding, K. E., The PshX subunit of the photochemical reaction center from Heliobacterium modesticaldum acts as a low-energy antenna. *Photosynth Res* **2021**. https://www.ncbi.nlm.nih.gov/pubmed/34480322

4. Johnson, W. A.; Redding, K. E., Reconstitution of the heliobacterial photochemical reaction center and cytochrome c553 into a proteoliposome system. *Photosynth Res* **2020,** *143* (3), 241-250. https://www.ncbi.nlm.nih.gov/pubmed/31838634

5. Song, Y.; Sechrist, R.; Nguyen, H. H.; Johnson, W.; Abramavicius, D.; Redding, K. E.; Ogilvie, J. P., Excitonic structure and charge separation in the heliobacterial reaction center probed by multispectral multidimensional spectroscopy. *Nat Commun* **2021,** *12* (1), 2801. https://www.ncbi.nlm.nih.gov/pubmed/33990569

6. Leung, S. W.; Baker, P. L.; Redding, K. E., Deletion of the cytochrome bc complex from Heliobacterium modesticaldum results in viable but non-phototrophic cells. *Photosynth Res* **2021,** *148* (3), 137-152. https://www.ncbi.nlm.nih.gov/pubmed/34236566

Growing and Maintaining Thylakoids in Chloroplasts of Land Plants

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Overall goals of the project –

The primary goal of the project is to characterize membrane contact sites between thylakoid and inner envelope membranes. Thylakoid membrane lipids are the most abundant in the world, due entirely to the importance of multiplying the surface on which photosynthesis occurs. Thylakoids do not synthesize their own lipids; instead they are made in the surrounding chloroplast envelope membranes. Thus, lipid transport is a prerequisite for self-assembly and self-repair of the photosynthetic thylakoid membrane. Nevertheless, we know few molecular components directly involved in transporting lipids from chloroplast inner envelope membrane to the thylakoid membrane. Multiple mechanisms have been hypothesized, and among them is lipid transfer at thylakoid/inner envelope membrane contact sites. Membrane contact sites are consistent with several observations about thylakoids, including fast, ATP-independent lipid transport rates, the direct observation of membrane contact sites in cyanobacteria and land plant chloroplasts, and the multiple observed metabolic connections between the two membranes. We have developed in-vivo biosensors of these contact sites and our long-term goal is to understand lipid transport at contact sites between the thylakoid and inner envelope membranes. Specific objectives are to (1) investigate the conditions increasing contact sites, and (2) investigate the sub-organellar location of candidate membrane-contact site proteins.

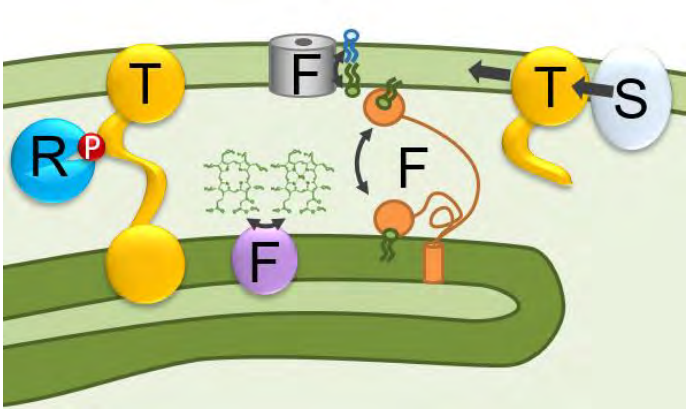
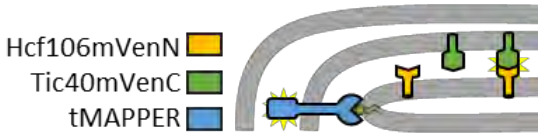


Figure 1. Potential anatomy of a thylakoid/inner envelope membrane contact site. Typical membrane contact sites include tether proteins (T), functional proteins (F), regulatory proteins (R) and sorter proteins (S). Individual proteins may have more than one function. Expected functional proteins include those involved in glycerolipid transport, isoprenoid biosynthesis, chlorophyll repair, and carotenoid biosynthesis.

Significant achievements of the past 2 years –

- Two independent visualization systems for membrane contact sites between the thylakoid and inner envelope membranes were developed. The first system has fused thylakoid integral membrane protein Hcf106 and inner envelope integral membrane protein Tic40 to self-assembling fragments of mVenus (mVenN and mVenC). It is characterized by a

uniquely punctate fluorescent signal distinct from expression of controls with both portions of mVenus targeted at either inner envelope or thylakoid membranes. Correct targeting of the fragments is supported by chloroplast fractionation. The second system, based off the ER-plasma membrane contact site sensor "Mapper" fuses an enzymatically-dead version of SENSITIVE TO FREEZING 2 acting as an MGDG-binding protein to a rigid inner envelope anchor. Various versions of the anchoring system yield progressively more or fewer puncta.



- These contact site visualization systems are transformed into *Arabidopsis thaliana*.
- Created 19 plasmids expressing potential contact site proteins fused to mKOκ for co-expression with either visualization system.
- Screened 11 constructs expressing potential lipid-transferring enzymes putatively located in the chloroplast. Identified two in the chloroplast.
- Proteomics of a chloroplast fraction enriched in membrane contact sites identified a number of putative contact site proteins.
- Literature supports inhibition of vesicle transport in the chloroplast when chilled. We have begun screening cold stress conditions for an increase in membrane contact sites by classic electron microscopy.

Objectives for the coming year –

- A major effort will be screening visualization systems for their stability in *A. thaliana* transgenic lines and identifying lines at multiple expression levels.
- Generated lines will be subjected to TEM immunogold labeling to confirm the presence of contact site markers at contact sites.
- We will co-express putative contact site proteins and thylakoid/inner envelope contact site markers in *Nicotiana benthamiana* to identify contact site components.
- DNA resources for screening additional potential contact site proteins will continue to be generated.
- We will finish determining if cold increases the number of contact sites, then use that condition to probe if either contact site marker allows dynamic tracking of fluctuations in membrane contact site numbers or size.

Accessing Structure and Dynamics of Photosynthetic Pigment-Protein Complexes by Time-Resolved Circular Dichroism Spectroscopy

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Overall goals of the project:

- Develop and use time-resolved circular dichroism (TRCD) spectroscopy to photosynthesis studies;
- Apply TRCD to investigate photosynthetic Fenna Matthews Olson (FMO) antenna complex, wild type and site-directed mutants;
- Develop and validate computational methods for quantitative prediction of electronic structure and energy transfer from *first principles* based on x-ray structures of photosynthetic proteins;
- Investigate future potential of TRCD in direct resolution of PS I RC primary charge separation.

Significant achievements of the past 2 years

(i) TRCD spectroscopy has been developed and shown to provide critical experimental data on electronic structure and dynamics of triplet energy transfer within the FMO complexes (Fig. 1). Experiments and first-principle modeling of wildtype FMO reveal that FMO pigments form three isolated pools with efficient triplet energy transfer within each pool. Pigment pool consisting of pigments #3, #4, #5, #6, and #7 is shown to decay with lifetime of ~50 μs, with the quencher being pigment #3. The pigment pool (#1, #2) decays to the ground state within ~0.4 μs, possibly because of a water molecule loosely bound to BChl #2. BChl #8 appears to be out of an efficient triplet energy transfer range with the rest of FMO, but its triplet population is too low to be detected.

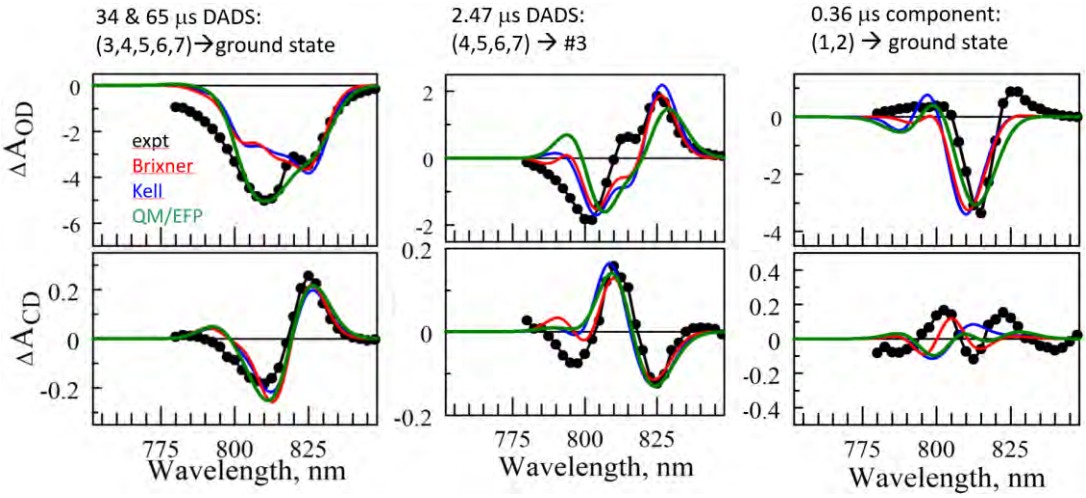

Fig. 1. Decay-associated difference spectra (DADS) of wildtype FMO reveals four major decay components (black dots) that are ascribed to triplet energy transfer between pools of pigments and modeled using Hamiltonians proposed by Kell et al. (*JPCA. 2016, 120, 6146*) (Kell, blue lines), Brixner et al. (*Nature, 2005, 434, 626*) (Brixner, red lines), and derived from first principles Kim et al. (*J. Phys. Chem. Lett. 2020, 11, 1636*) (QM/EFP, green lines).

(ii) Computational *first principles-based* procedure for predictive modeling of excitonic structure and spectroscopy of the FMO complex including site-specific mutants is developed and shown to reproduce major experimental features (*Kim et al., J. Phys. Chem. Lett. 2020, 11, 1636*), including TRCD data (Fig.1).
(iii) Computational modeling of FMO mutant at site #7 was capable to explain a previously observed mismatch between empirical modeling and experimentally measured changes in the absorption and CD spectra (*Saer et al., BBA 1858, p.288, 2017*). It is shown that in this mutant, structural rearrangements caused by mutation govern changes in excitonic structure, causing shifts in transition energies of several pigments but not pigment #7.
(iv) Photosystem I reaction center (PS I RC) mutants have been studied by time-resolved spectroscopy in search for possible candidates for future TRCD studies. Pump-probe data on PS I RC accessory pigment mutant suggests that when accessory pigment in one branch is perturbed by a mutation, the electron transfer is redirected via unperturbed branch, while simultaneous mutation on both sides leads to significant accumulation of triplet excited state on accessory pigment(s) severely affecting electron transfer efficiency.
(v) The interaction between Ferredoxin-NADP+ reductase and cytochrome b_6f has been investigated by means of isothermal titration calorimetry and CD spectroscopy, revealing no efficient coupling between these proteins and arguing against efficient 'cyclic' electron transport pathway.

