2013 Photosynthetic Systems Research Meeting

Westin Annapolis Hotel
Annapolis, MD
November 3-6, 2013

U.S. DEPARTMENT OF ENERGY | Office of Science

Office of Basic Energy Sciences
Chemical Sciences, Geosciences & Biosciences Division

2013 Photosynthetic Systems Research Meeting

Program and Abstracts

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Foreword

This volume provides a record of the third biennial meeting of the Principal Investigators (PIs) funded by the Photosynthetic Systems program and is sponsored by the Chemical Sciences, Geosciences, and Biosciences Division of the Office of Basic Energy Sciences (BES) in the U.S. Department of Energy (DOE). Photosynthetic Systems and Physical Biosciences are the two complimentary programs within BES that fund basic research in energy-relevant biological sciences. These two programs along with the Solar Photochemistry program comprise the DOE BES Photochemistry and Biochemistry Team.

As illustrated by the abstracts in this volume, you – the scientists supported by this program – are generating important fundamental knowledge of natural photosynthesis. Because of your productivity, creativity, and commitment to world-class research, Photosynthetic Systems is a vibrant, innovative program that tackles long-standing questions and explores new scientific frontiers in photosynthesis research and the processes of energy capture, conversion, and storage in biological systems. This research is central not only to DOE's mission but also for addressing many of the challenges facing our nation and, indeed, the world.

The primary purpose of this meeting is to foster exchange of scientific information on your DOE-funded work and build a robust community of photosynthesis researchers. Accordingly, the meeting format is designed to promote sharing of new ideas and methodologies between you and your colleagues; facilitate cooperation and collaboration among research groups; stimulate creativity and challenge you with new ideas and paradigms; and provide opportunities for you to interact with DOE Program Managers and staff.

We are looking forward to the presentations of your impressive individual research programs. Whether you are delivering a talk or presenting a poster, we sincerely appreciate your contribution. Your commitment is inspiring and we both learn from and share the excitement of your scientific achievements.

In closing, we also want to thank Diane Marceau from DOE BES and Connie Lansdon and Tim Ledford from Oak Ridge Institute for Science and Education (ORISE) for their invaluable help in planning and successfully executing the many logistical tasks associated with this meeting.

B. Gail McLean, Program Manager, Photosynthetic Systems, DOE BES
Team Lead, Photochemistry and Biochemistry Team, DOE BES
Robert J. Stack, Program Manager, Physical Biosciences, DOE BES

Agenda

AGENDA

2013 Photosynthetic Systems Research Meeting
Westin Annapolis Hotel, Annapolis, MD
November 3-6, 2013

Sunday, November 3

3:00 – 6:00 p.m. Registration
6:00 – 6:30 Reception (No Host)
6:30 – 7:30 Dinner at Westin Annapolis Hotel

Monday, November 4

7:30 – 8:30 a.m. Continental Breakfast

Session I

8:30 – 9:00 Welcome and Photosynthetic Systems Program Update
Gail McLean, DOE BES

9:00 – 10:00 The Photosynthetic Antenna Research Center (PARC)**
Robert Blankenship, Washington University in St. Louis

10:00 – 10:30 Break

Session II *Moderator: **Gary Brudvig**, Yale University*

10:30 – 11:00 The Fate of Triplet States and Photoprotection in Photosynthesis: From Monomeric (B)Chls to Pigment-Protein Complexes
Sergei Savikhin, Purdue University

11:00 – 11:30 Resonant and Non-Resonant Hole-Burning Study of BChls in Excitonically Coupled Photosynthetic Systems
Ryszard Jankowiak, Kansas State University

11:30 – 12:00 The Homodimeric Type I Reaction Center of *Heliobacterium modesticaldum*
Kevin Redding, Arizona State University
John Golbeck, Pennsylvania State University

12:00 – 12:30 Light Energy Transduction in Green (Sulfur) Bacteria
Donald Bryant, Pennsylvania State University

12:30 – 1:30 Lunch

1:30 – 4:00 **Free/Discussion Time** (Put up odd-numbered posters in room TBD)

Session III *Moderator: **Judy Callis,** University of California, Davis*

4:00 – 4:30 Dissecting and Imaging the Regulation of Photosynthetic Light Harvesting
Krishna Niyogi, Lawrence Berkeley National Laboratory

4:30 – 5:00 Global Analysis of Redox Regulation of Protein Thiols in Cyanobacteria
Himadri Pakrasi, Washington University in St. Louis

5:00 – 5:30 Chloroplast Retrograde Signaling During Development and High Light Stress
Joanne Chory, The Salk Institute

5:30 – 6:00 Systems Level Analysis of Chloroplast Iron Homeostasis
Sabeeha Merchant, University of California, Los Angeles

6:00 – 6:30 Reception (No-Host)

6:30 – 7:30 Dinner at the Westin Annapolis

Poster Session I

7:30 – 9:30 Odd Numbered Posters

Tuesday, November 5

7:30 – 8:30 a.m. Continental Breakfast

Session IV: Physical Biosciences Program Corner

Moderator: ***Robert Stack****, Program Manager, Physical Biosciences*

8:30 – 9:00 Analyses of Hyperosmotic-Induced Ca^{2+} Responses in *Arabidopsis thaliana* to Understand Early Osmo-Sensory Mechanisms in Plants
Aaron B. Stephan, University of California, San Diego

9:00 – 10:00 Two-Dimensional Electronic Spectroscopies for Probing Electronic Structure and Charge Transfer: Applications to Photosystem II
Jennifer Ogilvie, University of Michigan

10:00 – 10:30 Break

Session V *Moderator:* ***Marilyn Gunner****, City College of New York*

10:30 – 11:00 The Use of Mass Spectrometry in the Study of the Structure and Function of Photosystem II
Terry Bricker, Louisiana State University

11:00 – 11:30 Taking Snapshots of Photosynthetic Water Oxidation with an X-ray Laser
Junko Yano, Lawrence Berkeley National Laboratory

11:30 – 12:00 Elucidating the Principles that Control Proton-Coupled Electron Transfer Reactions in the Photosynthetic Protein, Photosystem II
K.V. Lakshmi, Rensselaer Polytechnic Institute

12:00 – 12:30 Immunophilins and Their Functions in the Assembly of Photosynthetic Complexes
Sheng Luan, University of California, Berkeley

12:30 – 1:30 Lunch

1:30 – 4:00 **Free/Discussion Time** (Put up even-numbered posters in room TBD)

Session VI *Moderator:* ***Christoph Benning,*** *Michigan State University*

4:00 – 4:30 Chloroplast and Thylakoid Biogenesis
Steven M. Theg, University of California, Davis

4:30 – 5:00 Photobiological Solar Fuels
Maria Ghirardi, National Renewable Energy Laboratory

5:00 – 5:30 Biochemical Integration of Metabolic Networks Critical for Energy Transformation in *Chlamydomonas reinhardtii*
Arthur R. Grossman, Carnegie Institution for Science and
Matthew C. Posewitz, Colorado School of Mine

5:30 – 6:00 Enhancement of Source-Sink Relationships by Manipulation of Two Starch Regulatory Enzymes, ADPglucose Pyrophosphorylase and Phosphorylase
Tom Okita, Washington State University

6:00 – 6:30 Reception (No-Host)

6:30 – 7:30 Dinner at Westin Annapolis

Poster Session II

7:30 – 9:30 Even Numbered Posters (No-Host)