Objectives for the coming year:
- Computational *first principles* modeling will be utilized for interpretation of the measured TRCD data on FMO mutants;
- The newly developed *femtosecond* TRCD spectrometer has shown sufficient sensitivity to detect kinetics of CD signal and will be applied to test its capabilities with FMO as a test sample.

Publications of the last 2 years supported by the award:
- Kim Y, Morozov D, Stadnytskyi V, Savikhin S, Slipchenko LV. Predictive First-Principles Modeling of a Photosynthetic Antenna Protein: The Fenna–Matthews–Olson Complex, *J. Phys. Chem. Lett. 11* (5), 1636-1643 (2020) DOI: 10.1021/acs.jpclett.9b03486.
- Kaliakin DS, Nakata H, Kim Y, Chen Q, Fedorov DG, Slipchenko LV. FMOxFMO: Elucidating Excitonic Interactions in the Fenna–Matthews–Olson Complex with the Fragment Molecular Orbital Method, *J. Chem. Theory Comput.* 16, 1175-1187 (2020) DOI: 10.1021/acs.jctc.9b00621.
- Kurashov V, Milanovsky G, Luo L, Martin A, Semenov AYu, Savikhin S, Cherepaov DA, Holbeck JH, Wu X. Conserved residue PsaB-Trp673 is essential for high-efficiency electron transfer between the phylloquinones and the iron-sulfur clusters in Photosystem I. *Photosynth Res* 148, 161–180 (2021) DOI: 10.1007/s11120-021-00839-x.

Submitted:
- Zakharov SD, Savikhin S, Misumi Y, Kurisu G, Cramee WA, Isothermal Titration Calorimetry of Membrane Protein Interactions: FNR and the Cytochrome b6f Complex. Submitted to *Biophysical J.*
- Watanabe Y, Washer BM, Zeller M, Savikhin S, Slipchenko L, Wei A. Copper(I)–Pyrazolate Complexes as Solid-State Phosphors: Tunable Emissions via a Remote Steric Effect. Submitted to *Nature Chemistry*

Spectroscopic studies of protein-protein association in model membranes

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Overall goals of the project –
The research 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 transient absorption spectroscopy.

Significant achievements 2019-2021 –
Energy transfer in purple bacteria. In purple bacteria, networks of light-harvesting complex 2 (LH2) capture and transport energy to a dedicated protein, LH1-RC, for charge separation. Previous measurements of inter-protein energy transfer were performed on native membranes, which averaged over heterogeneous protein organizations. We incorporated LH2 and a spectrally-shifted variant, LH3, from *Ph. molischianum* into a double-loaded nanodisc, which held each protein pair in a homogeneous environment. Using cryogenic electron microscopy, we measured the inter-protein distance, and found ~2.5 nm separation between the bacteriochlorophyll. Using femtosecond transient absorption spectroscopy, we measured the LH3-to-LH2 energy transfer timescale, and found a value of 5.7 ps, in agreement with theoretical calculations. Due to the confined environment of the nanodisc, this timescale represents the fastest possible protein-to-protein energy transfer, and, more generally, illustrates the utility of the nanodisc platform as a model system for future studies of the antenna protein network.

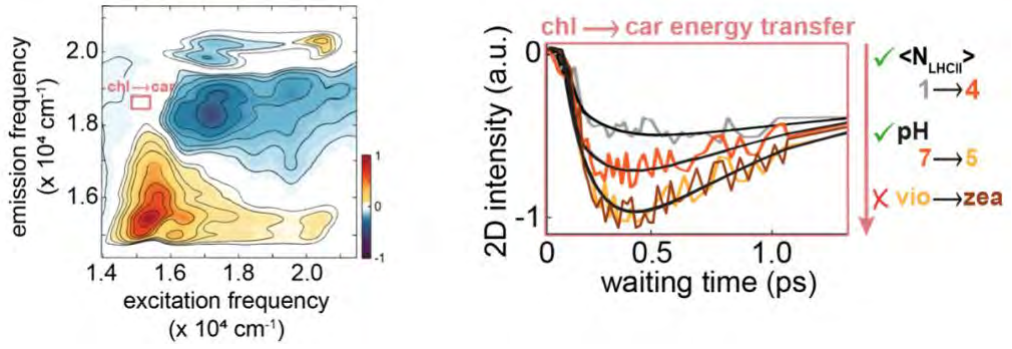

Figure. Chlorophyll-to-carotenoid energy transfer in LHCII. (left) Ultrabroadband 2D electronic spectrum of LHCII encompasses both the chlorophyll and carotenoid transitions, including chlorophyll-to-carotenoid energy transfer (chl→car; pink box). (right) Waiting time traces of the region indicated by the pink box for LHCII in nanodiscs. An increase in average LHCII content ($<N_{LHCII}>$) from one to four led to faster energy transfer, a pH drop increased the amplitude of the energy transfer component, and carotenoid conversion from violaxanthin (vio) to zeaxanthin (zea) had no effect.

Dissipation in green plants. Green plants have evolved mechanisms to prevent photooxidative damage by excess energy, known as non-photochemical quenching (NPQ). Activation of NPQ involves a drop in luminal pH, conversion of the carotenoid violaxanthin to zeaxanthin, and

clustering of the primary antenna protein, light-harvesting complex II (LHCII) into arrays. Although array formation has been mimicked by large aggregates prepared through detergent removal, these structures are non-physiological, heterogeneous, and poorly controlled. We systematically increased the number of LHCII complexes within a nanodisc from one to four, and observed a corresponding increase in spectroscopic signatures of protein-protein interaction. Using time-resolved spectroscopy, we showed that fluorescence quenching and dissipative chlorophyll-to-carotenoid energy transfer increased with protein-protein interaction. The presence of protein-protein interactions was also found to activate pH-dependent quenching and dissipative energy transfer, although no zeaxanthin-dependent effects were identified (Figure). These results indicate that the underlying mechanism of action of each parameter implicated in NPQ is likely distinct, potentially enabling maximum flexibility to respond to variable levels of sunlight.

Objectives for the coming year –

- We incorporated LH2 and LH3 in a larger membrane nanodisc, which has a protein density closer to the purple bacterial membrane under high light. Structural characterization showed a larger inter-chlorophyll distance. Transient absorption studies will explore the role of this increased separation on protein-to-protein energy transfer.
- We also incorporated LH1-RC into a nanodisc. A comparison of LH1 to RC energy transfer for complexes in nanodiscs and in detergent will show how the larger ring structure of LH1 is affected by the membrane, as well as if the charge transfer dynamics of the embedded RC are protected by the surrounding protein.

Publications in 2020/2021 supported by the award –

1. A. L. Tong, O. C. Fiebig, M. Nairat, D. Harris, M. Giansily, A. Chenu, J.N. Sturgis, **G. S. Schlau-Cohen**, "Comparison of the energy transfer rates in structural and spectral variants of the B800-850 complex from purple bacteria." *J Phys Chem B* **124**, 8 (2020).
2. M. Son, A. Pinnola, S. C. Gordon, R. Bassi, **G. S. Schlau-Cohen**, "Observation of dissipative chlorophyll-to-carotenoid energy transfer in light-harvesting complex II in membrane nanodiscs." *Nat Communs* **11**, 1 (2020).
3. M. Son, A. Pinnola[#], **G. S. Schlau-Cohen[#],** "Zeaxanthin independence of photophysics in light-harvesting complex II in a membrane environment." *BBA-Bioenergetics* **5-6**, 1861 (2020).
4. 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).
5. 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).
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).
7. 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).

A dual-function chaperone coordinates biogenesis of the light harvesting complex

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Overall project goal: The biogenesis of chlorophyll a,b-binding proteins (LHCPs), which comprise the light harvesting complex in green plants, is a major rate-limiting step in biomass production from solar energy. As the most abundant membrane protein on earth, the biogenesis of LHCP also provides an excellent model system to understand how molecular chaperones protect and guide highly aggregation-prone membrane proteins during their folding and assembly in the cell. The overall goal of this project is to understand the mechanism and regulation of membrane protein biogenesis at high resolution. The specific goal of this project is to understand the interaction, dynamics, and regulation of cpSRP43, a chaperone that mediates the protected and coordinated transport, folding and assembly of the light harvesting complex in green plants (Figure 1).

Significant achievements: Using a combination of NMR, EPR, and other biophysical methods, we uncovered extensive conformational dynamics in cpSRP43. We showed that a disorder-to-order transition in cpSRP43 enables its chaperone activity towards the LHCPs to be turned 'on' by an activator, cpSRP54 in the stroma, thus enabling effective capture and protection of LHCPs *en route* to the Alb3 translocase at the thylakoid membrane (Fig. 1b). In collaboration with the Grimm group, we further demonstrated a new role of cpSRP43 in stabilizing multiple enzymes in the tetrapyrrole biosynthesis (TBS) pathway, thus ensuring the appropriate supply of chlorophylls for LHCPs during the assembly of the light harvesting complex (Fig. 1a). The chaperone activity of cpSRP43 towards the TBS enzymes displays distinct molecular requirements from that towards the LHCPs and is particularly crucial for the thermoprotection of TBS activity during heat stress (Fig. 1c). Our results suggest an attractive model in which the toggling of cpSRP43 between two distinct chaperone activities provides a post-translational mechanism for coordinating between the two branches of light harvesting complex biogenesis and for thermoprotection during heat stress.

Objectives in the near future: Our next goals are: (i) To understand the molecular mechanism by which the chaperone activity of cpSRP43 is turned 'off' by the Alb3 translocase at the thylakoid membrane, thus enabling spatially regulated LHCP release at the site of its translocation; and (ii) to decipher how cpSRP43 recognizes its two distinct classes of client proteins, the LHCPs and the TBS enzymes.

Publications of work supported by the award:

1. Siegel, A.‡, McAvoy, C.Z.‡, Lam, V.Q., Liang, F.C., Kroon, G., Miaou, E., Griffin, P.R., Wright, P.E., Shan, S.O., (2020) *J. Mol. Biol.*, 432, 100768. Doi: 10.1016/j.jmb.2020.11.007. "A disorder-to-order transition activates an ATP-Independent Membrane Protein Chaperone".

‡equal contribution.

2. Ji, S‡., Siegel, S. ‡, Shan, S., Grimm, B.*, and Wang, P.* (2021) *Nature Plants*, DOI: 10.1038/s41477-021-00994-y. "Chloroplast SRP43 autonomously protects chlorophyll biosynthesis proteins against heat shock".
‡ equal contribution.

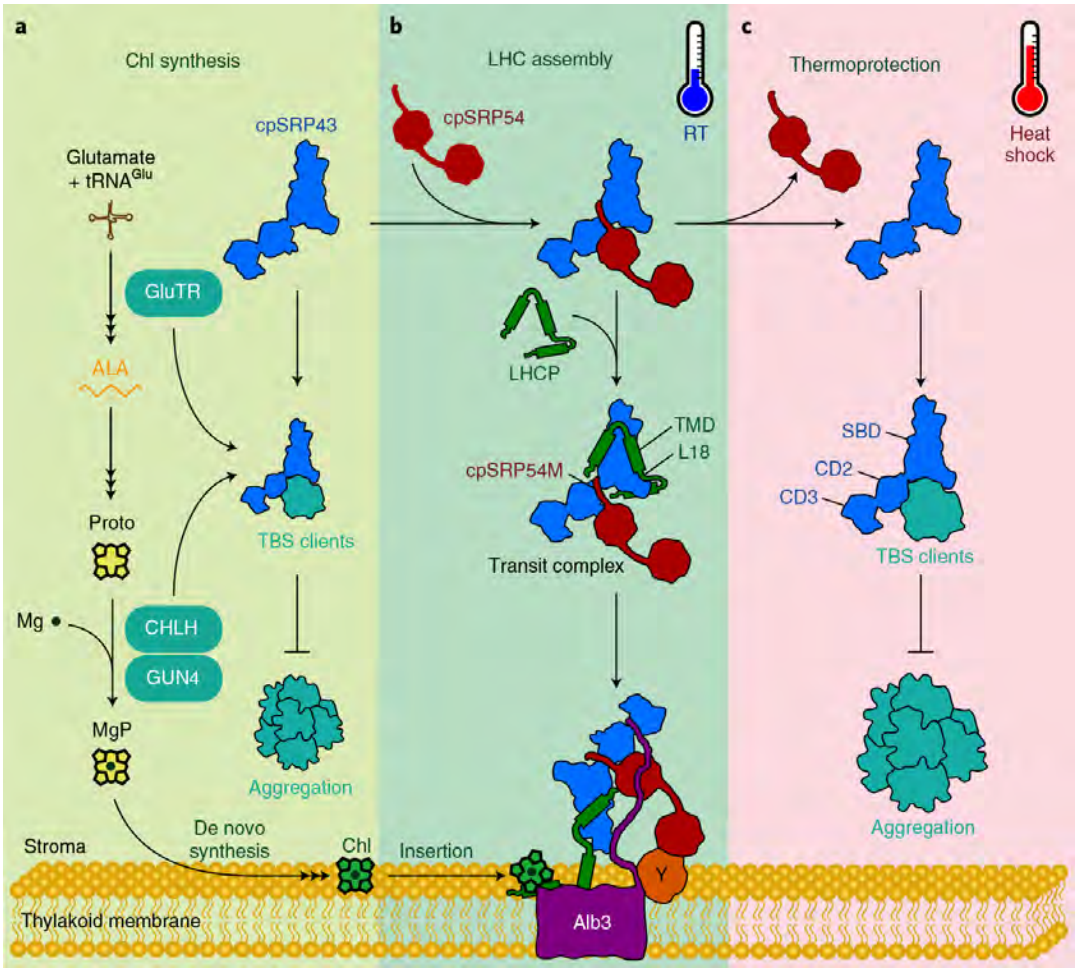

Figure 1. Dual chaperone functions of cpSRP43 mediate the protected and coordinated assembly of light harvesting complex. (**a**) Free cpSRP43 protects and stabilizes multiple TBS enzymes in the chlorophyll biosynthesis (TBS) pathway. (**b**) When cpSRP43 is bound to cpSRP54, it becomes a dedicated chaperone to capture and protect LHCPs from aggregation. Via interaction with the receptor cpFtsY, LHCP is delivered to the Alb3 translocase at the thylakoid membrane. Alb3 induces LHCP release from cpSRP43 to initiate its folding and assembly, a process that also requires the binding of chlorophyl molecules. (**c**) Under heat stress, cpSRP54 is released from cpSRP43, dedicating a larger pool of cpSRP43 for the thermo-protection of TBS enzymes.

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

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Overall goals of the project.

The amount of sunlight available for photosynthesis sometimes exceeds the amount of energy plants can use. This access energy has to be safely dissipated through non-productive biological processes. The ultimate goal of this project is to understand whether we can utilize that otherwise unused excess energy. In our previous work, we showed that, in principle, it is possible to attach a catalyst to photosystem I and generate H_2 using light. In the first thrust of this project, we take advantage of these constructs to investigate details of the coupling between photosystem I and an H_2-producing enzyme called [FeFe] hydrogenase. Through this research, we aim to gain an understanding of the catalytic mechanism of the latter by utilizing the photosystem's ability to generate a charge-separated state on the nanosecond time scale. In the second thrust of this project, we will research efficient and robust tethering of the [FeFe] hydrogenase to photosystem I in cyanobacteria. Our strategy is to genetically fuse parts of the photosystem I complex with a recently discovered oxygen-tolerant [FeFe] hydrogenase. Our rationale is that such chimeric proteins may potentially result in the natural incorporation of the photosystem I-hydrogenase link using the inherent genetic machinery of the cell. This work will highlight successful design strategies to guide the future development of photosynthetic biohybrids. Uncovering the principles governing the utilization of otherwise unusable energy will significantly further our understanding of cyanobacterial photosynthesis.

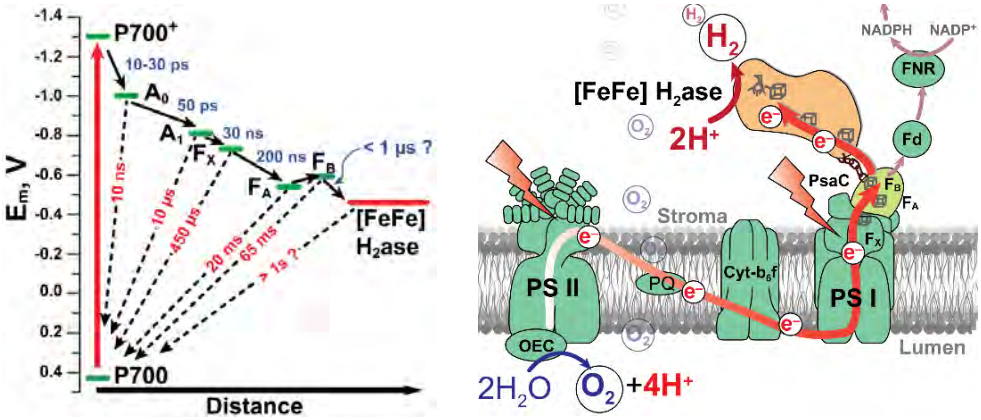

Figure 1. Hypothetical electron transfer scheme for PSI-[FeFe] H_2ase constructs (left), schematic depiction of linking PSI with an O_2-tolerant [FeFe] H_2ase for H_2 production using excess energy (right).

Significant achievements of the past 2 years

- We used a combination of spectroscopy, electrochemistry, and density functional theory (DFT) to uncover the intrinsic properties of the active center of [FeFe] hydrogenase from *Clostridium beijerinkii* (*Cb*HydA1) that leads to oxygen tolerance. These results demonstrate that *Cb*HydA1 has remarkable stability in the presence of oxygen, which will drive future efforts to engineer robust PS I–*Cb*HydA1 chimeric nanoconstructs.
- We succeeded in expressing both *Cb*HydA1^{WT} and one of its maturation factors (HydF) in *Synechocccus sp.* PCC 7002 at the same time. These genetic engineering experiments provide an important foundation for further investigation of *in vivo* coupling of HydA and PSI.
- We engineered and successfully expressed a chimeric construct consisting of [FeFe] hydrogenase from *Clostridium acetobutilicum* (*Ca*HydA) and PsaE in *E.coli*. Isolated constructs showed nearly quantitative binding of the PsaE-HydA fusion protein to PSI, as observed by time-resolved experiments.
- We succeeded in engineering and expressing a PsaC-*Cb*HydA1$^{\Delta 1\text{-}195}$ construct in *E.coli*. Electron paramagnetic resonance data indicate the presence of FeS cluster in both PsaC and HydA subdomains of the fused protein.

Objectives for the coming year

- Investigate H_2-generation reaction pathways of PSI-[FeFe] H_2ase constructs by time-resolved IR spectroscopic measurements.
- Innovate ultra-rapid freeze quench techniques for investigation of the long-range electron transfer pathways in the PSI-HydA constructs by EPR spectroscopy.
- Investigate O_2 tolerance and H_2-producing activity of PsaE-HydA and PsaC-HydA chimeric nanoconstructs.
- Study and optimize expression of maturation factors HydE, HydF, and HydG in *Synechocccus sp.* PCC 7002 in the background expression of *Cb*HydA1.

Publications of the last 2 years supported by the award

1. Corrigan, P., Tirsch, J. and Silakov, A. Investigation of the unusual ability of the [FeFe] Hydrogenase from *Clostridium beijerinkii* to access an O_2-protected state. *J. Amer. Chem. Soc.* (2020) 142, 12409.
2. 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.
3. 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 Chl_{3A} in the $M688H_{PsaA}$ Genetic Variant of Photosystem I. *BBA - Bioenergetics* (2021), 1862, 7, 148424

Exploring Rubisco Activase Limitations to C4 Carbon Capture

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Overall goals of the project – C_4 plants use carbon concentrating mechanisms that place Rubisco in a relatively high-CO_2, low O_2 environment. This decreases the wasteful oxygenation side reaction and photorespiration, while maintaining photosynthesis under stress conditions that are deleterious to C_3 plants. One significant unanswered question about the regulation of C_4 photosynthesis relates to Rubisco Activase (RCA), a AAA+ ATPase that removes inhibitors from the Rubisco active site, enabling the binding of the RuBP substrate (Fig. 1). Data from several groups suggest intricate regulatory relationships between the levels of inhibitor and the stability of RCA, as well as between the expression level and/or activity of RCA, and the amount of Rubisco present. In other words, C4 plants appear to have intrinsic limitations to Rubisco activation due to RCA accumulation and/or activity. Our work explores the relationship between RCA and Rubisco abundance and activation, through manipulation of RCA *in vivo* in the C_4 model *Setaria viridis*, as well as in maize. Another aspect of the project relates to metabolic exchange between bundle sheath and mesophyll cells in maize; this topic will not be covered at the PI meeting.