Wednesday, November 6

7:30 – 8:30 a.m. Continental Breakfast

Session VII *Moderator:* ***Katherine Osteryoung****, Michigan State University*

8:30 – 9:00 Molecular Regulation of Photosynthetic Carbon Dioxide Fixation in Nonsulfur Purple Bacteria
Robert Tabita, Ohio State University

9:00 – 9:30 Structure/Function of the Novel Proteins LCIB and LCIC in the Chlamydomonas CCM
Martin Spalding, Iowa State University

9:30 – 10:00 Integration of the Light and Dark Reactions of Oxygenic Photosynthesis
Robert L Burnap, Oklahoma State University

10:00 – 10:30 Break

Session VIII *Moderator:* ***Robert Gennis,*** *University of Illinois*

10:30 – 11:00 Photosynthetic Hydrogen: Is a Tyrosine Radical Involved in Making the [FeFe] Hydrogenase Catalytic H-Cluster?
David Britt, University of California, Davis

10:30 – 11:00 Multi-Heme Hybrid Protein Architectures for Solar Chemical Energy Conversion
David Tiede, Argonne National Laboratory

11:30 – 12:00 Photobiohybrid Solar Fuels
Paul King, National Renewable Energy Laboratory

12:00 – 1:00 Lunch

Session IX

1:00 – 2:30 Photosynthesis Science Discussion and Investigator Feedback

** Energy Frontier Research Center-supported project

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Session I

Cyanobacterial Megacomplexes

Robert E. Blankenship[a,b,c], Haijun Liu[a,b], Hao Zhang[b,c], Dariusz M. Niedzwiedzki[b,c], Michael L. Gross[c],

[a]Department of Biology, Washington University in St. Louis, St. Louis, MO 63130
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[c]Department of Chemistry, Washington University in St. Louis, St. Louis, MO 63130

In photosynthetic organisms, photons are harvested by light-harvesting complexes and transferred to reaction centers where photochemistry takes place, leading to long-term energy storage. We have isolated and characterized a megacomplex composed of a phycobilisome (PBS) and its energy acceptors, Photosystem II (PSII) and Photosystem I (PSI) in the cyanobacterium *Synechocystis* PCC6803. Techniques utilized include protein cross-linking along with biochemical analysis, mass spectrometry and ultrafast spectroscopy. The two terminal energy emitters of the PBS, ApcE and ApcD, are closely associated with components of PSII and PSI respectively. The PBS core fully covers PSII, but only overlaps partially with PSI. Time-resolved spectroscopy indicates that there is efficient energy transfer between the PBS and two photosystems in the megacomplex. The structural information on the proteins and cofactors and their interactions within the megacomplex provides a basis for understanding how phycobilisomes transmit excitation energy to reaction centers and how the energy balance of two photosystems is achieved, to adapt organisms to varying ecophysiological conditions and operate at maximum efficiency.

A key additional question is how this system responds to saturating light conditions and thus avoids light-induced damage. An orange carotenoid protein (OCP) is involved in photoprotection of cyanobacteria. However, the mechanism of how and where OCP interacts with the megadalton phycobilisome antenna complex is not clear. We monitored OCP conformational changes between low light and saturating light conditions using native mass spectrometry, ion mobility mass spectrometry and located the interaction interfaces between OCP and the pycobilisome by protein cross-linking.

This work was carried out at the Photosynthetic Antenna Research Center (PARC) and was supported by the Energy Frontier Research Center program of the Office of Basic Energy Sciences, US Department of Energy (Grant No. DE-SC 0001035).

Session II

The fate of triplet states and photoprotection in photosynthesis: from monomeric (B)Chls to pigment-protein complexes

Sergei Savikhin, Principal Investigator
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Overall research goals: : The research objective is to investigate novel photoprotection mechanism in strongly coupled (bacterio)chlorophyll complexes by: (1) time resolved and steady state spectroscopy, (2) infrared phosphorescence detection and (3) EPR techniques. The goals is to fully understand this exceptionally efficient intrinsic photoprotection discovered recently by our group and develop framework that would allow to predict this photoprotection in natural system from their structure and design robust artificial systems that incorporate this novel mechanism.

Significant achievements 2011-2013:

(i) The infrared time-gated phosphorescence spectrometer, built in the course of this project, was successfully used to characterize triplet state energies of *all* ten naturally occurring (bacterio)chlorophyll (BChl) molecules. (Fig. 1). This is the most comprehensive study of triplet states in (B)Chls up to date.

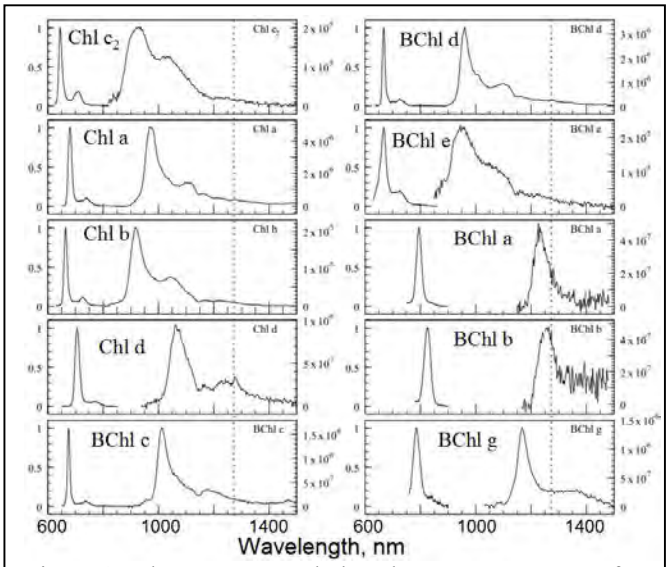

Figure 1. Fluorescence and phosphorescence spectra of ten (B)Chls. Dashed line: singlet oxygen energy.

(ii) The fate of triplet excited states in the Fenna Matthews Olson (FMO) complex was studied by means of time-resolved optical spectroscopy (Fig. 2) and structure based model exciton simulations. FMO contains a number of strongly coupled BChl *a* pigments, but no carotenoids. Yet this complex is extremely stable. We show that upon excitation, triplet state can be formed concurrently on several different pigments, and consequently transferred (within few microseconds) to the lowest energy pigment. The triplet state energy of this pigment appears to be below the energy of singlet oxygen preventing the formation of the highly reactive oxygen species and this explains the high stability of the complex. Lowest energy triplet state lifetime in FMO is found to be 50 µs and is not affected by the presence of oxygen in the sample.

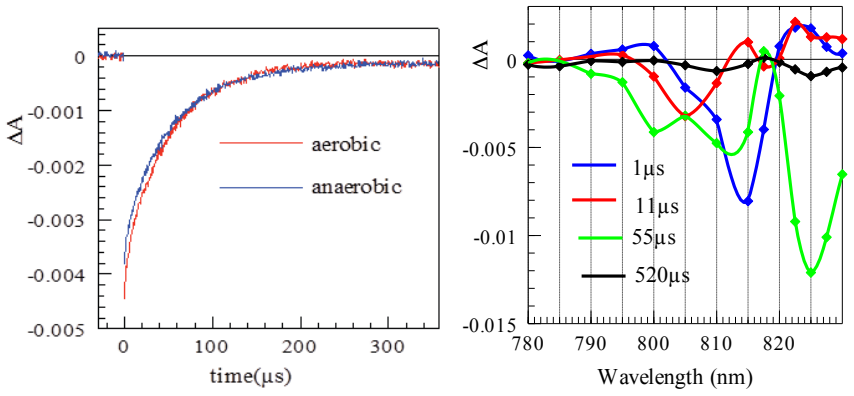

Figure 2. Left: Absorption difference kinetics of FMO probed at 810 nm in aerobic and anaerobic conditions shows independence of BChl *a* triplet state life time on oxygen. Right: Decay-associated difference spectra of FMO at 20 K after excitation at 775 nm reveal triplet energy transfer between individual pigments.