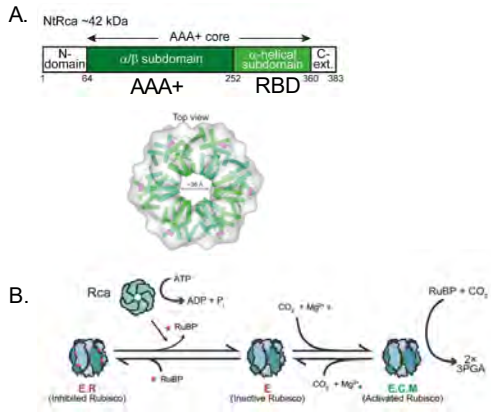

Fig. 1. RCA Structure and Function. A. Domain structure of the RCA protein (top) including the approximate location of the AAA+ heat-sensitive and rubisco binding domain (RBD) and a model of the hexameric ring structure (bottom; Bhat et al., 2017). B. The function of RCA to remodel inhibited Rubisco (ER) to release bound sugars (e.g. *RuBP). Inactive Rubisco (E) can bind CO2 and Mg2+ to form the activated holoenzyme (ECM; Ng et al., 2020).

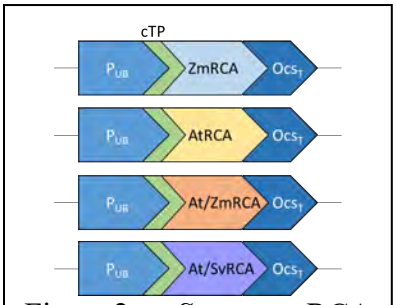

Fig. 2. *Setaria* RCA transgene constructs. P_{UB} – ubiquitin promoter; cTP – chloroplast transit peptide; Zm – maize; At – Agave; Sv – *Setaria*; Ocs_T – octopine synthase terminator.

Significant achievements of the past 2 years – In maize, we have combined lines programmed to overexpress RCA with lines known to overexpress Rubisco. The RCA overexpression lines did not accumulate excess RCA although the transgene is expressed, which could reflect an unfortunate aspect of transgene design, or more likely an endogenous regulatory mechanism. To test these two possibilities, additional RCA constructs were made using a different promoter for overexpression in either maize or *Setaria* using endogenous RCA, and in a second approach RCA from heat-tolerant Agave or RCA chimeras (see below) were expressed that might avoid regulatory limitations (Fig. 2). Additionally, in *Setaria*, we have used gene editing to create null or near-null

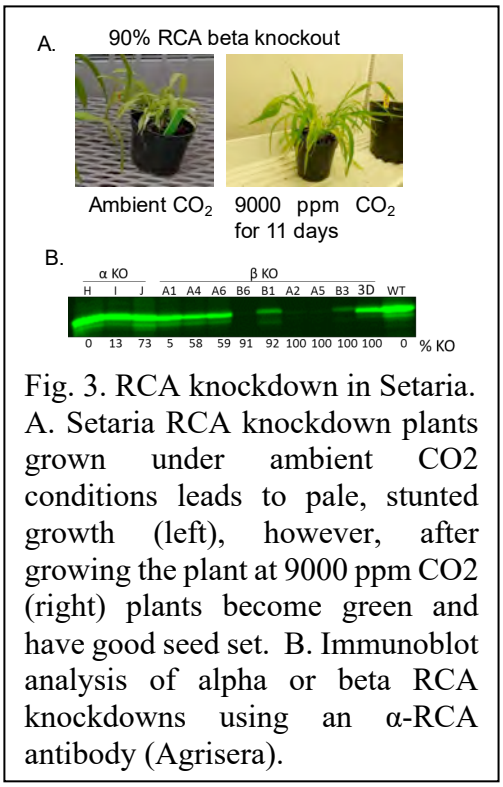

Fig. 3. RCA knockdown in Setaria. A. Setaria RCA knockdown plants grown under ambient CO2 conditions leads to pale, stunted growth (left), however, after growing the plant at 9000 ppm CO2 (right) plants become green and have good seed set. B. Immunoblot analysis of alpha or beta RCA knockdowns using an α-RCA antibody (Agrisera).

alleles of both forms of RCA, α and β (Fig. 3). β is the major form of RCA in both maize and *Setaria*, and the null allele plants are unable to grow outside of an imposed high CO_2 environment, illustrating that β is essential for photosynthesis, and thus that α is not interchangeable at least at its normal expression level. Additional work has been performed to express recombinant RCA of both forms as well as chimeric RCA β with Agave RCA domains, and to develop the relevant *in vitro* assays for activity.

Objectives for the coming year – Our RCA work will focus primarily on the *Setaria* model due its faster generation time and ease of transformation. A major objective is careful phenotypic exploration of the RCA mutants, especially the α null, given that the function of this redox-regulated isoform is very poorly understood. For example, α may be required for optimal performance under conditions where oxidative stress occurs. We will be determining the activation state of Rubisco, and metabolic and photosynthetic parameters under a variety of environmental conditions including light transitions and heat stress. The β null will also be phenotypically characterized, including looking for any evidence that the α form is induced in this background, and whether this expression can compensate partially or fully for the β null allele. The β null will also serve as a scaffold to express modified forms of β RCA, either in the presence or absence of the α form. We have engineered a chimeric β-RCA with the AAA+ domain from *Agave tequilana* and the Rubisco recognition domain from *Setaria*. This AtSv chimera is predicted to support higher photosynthetic rates at elevated temperatures due to its thermotolerant AAA+ domain, and as a partially foreign protein it may also overcome intrinsic limitations to RCA overexpression, and thus increase Rubisco activation overall. These *in vivo* experiments will be paired with *in vitro* assays of the relevant proteins. For example, the efficiency of AtSv RCA to activate Sv Rubisco can be compared to *in vivo* results. Together this information will help lead to an increased understanding of the interplay between RCA and Rubisco that limits photosynthesis in C_4 plants.

Publications of the last 2 years supported by the award –

Rubisco production in maize mesophyll cells through ectopic expression of subunits and chaperones. Hotto AM, Salesse-Smith C, Lin M, Busch FA, Simpson I, **Stern DB.** J Exp Bot. 2021 Jun 22;72(13):4930-4937. doi: 10.1093/jxb/erab189.

References:

Bhat et al. (2017) Rubisco Activases: AAA+ Chaperones Adapted to Enzyme Repair. Frontiers in Molecular Biosciences (4); article 20.

Ng et al. (2020) Rubisco activase requires residues in the large subunit N terminus to remodel inhibited plant Rubisco. J. Biol. Chem. 295(48): 16427-16435.

Hydrophobic mismatch is a key factor in protein transport in the Tat pathway

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Overall goals of the project –

The overarching goal of this project is to elucidate the mechanism of action of the Tat protein translocation machinery. The Tat pathway was first discovered in chloroplast thylakoids, and later determined to be active in bacteria, archaea, and plant mitochondria. They transport a number of proteins essential for photosynthesis into thylakoids, including PsbP and PsbR. The Tat pathway is unusual in that it transports folded proteins, without significant ion leakage, and using only the protonmotive force for energy. Rather than substrate proteins transiting through a proteinaceous membrane, experiments point to a unique mechanism of transport involving membrane thinning and transient bilayer breakdown, resulting in the formation of transient toroidal pores.

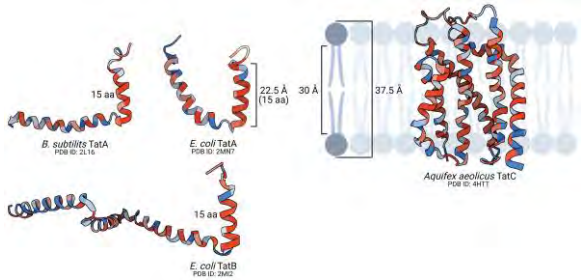

Figure 1. Hydrophobic mismatch between Tat subunits and membrane bilayer.

Significant achievements of the past 2 years –

We have made significant progress in two sets of experiments demonstrating a membrane-destabilization mechanism for Tat protein transport. First, we have monitored ion leakage via the carotenoid electrochromic shift during protein translocation on the Sec- and Tat pathways. Whilst no leak could be detected in during Tat operation, even when the TatABC complex was constitutively assembled, we measured ion leakage during Sec-mediated protein transport. The contrast between the membrane conductivity profiles during transport on these two pathways point to a fundamental difference in their translocation mechanisms.

In a second project, which is the subject of this poster, we have considered the effect on Tat protein transport of the hydrophobic mismatch established between the membrane and the short TatA and TatB transmembrane helices (TMHs) (Fig. 1). In the membrane-destabilization model, one action of the translocation machinery is to thin the membrane, paving the way for subsequent bilayer breakdown. In this view, the hydrophobic mismatch could be seen as conducive to the thinning process. We found that lengthening either TatA or TatB TMHs caused a decrease in Tat activity when measured by pulse-chase of the Tat substrate Sufi in E. coli (Fig. 2) Similarly, further decreasing the TMH lengths of Tat A or TatB also resulted in reduced Tat activity (Fig. 2). Thus, we conclude that the TMHs in both TatA

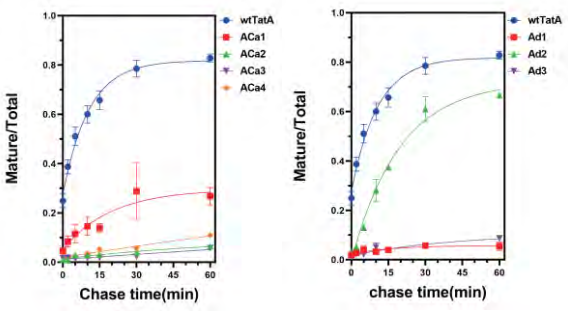

Figure 2. Pulse-chase of SufI with modified TatA THM lengths. Left panel, additions of 1, 2, 3, or 4 amino acids at the C-terminus of the TMH; right panel, deletions of 1, 2, 3, or 4 amino acids at the C-terminus of the TMH.

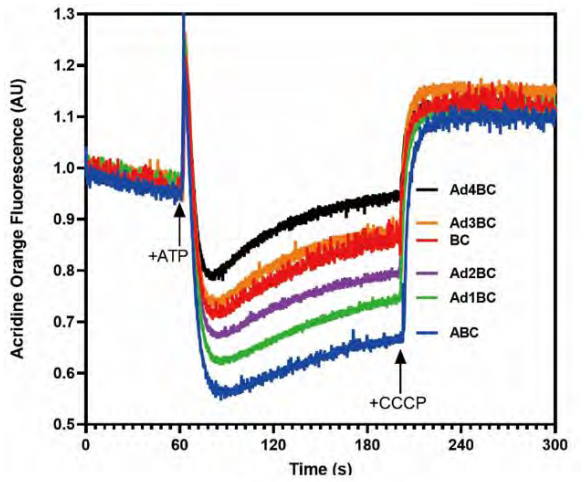

Figure 3. Measurement of the pH gradient in IMVs with shortened TatA TMH. Images from top to bottom show mutants shortened by 4, 3, 2, and 1 amino acids from the C-terminus of the TMH; BC indicates the TatA null. Gradient formation was initiated by addition of ATP at 60 s.

and TatB are evolutionarily tuned to the optimum 15 amino acids of the wild-type proteins, balancing the membrane destabilizing effect of even shorter TMHs, as seen in Fig. 3, and the potentially stabilizing effect of longer TMHs, as seen in Fig. 2.