Note that triplet state dynamics in FMO suggests also, that the lowest singlet excitonic state in this complex is shared among 3 pigments, and not 2 as predicted based on previous exciton simulations. It is also shown, that triplet excitons are not formed in FMO, and the photoprotection mechanism in these carotenoid-free complxes is based on lowering of the triplet state energy of BChl *a* below that of singlet oxygen by protein environment.

(iii) Triplet states of thin films of a slip-stacked perylenediimide were characterized using phosphorescence. This information is important for designing robust and efficient solar cells based on these compounds (collaboration with Dr. Wasielewski's group).

Science objectives for 2013-2014:

- The triplet states of the (B)Chl will be modeled using quantum mechanical approach (density functions). The preliminary modeling shows reasonably good agreement with the experiment, and addition of empirical energy shift term would allow to predict the triplet states of (B)Chl like molecules using that computational approach.
- The dimers and multimers of (B)Chl will be constructed and their phosphorescence will be characterised and compared with computational models.
- An attempt will be made to (i) characterize FMO triplet state energy through phosphorescence measurement and (ii) apply quantum mechanical model to compute these energies from the structure.
- Chlorosome and artificial (B)Chl aggregate triplet state properties will be revisited. So far triplet phosphorescence of chlorosoemes could not be detected. However, in light of additional information we learned from (B)Chl modeling and direct measurements the hope is to develop a comprehensive theoretical description of triplet states in these structures.

References to work supported by this project 2011-2013:

1. Xu, W., Y. Wang, E. Taylor, A. Laujac, L. Gao, S. Savikhin, P.R. Chitnis, Mutational analysis of photosystem I of Synechocystis sp. PCC 6803: the role of four conserved aromatic residues in the j-helix of PsaB. PLoS ONE, 2011. 6: p. e24625.
2. Chauvet, A., N. Dashdorj, J.H. Golbeck, W.T. Johnson, S. Savikhin, Spectral resolution of the primary electron acceptor A_0 in photosystem I. J. Phys. Chem. B, 2012. 116: p. 3380-3386
3. Chauvet A., Sarrou J, Lin S, Romberger SP, Golbeck JH, Savikhin S, Redding KE. Temporal and spectral characterization of the photosynthetic reaction center from Heliobacterium modesticaldum. Photosynth. Res. 2013, 116, p. 1-9
4. Jankowiak R, Savikhin S. Mechanism of Primary Charge Separation in hotosynthetic Reaction Centers. Book chapter in: The Biophysics of Photosynthesis, eds. John Golbeck and Art van der Est (in press).
5. S. W. Eaton, L. E. Shoer, S. D. Karlen, S. M. , B. S. Veldkamp, C. Ramanan, D. A. Hartzler, S. Savikhin, T. J. Marks, M. R. Wasielewski. Singlet Exciton Fission in Polycrystalline Thin Films of a Slip-Stacked Perylenediimide. Submitted to Journal of the American Chemical Society.
6. Kihara S., Hartzler D., Savikhin S. Oxygen concenration within functioning photosynthetic membrane. Submitted to Biophysical Journal.
7. Hartzler D., Blankenship R.A., Savikin S. Triplet energies of ten (bacterio)chlorophills measured by infrared phosphorescence. In preparation (to be submitted to Chem Phys B)
8. Kihara S, Blankenship RA, Savikhin S. The fate of triplet excitations in Fenna Matthew Olson complex. In preparation (to be submitted to Chem Phys B)

Resonant and Non-Resonant Hole-Burning and Delta Fluorescence Line-Narrowing Study of BChls in Excitonically Coupled Photosynthetic Systems

Ryszard Jankowiak, Principal Investigator; Grant No. DE-FG02-11ER16281; Period: 2011-2014
Department of Chemistry, Kansas State University, Manhattan, KS 66506;
Email: ryszard@ksu.edu; Web: http://www.kstate.edu/chem/people/faculty/jankowiak.html

Overall research goals:

The research objectives are to study excitonically coupled bacteriochlorophylls (BChls) that are ubiquitous in bacterial photosynthetic complexes. Hole-burning (HB) and delta fluorescence line-narrowing (ΔFLNS) spectroscopies and modeling studies are used to provide additional insight into the excitonic structure, electron-phonon and vibronic couplings, and excitation energy transfer (EET)/electron transfer (ET) processes in: i) various model protein systems; ii) the Zn-reaction center (RC) and its mutants; iii) *Rb. sphaeroides* RC and its mutants; and iv) various FMO complexes, found in anoxygenic green sulfur bacteria. Deeper insight into the origin of hole-burned (HB) spectra will provide a better framework for probing the electronic structure of complex biological systems via HB/ΔFLNS spectroscopies. Our approach will provide a more complete picture of the EET/ET processes in various photosynthetic systems.

Significant achievements 2012-2013:

- We provided a critical assessment of typical phonon spectral densities, $J(\omega)$, used to describe linear/nonlinear optical spectra in photosynthetic complexes [3]. We showed that many densities (especially the Drude-Lorentz/constant damping Brownian oscillator) display qualitatively wrong behavior when compared to experiment. We proposed that a lognormal distribution can be used to fit experimental data and exhibits desired attributes for a physically meaningful phonon $J(\omega)$, in contrast to several commonly used spectral densities which exhibit low frequency behavior in qualitative disagreement with experiment.
- We developed analytical formulas to describe the FLN spectra of weakly coupled dimers in the presence of EET. This model can be extended to multiple chromophores. Modeling studies show that the FLN spectra (including absorption and emission spectra) calculated for various model systems are in good agreement with spectra calculated by: *i)* the simple convolution method and *ii)* the more rigorous treatment using the Redfield approach [5].
- We showed that the widely used reorganization energy (E_λ) of 35 cm^{-1} in various modeling studies of the FMO two-dimensional electronic spectra from *C. tepidum* is overestimated by a factor of ~3, while its value has important implications for the contributions to the coherence rate. We showed, using both nonresonant and resonant HB spectra, and modeling studies, that the nature of the so-called 825nm absorption band of the FMO trimer, contrary to the presently accepted consensus, cannot be explained by a single transition. To explain the shape of emission and HB spectra, a downward uncorrelated EET between trimer subunits should be taken into account. That is, after light induced coherences vanish within each monomer, the uncorrelated EET between the lowest exciton levels of each monomer takes place due to static structural inhomogeneities in the trimer [6,7].

Science objectives for 2013-2014:

- Complete the theoretical description of various optical spectra (including HB spectra) of Chl *a* WSCP from cauliflower and *Lepidium*. Prove that both WSCP from *Lepidium virginicum* and cauliflower contain four Chl *a* and demonstrate that a slow protein relaxation between energetically inequivalent conformational substates within the lowest exciton state proposed for WSCP-C in Refs [Pieper et al. JPC B 2011, 115, 4053; Schmitt et al., JPC B 2008, 112, 13951] is not applicable to WSCP complexes.