Objectives for the coming year –

We are testing the competing "iris" model of the Tat pathway in which Tha4 provides the channel for protein transport that squeezes around the substrate during transit. To this end we are measuring the so-called "Tha4 sufficiency", the minimum amount of Tha4 required to translocate a protein. This iris model requires that the Tha4 sufficiency point varies as a function of substrate size. We are currently measuring the Tha4 sufficiency point by examining the kinetics of substrate transport at varying substrate concentrations, a technique pioneered by Celedon and Cline [1]. We will continue these experiments with substrates of different sizes.

Publications of the last 2 years supported by the award –

Gallaher, S.D., Craig, R.J., Ganesan, I., Purvine, S.O., McCorkle, S.R., Grimwood, J., Strenkert, D., Davidi, L., Roth, M.S., Jeffers, T.L., Lipton, M.S., Schmutz, J., Theg, S.M., Blaby-Haas, C.E., and Merchant, S.S. (2021) Widespread polycistronic gene expression in green algae. Proc Natl Acad Sci U S A 2021 Feb 16;118(7):e 2017714118. doi: 10.1073/pnas.2017714118. PMID: 33579822.

Asher A.H. and Theg, S.M. (2021) Electrochromic shift supports the membrane destabilization model of Tat-mediated transport and shows ion leakage during Sec transport. Proc Natl Acad Sci U S A 2021 Mar 23;118(12):e2018122118. doi: 10.1073/pnas.2018122118.

Hao, B., Zhou, W., and Theg, S.M. (2021) Hydrophobic mismatch effect is a key factor in protein transport on the Tat pathway. bioRxiv 2021.05.26.445883; doi: https://doi.org/10.1101/2021.05.26.445883.

Literature cited –

1. Celedon, J.M. and K. Cline, *Stoichiometry for binding and transport by the twin arginine translocation system.* J Cell Biol, 2012. **197**(4): p. 523-34.

Mechanisms for tuning protein electron transfer investigated via site-specific linear and two-dimensional infrared spectroscopy

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Overall goals of the project

We aim to advance our understanding of protein electron transfer (ET), specifically addressing

(1) how the intramolecular interactions of the inner and outer sphere of the Cu site of blue copper proteins contribute to tuning ET

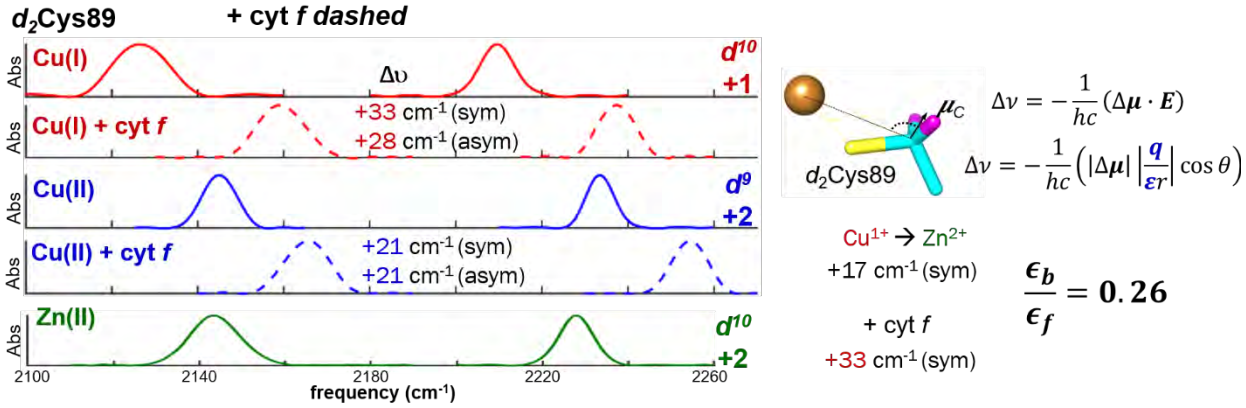
(2) how the formation and nature of the complexes with ET partner cytochrome *f* (cyt *f*) affect the Cu site in the BCP plastocyanin (Pc) and thereby tune redox properties

(3) how protein and/or solvent dynamics contribute to reorganization energy of ET

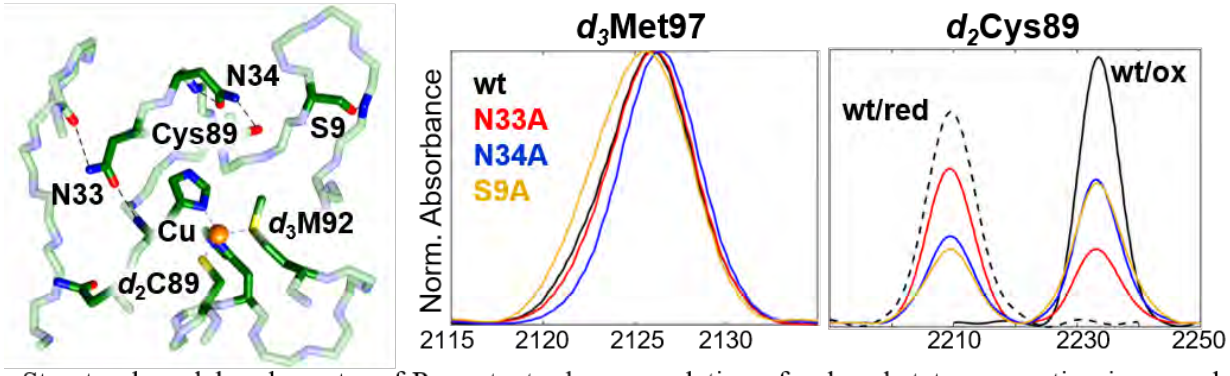
Toward addressing these questions, we have been developing site-specific linear and two-dimensional (2D) infrared (IR) spectroscopy as an approach toward high spatial and temporal investigation of proteins.

Significant achievements of the past 2 years

- Developed transparent window 2D IR spectroscopy as an approach to residue-specifically characterize dynamics within complexes of ET proteins. Showed that side chains within the interface remain highly dynamic within the complex.
- Developed C-D vibrational probes of the critical Cys89 ligand in the Cu center of Pc. Used the C-D probes to demonstrate substantial perturbation to the redox center environment, equivalent to a four-fold effective local dielectric change, upon complexation with partner cyt *f*.



- Applying C-D probes of Cys89 to characterize how the protein scaffold, the secondary sphere, influences the Cu redox center. Current data indicates that the scaffold stabilizes the oxidized state of BCP active sites.



Structural model and spectra of Pc mutants show population of reduced state, suggesting increased E_m and entatic control by Pc scaffold (Cu coordination geometry imposed by protein)

- Progress has been made to extend studies to time-resolved analysis of dynamics of ET through implementation of an approach for photo-triggered reduction of Pc.

Objectives for the coming year

- Assess effect of protein-protein complexation and influence of the secondary sphere of the Pc protein scaffold on controlling midpoint potentials and inter-protein ET. Toward this goal, we need to overcome challenges in implementing traditional assays for determining protein redox potentials and protein-protein crosslinking.
- Establish experiments using quantum cascade laser source to monitor response of active site using C-D probes at Cu ligands upon photo-triggering ET from Pc to cyt *f*.

Publications of the last 2 years supported by the award

Tumbic, G. Hossan, M.Y., Thielges, M.C. (2021) Protein Dynamics by 2D IR Spectroscopy, *Annu. Rev. Anal. Chem.* 14, 299.

Thielges, M.C. (2021) Transparent Window 2D IR Spectroscopy of Proteins, *J. Chem. Phys.* 155, 040903.

Ramos, S., Thielges, M. C. (2019) Site-Specific 1D and 2D IR Spectroscopy to Characterize the Conformations and Dynamics of Protein Molecular Recognition. *J. Phys. Chem. B.* 123, 551-3566. DOI: 10.1021/acs.jpcb.9b00969

Ramos, S. R., Le Sueur, A., Horness, R. E. Specker, J., Collins, J., Thibodeau, K., Thielges, M. C. (2019) Heterogeneous and Highly Dynamic Interface in Plastocyanin-Cytochrome *f* Complex Revealed by Site-Specific 2D IR Spectroscopy. *J. Phys. Chem. B* 213, 2114-2122. DOI: 10.1021/acs.jpcb.8b12157

Biohybrid and Selective Deuteration Approaches for Investigating Photosynthetic Electron Transfer and Charge Accumulation

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Overall research goals: Nature's photosynthetic mechanisms incorporate both large transmembrane protein complexes and small soluble redox proteins in a Z-scheme electron transport chain for photon capture and conversion, utilizing the resultant chemical energy for water splitting and carbon dioxide assimilation. Our goal for studying natural photosynthetic systems is to resolve fundamental principles and common underlying mechanisms of solar energy conversion. This information will provide knowledge required for the design of sustainable photosynthetic-inspired renewable and clean energy systems. In current work, we are creating custom biohybrid assemblies and utilize our group's unique EPR methods to track and interrogate the photosynthetic electron transfer between the soluble redox proteins in the latter part of the Z-scheme and in thylakoid membranes. This research addresses the important mechanism of how light energy, after conversion into chemical energy by reaction center (RC) charge separation, is subsequently utilized to drive NADPH formation via charge-accumulation. Our research strategy builds on our long-standing expertise in EPR spectroscopy of isotopically labelled proteins and incorporates our recent scientific advances in photosynthetic biohybrid chemistry. This work leverages our team's capabilities in bioinorganic and synthetic chemistries for photosynthetic protein network modification to enable spectroscopic characterization and to generate a deep, mechanistic understanding of the chemistry that controls light-driven ET pathways and charge accumulation in photosynthetic systems.

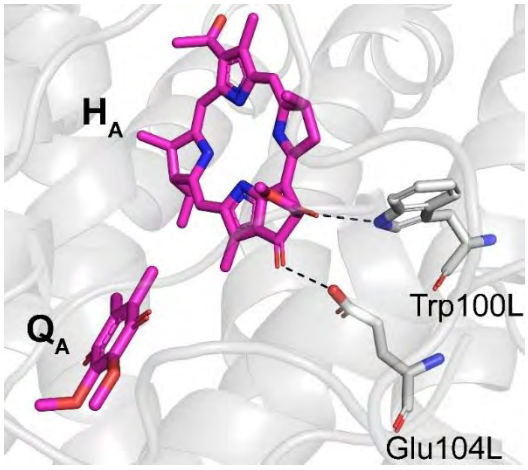

Figure 1. Structural position of Q_A, H_A, and amino acid residues Glu104L, Trp100L H-bonded to H_A as shown by dotted lines. These residues were identified by SCRP-ENDOR of isotopically labeled bRCs, and are believed to regulate the path the light-generated electron takes through the protein.