- Although there have been many studies regarding the excitonic structure and ET dynamics in bacterial RCs, some issues related to electrochromic effects and the position of the upper excitonic component of P (i.e., P_+) are yet to be fully understood. Preliminary results for the wild-type (WT) RC from *Rb. sphaeroides* and its mutants indicate that the large bleach near 810 nm in the absorption spectrum of the WT RC upon formation of the $P^+Q_A^-$ state is caused primarily by an electrochromic shift of the absorption band(s) of the monomeric BChls (B_A and B_B), rather than bleaching of the P_+ exciton band. Demonstrate that the P_+ transition in the WT RC from *Rb. sphaeroides* contributes to the absorption near 800 nm, while the P_+ excitonic component in the M214G mutant is blue shifted by about 10 nm.

- Complete the work on the optical lineshape function formulas involving multi-phonon transitions in HB spectra. Apply the models to analyze data obtained for various photosynthetic complexes.

Publications supported by this project 2012-2013:

1. R. Jankowiak, *"Probing Electron Transfer Times in Photosynthetic Reaction Centers by Hole-Burning Spectroscopy"* J. Phys. Chem. Lett., 3, 1684−1694 (**2012**).
2. B. Neupane et al., *"Electron Transfer in the Rb. Sphaeroides RC Containing Zn-Bacteriochlorophylls and its β-Zn-RC Mutant: HB Study"*, J. Phys. Chem. B 116, 3457 **(2012).**
3. A. Kell, X. Feng, M. Reppert, and R. Jankowiak, *"On the Shape of the Phonon Spectral Density in Photosynthetic Complexes"*, J. Phys. Chem. B 117(24):7317-23 (**2013**).
4. C. Lin et al., *"Fluorescence Line-Narrowing Difference Spectra: Dependence of Huang-Rhys Factor on Excitation Wavelength"*, Chem. Phys. Lett., 576:15-20 (**2013**).
5. C. Lin et al., *"Modeling of Fluorescence Line-Narrowed Spectra in Weakly Coupled Dimers in the Presence of Excitation Energy Transfer"*, J. Chem. Phys. (**2013**), submitted.
6. V. Zazubovich and R. Jankowiak, *"Biophotonics of Photosynthesis"*, an invited book chapter for Photonics, volume 4, *Biophotonics*; Ed.: David Andrews, Publisher Wiley, **(2013),** submitted.
7. S. Savikhin and R. Jankowiak, *"Mechanism of Primary Charge Separation in Photosynthetic Reaction Centers"*, in The Biophysics of Photosynthesis, Springer, Eds. John Golbeck and Art van der Est (**2013**), submitted.
8. N. Herascu et al., *"Modeling of various optical spectra in the presence of uncorrelated excitation energy transfer in dimers and trimers with weak inter-pigment coupling"*, J. Phys. Chem. B, (**2013**) to be submitted in November.
9. A. Kell et al., *"On the Controversial Nature of the 825 nm Exciton Band in FMO Complexes"*, J. Phys. Chem. Letters, (**2013**), to be submitted in November.

The Homodimeric Type I Reaction Center of *Heliobacterium modesticaldum*

Kevin E. Redding[1], Principal Investigator
John H. Golbeck[2], Principal Investigator
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Overall research goals: Our research objectives are to understand the structure, function and organization of homodimeric Type I reaction center found in *Heliobacterium modesticaldum*. Our goals include: (i) What are the 3-dimensional structures of the heliobacterial reaction center core and the F_A/F_B-containing polypeptides? (ii) Are the PshB1 and PshB2 polypeptides true subunits of the heliobacterial reaction center, or are they quasi-mobile acceptor proteins? (iii) Do other ferredoxin-like proteins function as electron acceptors from F_X? The overall idea is to 'jump start' the study of a simple Type I reaction center, so that with the acquisition of high-resolution X-ray crystal structures, mutagenesis techniques, biochemical protocols, and biophysical tools, structure/function studies can be carried out on a simplified photochemical reaction center that works at the reducing end of the biological redox scale.

Significant achievements 2011-2013:

(1) Purification of the Heliobacterial RC to homogeneity: crystallization and ultra-fast spectroscopy. We have extended the purification originally published for the HbRC by adding a second chromatography step (cation exchange chromatography). We have performed an extensive characterization of this purified HbRC core ($PshA_2$) with a wide range of analytical, biochemical, and spectroscopic techniques. The stoichiometry of cofactors within the HbRC was determined to be 19±3 BChl *g* : 2 BChl *g'* : 2 8^1-OH-Chl a_F : 1 4,4'-diaponeurosporene : 1.6 menaquinone (7:1 ratio of MQ-9:MQ-8). Flavodoxin reduction kinetics were very similar to the original prep. In single photon counting fluorescence experiments, a 25-ps component comprising 99% of decay and exhibiting an emission maximum at ~815 nm was assigned to excitation trapping. We have also confirmed that electron transfer from the 8^1-OH-Chl a_F cofactor (A_0) to the F_X cluster occurs with a time constant of ~700 ps, that it is unaffected by the presence or absence of quinones, and that it is blocked by the pre-reduction of F_X. While we have been able to crystallize the HbRC core in at least 2 different crystal forms, we have so far not observed diffraction with resolution better than 8-10 Å.

(2) Identification of the F_X [4Fe-4S] cluster as the terminal electron acceptor of the HbRC. We have shown that HbRC core directly reduces the soluble redox protein flavodoxin from cyanobacteria. Not only does this reaction not require the presence of the PshB polypeptides, but addition of either PshB1 or PshB2 suppresses it, likely due to competition for the electron on F_X. This is the first report of the F_X cluster participating in forward electron transfer to a completely soluble redox protein in any Type I reaction center and implies that the membrane-embedded PshA homodimer should be capable of donating electrons directly to a variety of soluble redox partners in heliobacteria.

(3) Characterization of the F_X [4Fe-4S] cluster. We had previously shown that the reduced cluster has an unusual EPR signal that was interpreted as a ground state of $S = 3/2$. This EPR signal has been confirmed in both HbRC cores reduced in the dark in strongly reducing conditions and in membranes illuminated under weakly reducing conditions. We have measured the reduction potential of the F_X cluster by EPR and optical spectroscopy and determined that it is about -500 mV (vs NHE), which is much less reducing than the F_X cluster of Photosystem I.

(4) Expression and purification of a soluble version of cytochrome c_{553}: This protein is the major electron donor to the HbRC and is attached to a lipid in *H. modesticaldum*. We have expressed a recombinant version of cyt c_{553} in which the lipid-attachment site is replaced by a hexahistidine tag

and purified it to homogeneity. Biochemical and spectroscopic characterization indicate that the recombinant protein behaves much like the native protein and possesses a low-spin heme with a reduction potential of +220 mV (vs. NHE).

(5) Demonstration that the HbRC can use menaquinone as a terminal electron acceptor: Illumination of membranes in the presence of ascorbate results in reduction of menaquinone (MQ) to menaquinol (MQH_2). There seems to be a competition between the light-driven reduction of the MQ pool and the oxidation of MQH_2 by the cyt b_6c complex, which determines the steady-state level of quinone reduction.

Science objectives for 2011-2013:

1. Produce recombinant proteins of candidate electron acceptors and test the ability of the HbRC to reduce them.
2. Finish experiments to test double-reduction of menaquinone by the HbRC in membranes and create an in vitro system using purified HbRC and recombinant cyt c_{553} with exogenous quinones. Screen for Q_B-type inhibitors that bind in the MQ site of the HbRC.
3. Test the idea that the F_X cluster can be doubly reduced.
4. Study the effect on primary charge separation when bacteriochlorophyll *g* is converted to a chlorophyll *a*-like molecule in the presence of molecular oxygen.
5. Produce first gene deletion mutants in *H. modesticaldum.*
6. Obtain first structure of the HbRC core by X-ray crystallography.

References to work supported by this project 2011-2013:

1. Jagannathan, B., Shen, G., and Golbeck, J.H. (2011) 'The evolution of type I reaction centers: the response to oxygenic photosynthesis', in *Functional Genomics and Respiration*, 33, Chapter 12, R. Burnap and W. Vermaas, eds., Springer, The Netherlands.
2. Romberger, S. P., and Golbeck, J. H. (2012) The F_X iron-sulfur cluster serves as the terminal bound electron acceptor in heliobacterial reaction centers. *Photosynth. Res. 111*, 285-290.
3. McConnell, M.D., J.B. Cowgill, P.L. Baker, F. Rappaport and K.E. Redding (2011) Double reduction of plastoquinone to plastoquinol in Photosystem 1. *Biochemistry* 50:11034-46.
4. Mula, S., M.D. McConnell, A. Ching, N. Zhao, H.I. Gordon, G. Hastings, K.E. Redding, and A. van der Est. (2012). Introduction of a hydrogen bond between phylloquinone PhQ_A and a threonine side-chain OH group in Photosystem I. *J. Phys. Chem. B* 116:14008-16.
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Light Energy Transduction in Green (Sulfur) Bacteria

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Overall research goals: Current research objectives are (1) to establish and utilize a genetic system to define the pathway for BChl *e* biosynthesis; (2) define the supramolecular structure(s) formed by BChl *e* and *f* in chlorosomes; (3) characterize the photosynthetic apparatus in the novel chlorophototroph, Chloracidobacterium thermophilum; (4) cultivate and characterize novel chlorophototrophs from hot spring microbial mats in Yellowstone National Park; (5) characterize enzymes involved in the synthesis of BChls and carotenoids.

Significant achievements 2011-2013: We identified the gene encoding the first committed step in BChl *c* biosynthesis, thereby completing this pathway, and we characterized three enzymes (BciA, BciB, and BchV) in this pathway. We established a genetic system in the *Chlorobaculum limnaeum* and produced mutant strains affecting carotenoid and BChl *e* biosynthesis. We produced and characterized a *bchU* mutant that produces BChl *f*, which does not occur naturally (Figure 1, left). We characterized the chlorosomes, FMO protein, and Type-1 reaction centers of *Chloracidobacterium thermophilum*, and showed that these reaction centers contain 3 Chls (BChl *a*, Chl *a*, and Zn-BChl *a*′). We defined the entire pathway for the important biomarker carotenoid okenone. We determined the structural organization of BChl *c* molecules in chlorosomes of a *bchQ bchR* mutant of *Cba. tepidum* and showed that the BChl molecules are organized in a different manner than in the wild type (Figure 1, right). Finally, we isolated new phototrophs from hot spring mats and obtained the first axenic culture of the novel chlorophototroph, *Cab. thermophilum*.

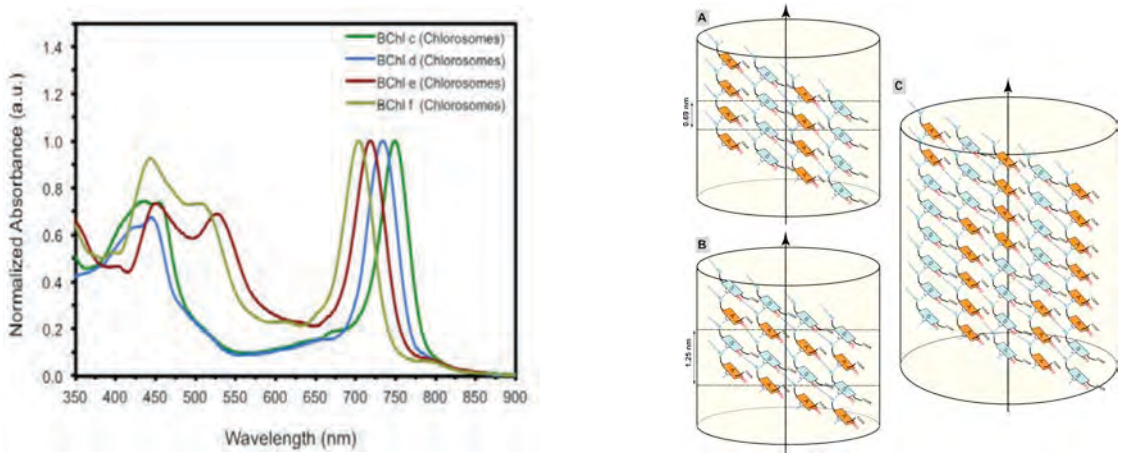

Figure 1. Left Panel: Comparison of the absorption spectra of chlorosomes containing four different BChls, including BChl *f*, which has not been observed in nature. Right Panel: Schemes showing the organization of BChls in a *bchQ bchR* mutant of *Cba. tepidum* (A, C) and (B) the wild-type strain of the same organism. In wild-type chlorosomes, the BChl *c* molecules form *syn-anti* dimer stacks that are parallel to the long axis of the chlorosome. In the *bchQ bchR* mutant, the BChls form all-*syn* and all-*anti* monomer stacks that stack parallel to the long axis of the chlorosome. As shown in part C to the right, these parallel stacks can be heterogeneous and contain both *syn* and *anti* stacked molecules.

Science objectives for 2013-2014: (1) We will continue our efforts to determine the supramolecular organization of BChls *e* and *f* in chlorosomes of *Cba. limnaeum*. (2) We will continue to define the biosynthetic pathway for BChl *e* biosynthesis and produce mutants that help to define the role of isorenieratene in the chlorosomes of BChl *e*-producing strains. (3) We will continue efforts to characterize the type-1 RCs of *Cab. thermophilum* and to establish the roles of the Zn-BChl *a*′ molecules (primary donor or primary acceptor). (4) We will continue to cultivate and characterize novel phototrophs from hot spring microbial mats of Yellowstone National Park.

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Session III

Regulation of Photosynthetic Light Harvesting

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Overall research goals: Photosynthetic light-harvesting systems are regulated, protected against photo-oxidative damage, self-assembled into supercomplexes, and rely on a subtle balance of electronic interactions to produce a highly robust system. In this project, we apply a multidisciplinary approach that combines genetic and biochemical techniques with ultrafast spectroscopy to understand the mechanisms by which oxygenic photosynthetic organisms regulate the efficiency of light harvesting in Photosystem II. In response to fluctuations in light intensity, nonphotochemical quenching (NPQ) mechanisms that regulate photosynthetic light harvesting are induced. In algae and plants, the qE type of NPQ is turned on and off rapidly by changes in thylakoid lumen pH, whereas the slower qI type downregulates PSII during long-term light stress. Our specific aims are (1) to investigate a novel type of violaxanthin de-epoxidase (VDE) enzyme in *Chlamydomonas*, (2) to identify the structural features that make LHCSR a qE-quenching protein instead of a light-harvesting protein, (3) to assess the roles of LHCSR (and PsbS) in other algae, (4) to gain new insight into qI in *Arabidopsis*, and (5) to develop predictive computational models of excitation energy transfer and NPQ.