Significant achievements 2020-2021:

Hydrogen-bond interactions regulate RC electron transfer pathway. Time-resolved high field EPR/ENDOR spectroscopy was used to probe the interaction of the entangled electron spin qubits with nuclear spins in the bacterial RC (bRC). Isotopically labelled RCs enabled high resolution of the Spin Correlated Radical Pair (SCRP)-ENDOR spectra. Two protein residue protons were identified in the pathway between the radical pair $P^+Q_A^-$ (Figure 1). We hypothesize that H-bonding interactions of these residues with the cofactor-protein interface regulate the electron transfer pathway through the protein during light-initiated charge separation.

Defining the electronic structure of P_{700}^+ and A_1^- in Photosystem I (PSI). HF EPR/ENDOR spectra of ^{15}N isotopically labelled PSI were obtained to resolve details about the electronic structures of the primary donor P_{700} and secondary electron acceptor A_1.

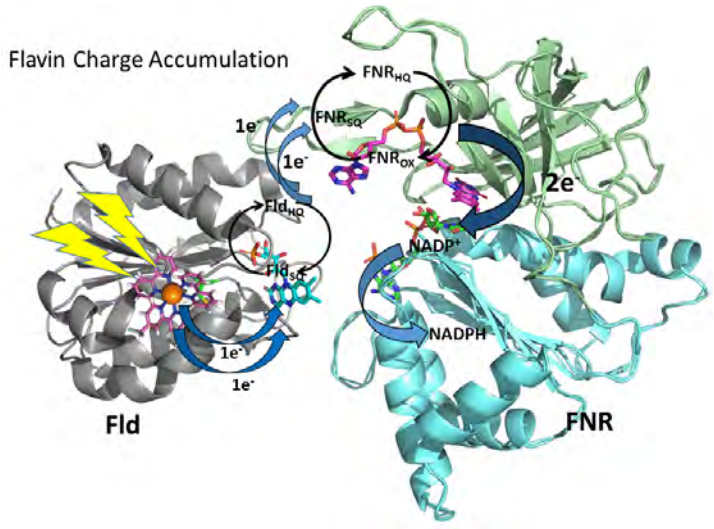

Figure 2. Scheme of photosynthetic electron transfer between RuFld and FNR highlighting the multielectron capabilities of the flavin cofactors. To initiate the interprotein electron transfer, Fld_{OX} must obtain two electrons from two sequential one electron transfer steps from the RuPS covalently bound to Cys54 of Fld. The Fld cofactor then cycles between the Fld_{SQ} and Fld_{HQ} redox states, transferring electrons one at a time to FNR. FNR pairs these single electrons in a single hydride transfer step to protein bound $NADP^+$ to form NADPH.

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.

Science objectives for 2021-2022:

- Mims-type proton ENDOR (matrix-ENDOR) to look at the protein environment around the semiquinone in selectively deuterated FNR.
- Image Pt nanoparticles bound to Photosystem I thylakoids from spinach, algae, and cyanobacteria using the analytical PicoProbe at ANL.
- Examine the structural dynamics of photosynthetic biohybrids with real time X-ray solution scattering and absorption spectroscopy.
- Employ unique combination of isotopically labeled cyanobacterial membranes, isolated proteins, and biohybrid strategies that enable EPR methods to characterize the coupling of RC primary photochemistry to secondary reaction sequences in supercomplexes and thylakoid membranes.

References to work supported by this project 2019-2021:

1. O. G. Poluektov, J. Niklas, L. M. Utschig, "Spin-correlated radical pairs as quantum sensors of bidirectional ET mechanisms in Photosystem I," *J. Phys. Chem. B*, **2019**, 123, 7536−7544. DOI: 10.1021/acsjpcb.9b06636.
2. U. Brahamachari, R. Pokkuluri, D. M. Tiede, J. Niklas, O. G. Poluektov, K. L. Mulfort, L. M. Utschig, "Interprotein electron transfer biohybrid systems for photocatalytic H_2 production," *Photosyn. Res.*, **2020,** 143, 183-192. DOI: 10.1007/s11120-019-00705-x.
3. L. M. Utschig "Preface to the special issue: photosynthesis-inspired biohybrid and biomimetic systems" *Photosyn. Res*., **2020**, 143, 97-98. DOI: 10.1007/s11120-019-00703-z.
4. 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. B* **2021**, 125, 4025–4030. DOI: 10.1021/acs.jpcb.1c00846.
5. J. Niklas, U. Brahamachari, L. M. Utschig, O. G. Poluektov, "D-band EPR and ENDOR of ^{15}N-labeled Photosystem I" *Appl. Mag. Reson.,* **2021**, in press.

Early steps of Photosystem II oxygen-evolving complex assembly are limited by proton release

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Overall goals of the project –

- We are studying the dependence of soluble proteins, pH, and chloride 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 transport of early PSII intermediates from the plasma membrane to the thylakoid membrane in cyanobacteria.
- We are using mass spectrometry and numerical simulations to quantify when and how PSII centers are targeted for repair.

Significant achievements of the past 2 years –

The PSII OEC is assembled from free Mn^{2+}, Ca^{2+}, and H_2O during biogenesis and repair in a process termed photo-assembly. Building this Mn_4CaO_5 active site requires multiple water deprotonation events to form μ-hydroxo and μ-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).

More recently, we aimed to investigate early photo-assembly intermediates spectroscopically. Using dual mode electron paramagnetic resonance (EPR) spectroscopy, we observe the binding and oxidation of Mn^{2+} and the accumulation of Mn^{3+} in isolated apo-OEC PSII membranes under controlled conditions. At the physiologically relevant pH value of 6.0, Mn^{3+} content increases with increasing chloride concentrations. This effect requires the presence of Ca^{2+}.

These combined studies show that chloride facilitated deprotonation events occur during photo-assembly both during the initial Mn^{2+} oxidation event *and* during the rate-limiting (light-independent) step that follows. The ability to efficiently shuttle away protons during metal cluster assembly and active catalysis is an essential design principle of PSII.

Objectives for the coming year –

Dual mode EPR studies will be extended using other anions, with the addition various extrinsic subunits, and with selected point mutations. High field EPR studies are being explored to more accurately characterize Mn^{3+} intermediates.

Our protein biochemistry objectives were delayed during the pandemic but are now making strong progress. Both candidate proteases and chaperones that may be involved in PSII assembly have been identified and are currently under investigation.

Publications of the last 2 years supported by the award –

Brandon P. Russell and David J. Vinyard, “Chloride facilitates Mn(III) formation during photo-assembly of the Photosystem II oxygen-evolving complex.” *in revision*.

Single Photon studies of Photosynthetic Light Harvesting

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Overall goals of the project –

Our long-term goal is to generate a complete and consistent microscopic understanding of the dynamical processes underlying the transduction of energy from sunlight to electron-hole pairs in photosynthetic systems, starting from the incidence of single photons from the ultraweak radiation field of sunlight on the photosynthetic. We approach this goal with new techniques using quantum light sources that allow experimental investigation of the absorption of individual photons and the subsequent spatio-temporal dynamics of the electronic excitation within a photosynthetic complex, coupled with theoretical studies that combine tools from quantum optics and stochastic processes with methods of open quantum simulation and non-linear spectroscopy.

Significant achievements of the past 2 years –

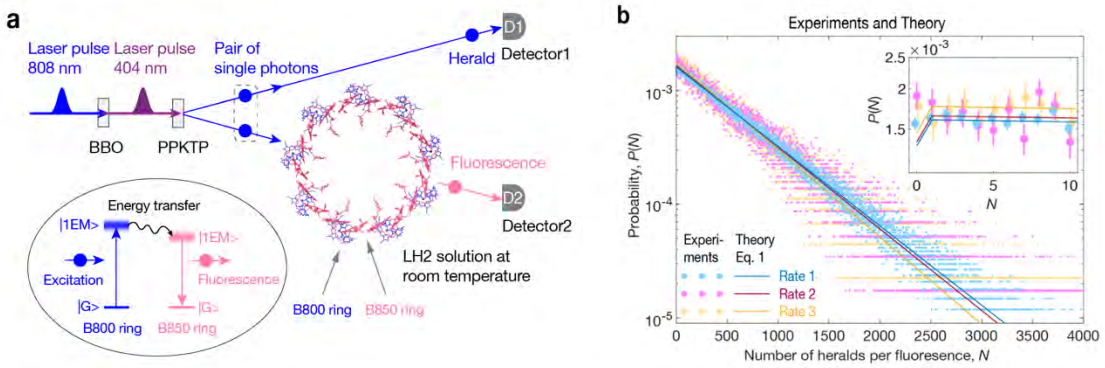

Figure 1. a) Photon Counting Quantum Light Spectroscopy experiment measuring correlations between fluorescent photons and individual heralded incident photons. b) Distribution of the number of heralds, N, between successive fluorescent photons for three incident photon rates. The non-zero value at N=0 is due to imperfect heralding efficiency. Simulations for larger integration times reduce the counting noise and reveal the maximum probability to be at exactly N=1.

- We developed a high-performance, modularized, and versatile photon counting quantum light spectroscopy (PCQLS) technique to investigate the fundamental nature of light absorption by light harvesting complexes. This exploits a heralded single photon source constructed by spontaneous parametric downconversion and allows timing, counting and correlation of individual fluorescent photons with heralds of individual incident photons. We used this to carry out a first time-resolved PCQLS experiment for LH2 that reveals the correspondence and time correlations between incident single photons absorbed by the B800 ring and subsequent fluorescent photons from the B850 ring. This allowed construction of the probability distribution of the number of heralds between successive fluorescent photons (Figure 1), which has a

maximum value at one herald, corresponding to one incident photon, followed by an exponential fall off with rate determined by the ratio of the experimental efficiencies for herald and fluorescent photon collection and detection. Detailed analysis with a stochastic model showed that a single fluorescent photon is produced by a single incident photon, thus providing the first truly microscopic measurement of a quantum efficiency for a single light harvesting complex.

- We completed a quantum trajectory study of the dynamics of single photon absorption and energy transport in Photosystem II, characterizing the significant differences between average dynamics and conditional evolution of the photosynthetic system given the detection of emitted fluorescent photons following absorption. We found that photon counting rates for fluorescence reflect excitonic coherence subsequent to single photon absorption, providing a new photon-counting witness of coherence in energy transfer. We have further expanded the quantum trajectory approach to include exciton-phonon coupling and have begun to explore the role of phonon jumps in the conditional dynamics, which we expect will reduce the extent of coherence.
- We developed a method to simulate the average state dynamics for excitation of a realistic light harvesting system under excitation by N-photon Fock (number state) pulses including coupling to phonons, demonstrated its use for LHCII, and showed that this approach leads to a universal scaling of absorption for light harvesting systems under the pulse bandwidth and time scales relevant to sunlight. We also showed that under weak fields relevant to sunlight, the average excited state dynamics following excitation with a fixed number of photons can be exactly reproduced by simulation with a coherent state have the same average number of photons.