Significant achievements 2011-2013: By complementing the *Arabidopsis npq1* mutation with the *NPQ1* gene of *Chlamydomonas*, we showed that the NPQ1 protein is indeed a functional VDE. Furthermore, we were able to complement both the *Chlamydomonas* and *Arabidopsis npq1* mutations with a carboxyl-terminal epitope-tagged NPQ1 protein, and cellular fractionation experiments showed that, unlike the plant-type VDE, NPQ1 is a stromal protein associated with thylakoid membranes. By site-directed mutagenesis, we identified three lumen-facing, acidic residues in LHCSR3 that are necessary for its function in qE. Mutagenesis techniques were established for the green alga *Chromochloris zofingiensis*, which has an unusually high NPQ capacity, and for the heterokont alga *Nannochloropsis oceanica*, and *npq* mutants of both species were isolated. We showed that the *soq1* mutant of *Arabidopsis* identifies a novel type of antenna qI that is distinct from previously characterized components of NPQ. We constructed an apparatus for measuring "fluorescence lifetime snapshots" during induction of qE in *Chlamydomonas*, and we have extended this technique to measuring whole leaves. We developed a kinetic model of qE that allows simulation of chlorophyll fluorescence yield experiments, as well as the first structure-based model of energy transfer in PSII supercomplexes.

Science objectives for 2013-2014:

- Establish assay for NPQ1 activity an *in vitro* and determine its cofactors and regulation.
- Investigate structure-function relationships in LHCSR3 by further site-directed and random mutagenesis.
- Determine the molecular basis for the *npq* mutants of *Chromochloris* and *Nannochloropsis*.
- Identify downstream targets of SOQ1 using biochemical and genetic approaches.
- Elucidate the mechanism of antenna qI in the *soq1* mutant.

- Refine the kinetic model for qE to include additional NPQ components and alternative electron transport pathways.
- Use the energy transfer model for PSII supercomplexes to generate testable hypotheses about sites and mechanisms of NPQ.

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Imaging the Dynamics of Photosynthetic Membrane Biogenesis, Regulation, and Repair

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Overall research goals: In plants and algae, a dynamic network of photosynthetic membranes forms inside chloroplasts, responds to changing light levels and spectral composition, regulates the balance of activities of the two photosystems, and repairs and replaces critical components as they are damaged by photo-oxidation. By integrating programs in plant genetics, biophysics, advanced imaging, ultrafast spectroscopy, engineering, and computational modeling, our goal is to obtain a detailed molecular description of the organization of the thylakoid membrane, the regulation of its composition, and the membrane dynamics occurring during its biogenesis, regulation and repair. We follow protein rearrangements in photosynthetic membranes using a range of cutting-edge imaging techniques, which are ideally suited to reveal the complex structural dynamics of the membrane at different levels of spatio-temporal resolution. The imaging approaches are integrated with parallel modeling and theoretical efforts, which will provide new insights into the fundamental biophysical mechanisms governing protein interactions and the partitioning of protein complexes in the photosynthetic membrane.

Significant achievements 2011-2013: Atomic force microscopy (AFM) was used to visualize photosystem II (PSII) supercomplexes in grana membranes from *Arabidopsis*. We developed a statistical image analysis pipeline to discriminate disordered from crystalline particles in an unbiased fashion and classify crystalline arrays according to their unit cell properties. We found evidence that interactions among light-harvesting antenna complexes are altered in the *soq1* mutant, which affects regulation of light harvesting. We acquired a high speed AFM (HS-AFM) that will allow us follow protein structural rearrangements under varying illumination in real time. Using a customized AFM with integrated fluorescence imaging, we directly characterized the dynamic changes in biophysical properties of the thylakoid membrane during a state transition. The elasticity (stiffness) of the thylakoid membranes increased immediately upon PSII-specific illumination, followed by a delayed height change. Electron microscopy was used to corroborate changes in membrane-to-membrane spacing. While the change in stiffness depended primarily on the transmembrane pH gradient, the height change required both a pH gradient and STN7-dependent phosphorylation. We are developing a novel near-field optical scanning microscopy that uses a cathodoluminescent film to combine the high spatial and temporal resolution of a scanning electron microscope with the non-invasiveness of light microscopy at length scales well below the diffraction limit. Fluorescence microscopy was used to identify *Chlamydomonas* mutants with altered formation or organization of the thylakoid network. Using a coarse-grained model of transmembrane alpha helices, we showed that hydrophobic mismatch between the membrane and the protein is the dominant factor determining transmembrane helix packing and the crossing-angle distribution of transmembrane helices.

Science objectives for 2013-2014:

- Use HS-AFM to record images of PSII supercomplex dynamics in thylakoid membranes.
- Obtain electron tomographic images of the altered PSII organization in the *soq1* mutant.
- Determine the effect of physical constraints on membrane remodelling during state transitions, using simultaneous AFM and pulse-amplitude-modulated fluorescence yield measurements.
- Develop photolithography and etching processes to manufacture free-standing membranes of our cathodoluminescent films.
- Use confocal fluorescence microscopy, electron microscopy, and electron tomography to investigate thylakoid membrane architecture in biogenesis mutants of *Chlamydomonas*, *Arabidopsis*, and maize.
- Implement an algorithm that allows for flow of lipid or protein material from the edges of a simulation box. Using this reservoir technique along with a dissipative particle dynamics algorithm that is at least 25 times faster than our previous approach, we can simulate lipid and protein flows in membrane systems that are as large as grana.

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Broad Light-dependent Redox Regulation of Protein Thiols in Cyanobacteria Revealed by Quantitative Site-Specific Proteomics Profiling

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Overall research goals: The central objective of this project is to determine the mechanisms of thylakoid lumen localized redox proteins and small molecules in photoprotection and repair of the photosynthetic apparatus. There are three specific aims in this proposal: (1) To develop a simple and rapid method for the isolation of functionally competent thylakoid membranes from cyanobacteria, followed by analysis of the proteome of the thylakoid lumen; (2) To identify and characterize glutathione transporters in cyanobacterial thylakoid membranes; and (3) To dissect the Redox Proteome of cyanobacterial thylakoid lumen.

Significant achievements 2012-2013: During photosynthesis, light is known to modulate the redox state of the photosynthetic electron transport chain. Redox regulation through cysteine oxidation modulates the activation or inactivation of enzymes linked to photosystems I and II, thus regulating various target processes. In this study, we have developed a novel proteomic approach for selective enrichment of oxidized thiols with quantitative isobaric labeling to quantify the extent of oxidation on individual thiols, as well as the redox dynamics of the thiol proteome under different conditions (light, dark, and in the presence of DCMU, a PSII inhibitor) in the cyanobacterium *Synechocystis* sp. PCC 6803.

In this study, the cells were cultured under continuous light, or shifted to darkness for 2 h, or exposed to DCMU for 2 h in light before harvesting. Cells were lysed and pelleted using 10% TCA. Free thiols were initially blocked with N-ethylmaleimide (NEM), and excess NEM was precipitated by cold acetone. The reversible oxidized cysteines (Cys) were reduced by DTT, and enrichment of Cys-proteins was carried out by using thiopropyl Sepharose resin. Following on-bead tryptic digestion and on-resin isobaric labeling for relative quantification using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

The proteomics results show consistent increases in the levels of cysteine thiol oxidation in the dark compared to the light condition. DCMU inhibition leads to further increase in the level of oxidation. In total, redox changes were observed in ~2600 peptides from ~1000 proteins, indicating the broad light-dependent redox regulation in this organism. Moreover, the level of thiol oxidation for many important proteins (such as thioredoxin, PSI and PSII reaction center proteins, and various oxidoreductases) and numerous other types of enzymes changed in response to the three different treatments. These results are consistent with earlier findings that specific electron carriers in the photosynthetic electron transport chain are increasingly oxidized during dark incubation than under light, and that disruption of the electron flow alters the redox state of the electron carriers. The observation of dynamic changes of redox states on individual cysteine residues provides important functional information as to their roles in redox regulation in photosynthesis and metabolism. In addition to establishing a new approach for quantifying redox dynamics, this work also provides novel information important to understand the redox biology in cyanobacteria.