Objectives for the coming year –

Experiments: (1) We plan to install the superconducting nanowire detectors that are due at the end of this month (November 2021). (2) We shall use these new detectors to measure the g2 function for LH2 fluorescence and then to implement pump-probe spectroscopy with entangled photon pairs from our downconversion setup. The many new experimental parameters available with our quantum light source, such as the frequency, bandwidth and spectral shape of the pump laser and the down-converted photons as well the time delay and degree of entanglement between the two photons constituting the pair will be explored for their capacity to reveal new information and to control the dynamics of the photosynthetic pigment-protein system.

Theory: (1) Further develop the theory of quantum trajectories with exciton-phonon coupling and determine the effect of this on the photon-counting witness of excitonic coherence. (2) Develop theory and simulations for both average state and conditional dynamics for pump-probe experiments with entangled photon pairs. We shall undertake simulations for our experiments with LH2 for analysis of the dynamics and the role of coherence in the light and in LH2.

Publications of the last 2 years supported by the award –

(1) “Photosynthesis begins with absorption of a single photon”, Q. Li, K. Orcutt, R. L. Cook, J. Sabines-Chesterking, A. L. Tong, G. S. Schlau-Cohen, X. Zhang, G. R. Fleming, and K. B. Whaley, submitted to Nature.

(2) “A Quantum trajectory picture of single photon absorption and energy transport in photosystem II”, R. L. Cook, L. Ko, and K. B. Whaley, arXiv:2110.013811.

(3) “Dynamics of photosynthetic light harvesting systems interacting with N-photon Fock states”, L. Ko, R. L. Cook, and K. B. Whaley, to be submitted.

Signal Transduction Pathways of Chloroplast Quality Control

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Overall goals of the project – 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. Such signaling would allow individual chloroplasts to control their own maintenance under dynamic conditions and 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 poorly understood.

To identify and characterize components of chloroplast quality control pathways, we have used the *Arabidopsis ferrochelatase two* (*fc2*) mutant as a genetic model system. 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 genetic screen identified suppressors of chloroplast degradation in *fc2* mutants demonstrating this turnover is a genetically controlled response to $^1O_2$. Two key processes important for initiating and propagating the signal have been identified. First, several mutants have shown chloroplast gene expression is necessary to initiate signaling, suggesting a product of the chloroplast genome is a signaling factor. Second, a suppressor mutation, *pub4* (affecting a cytoplasmic E3 ubiquitin ligase), has demonstrated ubiquitination of chloroplast proteins precedes degradation and may play a role in propagating the $^1O_2$ signal. As such, we hypothesize chloroplast-encoded factors allow $^1O_2$ to "mark" chloroplasts for repair or degradation by ubiquitinating chloroplast proteins. Therefore, the major questions in this proposal are **1)** How does $^1O_2$ initiate chloroplast quality control pathways? **2)** What is the role of ubiquitination? **3)** What is the role of this pathway under natural light stress? With the genetic system we have generated, we can address these questions and understand natural mechanisms protecting cells from the inherent dangers of energy capture.

Significant achievements of the past 2 years – *1. Plastid gene expression is necessary to initiate $^1O_2$ signaling* [3,4]. To determine what factors may be involved in initiating $^1O_2$ signaling and chloroplast quality control, we characterized additional mutations that block cell death in the *fc2* mutant. This led to the identification of three genes involved in plastid gene expression; *PENTATRICOPEPTIDE REPEAT PROTEIN 30* (*PPR30*), "mitochondrial" *TRANSCRIPTION TERMINATION FACTOR 9* (*mTERF9*), and *CYTIDINE TRIPHOSPHATE SYNTHASE 2* (*CTPS2*). PPR30 and mTERF9 are chloroplast nucleoid-localized proteins likely involved in post-transcriptional gene regulation. CTPS2 is a cytoplasmic enzyme catalyzing the rate-limiting step for de novo CTP and dCTP synthesis. Mutations affecting these three genes lead to a specific reduction in plastid gene expression and blocked the ability of $^1O_2$ to signal. We hypothesize that a plastid-encoded gene product (possibly a direct target of PPR30 or mTERF9) is part of the $^1O_2$ signaling mechanism (Fig. 1). Future work aims to determine the identity of this signaling factor.

2. *Degrading chloroplasts interact with the central vacuole, but are not dependent on the core autophagy machinery* [1,2,5]. We previously reported chloroplast degradation and cell death can be conditionally induced in *fc2* mutants under diurnal light cycling, due to the accumulation of

Protoporphyrin-IX and $^1O_2$. However, under constant light conditions, individual chloroplasts can be observed to degrade even in the absence of other cellular degradation, sometimes pushing into the central vacuole. To further define these degrading chloroplasts and the associated structures, we used three-dimensional transmission electron microscopy for imaging. Our results demonstrated that these structures frequently occur in otherwise healthy *fc2* cells. Such degrading chloroplasts also appear to swell and contain large plastoglobules, both hallmarks of stress and senescence. To determine what degradation machinery may be involved in the dismantling of damaged chloroplasts, we tested the possibility that canonical autophagy and autophagosomes are required. To this end, we introduced mutations affecting the core autophagy machinery (*atg* mutations) into the *fc2* background. The *fc2 atg* double mutants, however, showed no change in chloroplast degradation, suggesting that canonical autophagy is dispensable for this process. An analysis of mRNA transcripts suggested fission-type microautophagy (a poorly understood process in plants) may instead play a role in quality control.

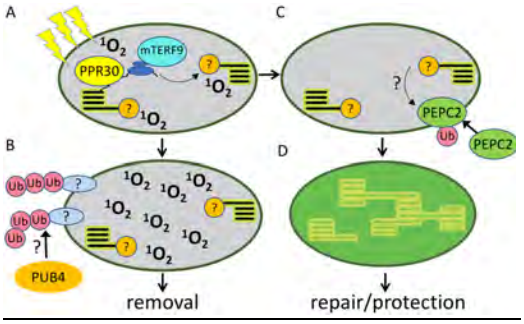

Fig. 1: Model for chloroplast quality control pathways. A) During photosynthetic stress, $^1O_2$ accumulates in specific sub-compartments of individual chloroplasts. Propagation of the $^1O_2$ signal requires chloroplast gene expression, possibly a target of PPR30 or mTERF9. B) This can lead to the polyubiquitination of unidentified chloroplast membrane proteins resulting in the removal of the chloroplast though a process independent of macroautophagy, involving the cytoplasmic E3 ubiquitin ligase PUB4 (at least indirectly). C) Alternatively, recruitment of PEPC2 to the chloroplast leads to D) chloroplast repair and/or protection.

Objectives for the coming year – <u>(1) Understand the mechanism(s) by which $^1O_2$ triggers chloroplast degradation</u>. We will test the hypothesis that $^1O_2$ triggers signaling from within stressed chloroplasts by completing our genetic screens to identify genes and protein systems involved, by exploring the role of chloroplast gene expression in propagating the $^1O_2$ signal, and by determining the site(s) of $^1O_2$ accumulation required to trigger signaling. This work will provide a mechanistic understating of ROS signals controlling chloroplast maintenance in dynamic environments.

<u>(2) Explore the roles of ubiquitination in chloroplast quality control.</u> We will test the hypothesis that ubiquitination of chloroplast proteins propagates the $^1O_2$ signal to control chloroplast maintenance by characterizing the role of PEPC2 (a candidate ubiquitination target) and by identifying additional ubiquitinated chloroplast proteins. This work will provide insight into how chloroplasts direct their own repair or degradation in response to photosynthetic damage.

Publications of the last 2 years supported by the award –

1. K. E. Fisher, P. Krishnamoorthy, M. S. Joens, J. Chory, J. A. J. Fitzpatrick, and J. D. Woodson. 2021. Singlet oxygen leads to structural changes to chloroplasts during degradation in the *Arabidopsis thaliana plastid ferrochelatase two* mutant. *bioRxiv* preprint. https://www.biorxiv.org/content/10.1101/2021.07.19.452378v1
2. M. D. Lemke, K. E. Fisher, M. A. Kozlowska, D. Tano, J. D. Woodson. 2021. 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.
3. K. Alamdari. K. E. Fisher, D. W. Welsh, S. Rai, K. R. Palos, A. D. L. Nelson, and J. D. Woodson. 2021. Chloroplast quality control pathways are dependent on plastid DNA synthesis and nucleotides provided by cytidine triphosphate synthase two. *New Phytologist*. 231(4):1431-48.
4. K. Alamdari, K. E. Fisher, A. B. Sinson, J. Chory, and J. D. Woodson. 2020. 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.
5. Y. Kikuchi, S. Nakamura, J. D. Woodson, H. Ishida, Q. Ling, J. Hidema, R. P. Jarvis, S. Hagihara, M. Izumi. 2020. Chloroplast Autophagy and Ubiquitination Combine to Manage Oxidative Damage and Starvation Responses. *Plant Physiology* 183:1531-44.
6. J. D. Woodson 2019. Chloroplast stress signals: regulation of cellular degradation and chloroplast turnover. *Curr Opin Plant Biol*, 52:30-37. ***Review.***

xpEnergy Conversion in Photosynthesis: Untangling the Sequence of Events During the $S_2 \rightarrow S_3$ Transition in Photosystem II

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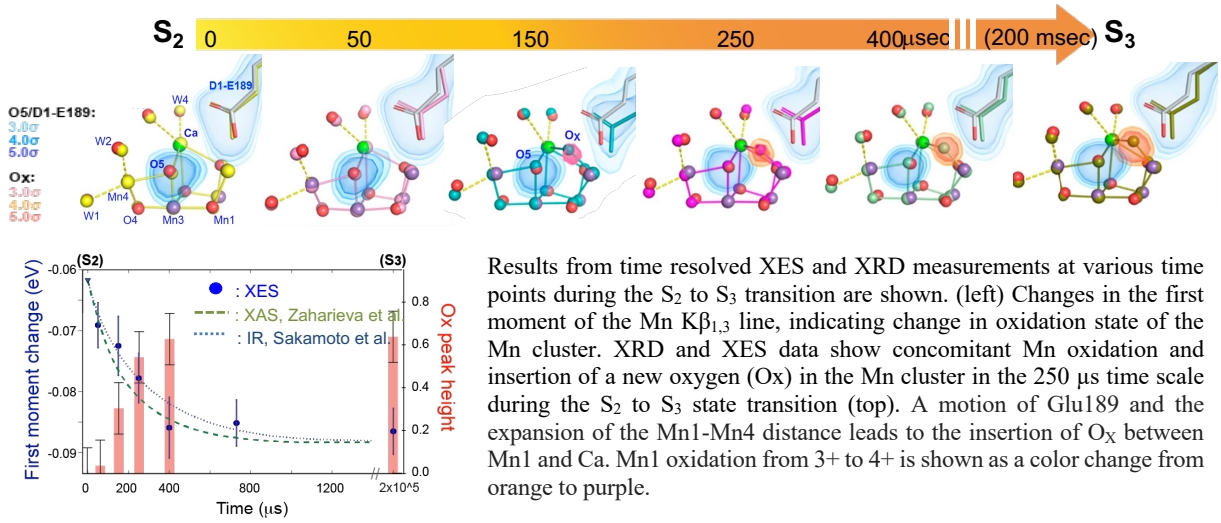
Overall goals of the project

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. We are also applying the new tools that we develop using synchrotrons and XFELs for addressing similar chemical questions in the field of energy conversion in inorganic systems.

Significant achievements (2020-2021)

Recently, we reported the room temperature structures of PS II in the four (semi-)stable S-states, S_1, S_2, S_3 and S_0, showing that a water molecule is inserted during the $S_2 \rightarrow S_3$ transition, as a new bridging O(H)-ligand between Mn1 and Ca. However, the sequence of events leading to this change were not known. To understand the sequence of events leading to the formation of this last stable intermediate state before O_2 formation, we recorded diffraction and Mn X-ray emission spectroscopy data at several time points during the $S_2 \rightarrow S_3$ transition, at room temperature using an XFEL, to collect a 'molecular movie' of the structural and oxidation state change steps in the 50 µs to 200 ms time scales after photon absorption. At the electron acceptor site, changes due to the two-electron redox chemistry at the quinones, Q_A and Q_B, are observed. At the donor site, tyrosine Y_Z and His190 H-bonded to it move by 50 µs after the 2nd flash, and Glu189 moves away from Ca. This is followed by Mn1 and Mn4 moving apart, and the insertion of O_X(H) at the open coordination site of Mn1. This water, possibly a ligand of Ca, could be supplied via a "water



Results from time resolved XES and XRD measurements at various time points during the S_2 to S_3 transition are shown. (left) Changes in the first moment of the Mn $K\beta_{1,3}$ line, indicating change in oxidation state of the Mn cluster. XRD and XES data show concomitant Mn oxidation and insertion of a new oxygen (Ox) in the Mn cluster in the 250 µs time scale during the S_2 to S_3 state transition (top). A motion of Glu189 and the expansion of the Mn1-Mn4 distance leads to the insertion of O_X between Mn1 and Ca. Mn1 oxidation from 3+ to 4+ is shown as a color change from orange to purple.

wheel" like arrangement of five waters next to the OEC that is connected by a large channel to the bulk solvent. XES spectra show that Mn oxidation (t of ~350 μs) during the $S_2 \rightarrow S_3$ transition mirrors the appearance of O_X electron density. This indicates that the oxidation state change and the insertion of water as a bridging atom between Mn1 and Ca are highly correlated. More recently, using a combined high-resolution structure, we have identified the O1 channel for water intake to the metal site, and the Cl1 channel as the proton exit channel that also includes a proton gate.

Objectives for the coming year (2022-2023)

We will collect simultaneous XRD/XES at a resolution of ~2 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. Mn L-edge studies of PS II are also a high priority.

Publications of the last 2 years (2020-2021) supported by the award

Reviews

1. 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 Rev. Physics*, **3**, 265-282.
2. Yano, J., Yachandra, V. K. (2021) Photosystem II: Water Oxidation, Overview. *Encyclopedia of Biochemistry*, 3rd Edition, https://doi.org/10.1016/B978-0-12-819460-7.00013-X. In Press.
3. Lafuerza, S., Retegan, M., Detlefs, B., Chatterjee, R., Yachandra, V. K, Yano, J., and Glatzel, P. (2020) New Reflections on Hard X-Ray Photon-in/Photon-out Spectroscopy. *Nanoscale* **12**, 16270-16284.

Refereed Publications

1. Hussein, R., Ibrahim, M., Bhowmick, A., Simon, P., Chatterjee, R., et al. (2021) Structural Dynamics in the Water and Proton Channels of Photosystem II During the S_2 to S_3 Transition. *Nat. Comm.* (In press).
2. Fransson, T., Alonso-Mori, R., Chatterjee, R., 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. Dyn.* (In press).
3. Ibrahim, M., Fransson, T., Chatterjee, R., Cheah, M. H., et al., (2020) Untangling the Sequence of Events During the $S_2 \rightarrow S_3$ Transition in Photosystem II and Implications for the Water Oxidation Mechanism. *Proc. Natl. Acad. Sci. USA* **117**, 12624-12635.
4. Sauter, N. K., Kern, J. F., Yano, J., Holton, J. M. (2020) Towards the Spatial Resolution of Metalloprotein Charge States by Detailed Modelling of XFEL Crystallographic Diffraction. *Acta Cryst. D*, **76**, 176–192.
5. Kroll, T., et al., (2020) Observation of Seeded Mn Kb Stimulated X-ray Emission Using Two-Color X-Ray Free-Electron Laser Pulses. *Phys. Rev. Letts.* **125**, 037404.
6. Lee, H. B., Marchiori, D. A., Chatterjee, R., Oyala, P. H., Yano, J., Britt, R. D., Agapie, T. (2020) S = 3 Ground State for a Tetranuclear $Mn^{IV}_4O_4$ Complex Mimicking the S_3 State of the Oxygen-Evolving Complex. *J. Am. Chem. Soc.* **142,** 3753–3761.
7. Mendez, D., Bolotovsky, R., Bhowmick, A., Brewster, A. S., Kern, J., Yano, J., Holton, J. M., Sauter, N. K. (2020) Beyond Integration: Modeling Every Pixel to Obtain Better Structure Factors From Stills. *IUCrJ.*, **7**, 1151-1167.

The Dynamics of Cyclic Electron Flow around PSI under Moderate and Acute High Temperatures in the Green Alga *Chlamydomonas reinhardtii*

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Other participant names as appropriate: Rachael Morgan-Kiss, Xin Wang

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Overall goals of the project
The overarching goal of this project is to fully describe the function of sustained PSI-driven cyclic electron flow (CEF) and assembly of PSI supercomplex in the Antarctic *Chlamydomonas* sp. UWO241 (UWO241) and the mesophile *Chlamydomonas reinhardtii* to long-term stress.

Significant achievements of the past 2 years –

1. In collaboration with the lab of Morgan-Kiss, we confirmed that UWO241 had higher CEF under high salt condition (700mM NaCl) than low salt condition (0.43mM NaCl) using our custom-designed spectroscopic instrument Ideaspec (purchased from the Kramer lab at MSU and we thank their help). We also found that UWO241 grown under high salt had higher NPQ and proton motive force than cells grown under low salt, but both high and low salt cultures had comparable O_2 evolution rates, indicating robust growth of high salt cultures. Based on these data, we propose that UWO241 maintains constitutively high CEF possibly with associated PSI-cytb_6f supercomplexes to support robust growth and strong photosynthetic capacity under high salinity. In collaboration with PI Morgan-Kiss, Co-PI Wang and others, our paper about this work was published in Plant Physiology in 2020.

2. The Antarctic *Chlamydomonas* sp. UWO241 (UWO241) has sustained CEF and PSI supercomplex under high salt conditions. The work from the PI Rachael Morgan-Kiss and Co-PI Xin Wang showed that the CEF-supporting PSI supercomplex contains PSBP1, but its roles in supercomplex formation and CEF induction are unknown. The PSBP family are thylakoid luminal proteins which are found in cyanobacteria, algae, and land plants; among these, PSBP1 functions in binding of the Mn cluster of the oxygen evolving complex and assembly/stability of PSII in land plants and green algae. Other PSBP proteins have proposed roles in PSII repair, PSI assembly, NDH-like subunit, or stress responses. The UWO genome contains two copies of PSBP1, named as *UWO-PSBP1A* and *UWO-PSBP1B*. These two genes are adjacent in the UWO genome in a tail-to-tail orientation, suggesting tandem gene duplication. UWO-PSBP1A is present in the PS1 supercomplex. Since genetic manipulation is currently unavailable in UWO241, we aim to study the function of UWO-PSBP1A in the model green alga *Chlamydomonas reinhardtii*. We generated CRISPR knockout lines of *CrPSBP1* in *Chlamydomonas reinhardtii*, and cloned *UWO-PSBP1A* gene. We are currently working on to complement the *Crpsbp1* CRISPR mutants using *CrPSBP1* or *UWO-PSBP1A*, to see if UWO-

PSBP1A can rescue the mutant phenotype (lower PSII efficiency and O_2 evolution rates than WT).

3. We identified conditions where the model green alga *Chlamydomonas reinhardtii* also has sustained CEF, which is 24-h treatment of moderate high temperature (35ºC) and acute high temperature (40ºC). We cultivated *Chlamydomonas reinhardtiii* under well-controlled conditions in photobioreactors at 25ºC first and then switched the culture temperature to 35ºC or 40ºC for 24-h, followed by 48-h recovery at 25ºC. We used P700 measurement to monitor CEF activities in algal cultures before, during, and after high temperature treatments. Our data showed that both 35ºC and 40ºC induced sustained CEF but with different dynamics and function. The CEF induced by 35ºC may be for fast growth/metabolism and could recover after returning to 25ºC for 8-h. But CEF induced by 40ºC may be mainly for photoprotection and could not recover after returning to 25ºC for 48-h. We hypothesize that CEF-supporting supercomplexes with different modifications may be formed during 35ºC or 40ºC high temperature treatments, showing different assembly/disassembly dynamics to support the need for energy or photoprotection in the model green alga *Chlamydomonas reinhardtii*. Our paper about this work is currently under review and the preprint can be seen here in bioRxiv: https://doi.org/10.1101/2021.08.17.456552

Objectives for the coming year –

1. We will complete complementation of *Crpsbp1* CRISPR mutants using *CrPSBP1* or *UWO-PSBP1A* to investigate the function of UWO-PSBP1A. We will also measure CEF activities in these mutants and their complementing lines to investigate how the deficiency of *CrPSBP1* or the complementation using *UWO-PSBP1A* affect CEF activity in *Chlamydomonas reinhardtii*.

2. We will isolate CEF-supporting PSI-supercomplexes during and after treatments of moderate (35ºC) or acute (40ºC) high temperature to investigate the dynamic assembly/disassembly of and components involved in the CEF-supporting PSI-supercomplexes in *Chlamydomonas reinhardtii*.

Publications of the last 2 years supported by the award –

1. 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. (Zhang Lab mentees with underlines)

2. 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*** (2021) Systems-wide analysis revealed shared and unique responses to moderate and acute high temperatures in green alga *Chlamydomonas reinhardtii*. Manuscript under review. bioRxiv: https://doi.org/10.1101/2021.08.17.456552 (Zhang Lab mentees with underlines; #, Equal contribution; * Corresponding author)

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