Science objectives for 2013-2014:

- We have made significant progress in developing a simple method to isolate right-side out thylakoid membranes from the cyanobacteria *Synechocystis* 6803 and *Synechococcus* UTEX 2973. As described above, we have also generated an initial detailed global map of the redox proteome of *Synechocystis* 6803.
- During the next six months, we will refine both of these methods to generate reliable data sets for two publications next year.

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Chloroplast Retrograde Signaling During Development and High Light Stress

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Overall research goals:

Chloroplast signals regulate hundreds of nuclear genes during development and in response to stress. Such communication involves accumulaton of metabolites produced within plastids, but the identities of these molecules, what regulates their production, and the signaling pathways are mostly unknown. One type of retrograde signal is used by plastids as they develop into chloroplasts in emerging leaves. When chloroplast biogenesis is blocked in young seedlings using plastid-specific translation inhibitors or mutants, the nucleus responds by greatly reducing the expression of hundreds of genes involved in photosynthesis or other aspects of chloroplast function. In earlier years of this grant, we performed a genetic screen in Arabidopsis and identified alleles of 7 *gun* (genomes uncoupled) genes that are needed to communicate with the nucleus during chloroplast development. Six of these genes implicate the chloroplast tetrapyrrole biosynthetic pathway, in particular enzymes around the branch point between heme and Chl biosynthesis, as a source of retrograde signals (Fig 1, *gun 2-6*).

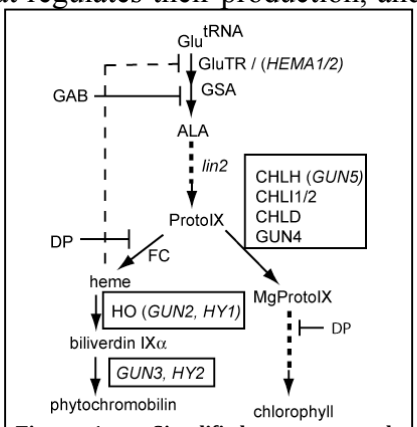

Fig. 1: Simplified tetrapyrpole biosynthesis pathway. Genes identified during gun mutant screens are boxed.

Reduced function of GUN genes (or overexpression of *GUN6*) leads to misexpression of photosynthesis-associated nuclear genes (*PhANGs*) when chloroplast development is blocked. We showed that increased flux through the Ferrochelatase I (FC1) branch of the heme pathway increases *PhANG* expression. The second ferrochelatase, FC2, co-localizes with FC1, but increased FC2 activity is unable to increase *PhANG* expression in undeveloped plastids. These data suggest a model where heme, specifically produced by FC1, may be used as a retrograde signal to coordinate *PhANG* expression with chloroplast development, suggesting that heme is a positive signal that coordinates nuclear and chloroplast gene expression when the plastid is functioning. In contrast, accumulation of Mg-ProtoIX or other tetrapyrrole intermediates may indicate that the plastid is damaged. These results demonstrate the importance of the tetrapyrrole pathway during chloroplast development (Woodson et al., 2011, *Curr Biol.*).

Chloroplasts are also sensory organelles that perceive abiotic stresses such as excess light. Several studies have implicated the accumulation of metabolites, singlet oxygen or hydrogen peroxide in response to excess light. We performed RNAseq studies under short periods of high light stress and identified a set of candidate regulatory elements enriched in promoters of genes induced by high light stress. Among these were heat shock elements, and other known light-regulatory elements. T-DNA insertion lines and overexpression studies allowed us to identify a subset of three heat shock transcription factors (of the greater than 20 predicted in the *Arabidopsis* genome) in the early response to excess light stress to coordinate gene expression (Jung et al., 2013).

Significant achievements 2011-2013:

- GUN1: GUN1 contains 10 PPRs and a second domain called the SMR (small MutS-related) domain. In bacteria, the SMR domain has endonuclease activity. We generated recombinant GUN1 protein and mutant proteins and showed that GUN1 possesses nuclease activity. In

plants, this nuclease activity is required for its function. Using whole genome RNA-sequencing analysis and genetic epistasis studies, we concluded that GUN1 acts in the same pathway as GUN2-6. This pathway may be distinct from stress-induced plastid signaling pathways.

- Forward and reverse genetic studies to identify regulatory circuits controlling high light stress: A second signaling pathway mediates signals derived from the reduction/oxidation (redox) of the photosynthetic electron transfer chain (PET) and affects both photosynthesis-related and stress-related gene expression. Several studies have provided evidence for a role of the redox state of the PQ pool as a sensor of EL and a regulator of nuclear gene expression. A screen for mutants that mis-express the *ELIP2* promoter helped us to identify mutations in 4 genes involved in light stress; these include: A putative protein with a G-patch domain (RNA-binding), a unique allele of the LOS4 gene involved in temperature stress; cryptochrome -1; and a transcriptional co-activator. Characterization of the molecular phenotypes of these mutants implicated alternative splicing of hundreds of genes in response to excess light. We are trying to order the genes into a network.
- To better understand why heme made by ferrochelatase 2 cannot rescue an *fc1* mutant, we identified T-DNA insertions into the *fc1* and *fc2* genes. Surprisingly, *fc2* mutants die when they encounter light; death is accelerated when nights are longer. We identified 25 suppressors of the "death" phenotype of *fc2;* current studies aim to understand what these genes are.

Science objectives for 2013-2014:

- We want to know the precise function of GUN1, and are following up on several leads to identify the nucleic acid or protein bound by the PPR domain of GUN1.
- We are attempting to identify the 2 proposed pools of heme, by purifying FC1 and FC2 complexes from plants. We have some candidates for possible components of the complexes.
- We will continue to clone the genes defined by the *fc2* suppressors.
- Follow-up studies on the 7 new genes found in the high light screen are underway.

The successful completion of the proposed projects should allow us to begin to engineer these crucial pathways. Since heat, cold, and high light stress share at least some common signaling elements, generation of new genotypes will ultimately influence our abilities to manipulate plant growth and development, and will aid in the understanding of the developmental control of photosynthesis.

References to work supported by this project 2011-2013:

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Systems level analysis of chloroplast iron homeostasis

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Overall research goals: Photosynthetic metabolism and output from the photosynthetic apparatus is dependent on resource (CO_2, light, mineral nutrients) availability and is also tightly connected to respiratory function. Iron is one nutritional resource that limits bioenergetics capacity and hence global productivity. We aim to understand iron-nutrition dependent programming of chloroplast and mitochondrial metabolism in photosynthetic eukaryotes. In this context, we use *Chlamydomonas reinhardtii* reference organism for discovering and understanding the regulatory circuits that connect carbon source, photon flux density and inorganic nutrients to the operation of the light and dark reactions of photosynthesis.

Significant achievements 2010-2013: Using the expression of genes for iron assimilation pathways as sentinels of iron status coupled with performance of the photosynthetic apparatus and biomass production, we established four operational stages of iron nutrition: iron-limited (< 0.5 μM iron chelate in the medium), iron-deficient (1-3 μM), iron-replete (20 μM) and iron-excess (> 50 μM). We used RNA-Seq and quantitative label-free proteomics methodologies to compare the transcriptomes and proteomes of iron-replete cells to those of iron-deficient and iron-limited cells, leading to the discovery of many new targets of nutritional iron-signalling, including some novel and conserved (in the plant lineage) responses. In addition, we documented a mechanism that maintains the function of the photosynthetic apparatus in iron-limited CO_2-grown cells but not in iron-limited acetate-grown cells. Besides the previously-described iron homeostasis factors, the responses to poor iron nutrition that are conserved across the plant lineage are: up-regulation of anti-oxidant pathways to counter enhanced production of reactive oxygen species arising from compromised function of the iron-containing electron transfer complexes, a novel organelle-localized protein (CGLD27) and other uncharacterized proteins, including one with a Kelch-repeat domain. Up-regulation of plastid anti-oxidant pathways includes enhanced accumulation (10-fold greater) of ascorbate (via both de novo synthesis and recycling), Vitamin E and superoxide dismutase activity (via maintenance of FeSOD and increased expression of a novel plastid-localized MnSOD3). Maintenance of FeSOD occurs by intracellular iron-recycling.

Science objectives for 2013-2014: We are now proposing to monitor the occupancy by iron of various iron proteins by combining quantitative proteomics with elemental profiling to understand the mechanisms that operate to distribute iron within the cell. We are also monitoring the transition from CO_2 to acetate to understand how nutritional iron signaling is integrated with carbon metabolism and we are using reverse genetics to understand the function of iron-nutrition responsive proteins that are conserved in the green lineage.

Poster Session I

Energy Transfer and Radiationless Decay in Light-Harvesting Proteins

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Overall research goals: This project employs two-dimensional (2D) electronic spectroscopy and transient-grating spectroscopy with optical heterodyne detection to determine the structural and physical mechanisms that control and optimize the excitation energy transfer channels involved in photosynthetic light-harvesting. The planned work will focus on the structurally well-characterized peridinin–chlorophyll *a* protein (PCP). The overall research plan will compare the photophysics of peridinin in solution with that of the PCP complex in order to distinguish the effects of the protein/chromophore environment on excited-state motions and nonradiative decay mechanisms.

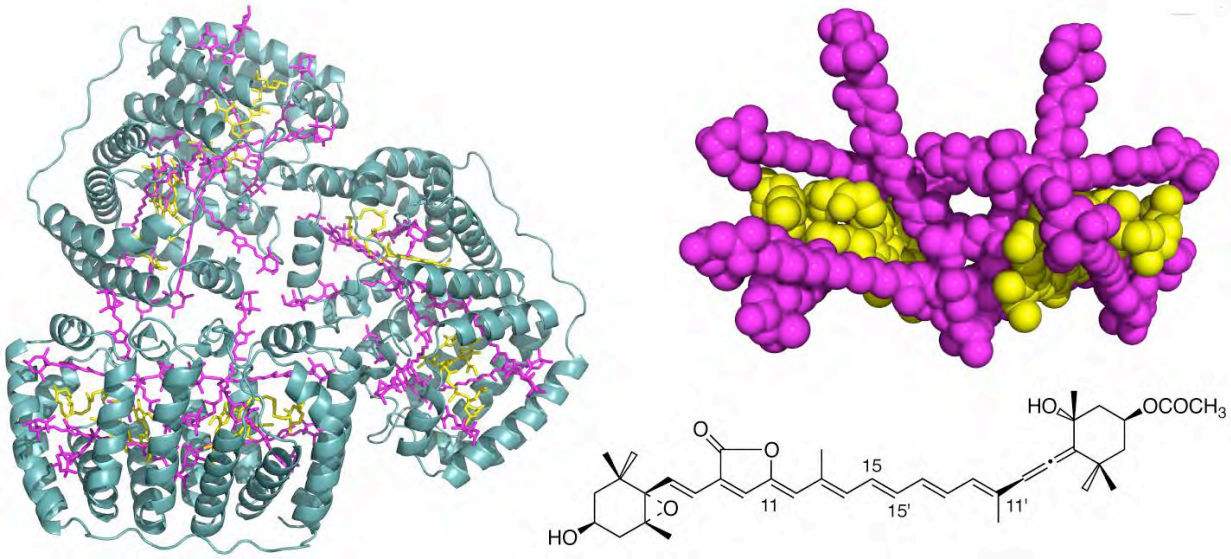

Figure 1. *Left:* Trimeric complex of the peridinin–chlorophyll *a* protein from *Amphidinium carterae* (1PPR.pdb). *Right:* Space-filling rendering of the chromophores in a single subunit: peridinin (magenta) and chlorophyll *a* (yellow). *Lower right:* structure of peridinin.

Significant achievements in preliminary work: Femtosecond transient-grating spectroscopy with optical heterodyne detection was employed to observe separately the time evolution of the absorption and dispersion components of the third-order nonlinear optical signal following resonant excitation of the S_2 ($1B_u^+$) states of β-carotene in benzonitrile and peridinin in methanol using 40-fs pulses centered at 520 nm. The absorption and dispersion components exhibit distinctively different time profiles owing to the population of intermediate states. An initial intermediate state is populated on an ultrashort (<30 fs) time scale in both carotenoids; judging from the rise time of a long-lived (~10 ps) S_1 ($2A_g^-$) state absorption band, the intermediate state decays to form the S_1 ($2A_g^-$) state in 142 fs for β-carotene and in 615 fs for peridinin. Owing to the fast red-shifting of the stimulated emission part of the S_2-state transient grating signal, we suggest that the intermediate state arises from vibrational displacements on the S_2-state potential surface that eventually yield twisted or bent conformations. Motions of the molecule of this type in the S_2-state would contribute to a mixing of the diabatic S_2 and S_1 electronic states and would impact the efficiency of energy

transfer from the S_1 state to the (B)Chl Q_y state in photosynthetic light-harvesting proteins. The time-resolved transient-grating spectra obtained for PCP complexes suggest a more rapid formation of the intermediate than for peridinin in methanol. This finding suggests that the conformation of the peridinin chromophore is controlled in the binding site to optimize the formation of the intermediate upon excitation of the S_2 state.

Science objectives for 2013–2014:

- The intramolecular charge-transfer (ICT) character of the intermediate state formed from the S_2 state of peridinin in a range of solvents will be assessed in 2D and transient-grating experiments. This objective will help us determine the nature of the ICT state and the mechanism of its formation.
- Broadband 2D spectra will be acquired from the PCP complex in order to observe the states that mediate energy transfer from peridinin to Chl *a*.

Investigation into interaction of methanol with the Oxygen Evolving Complex of Photosystem II: Implications for identity of catalytically relevant waters.

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Methanol and other small primary alcohols are thought of as substrate analogs for Photosystem II (PSII), the water splitting enzyme in cyanobacteria and higher plants, yet it does not inhibit oxygen evolution by more than 10% even up to a concentration of 5 M (1). To evaluate probable sites for methanol binding to the oxygen-evolving complex (OEC), we utilized pulse electron paramagnetic resonance methods (EPR) to measure couplings from ^{13}C-labeled methanol to the Mn_4 cluster poised in the S_2 state. These results are compared to those from an analogous study of (2-OH-3,5-Cl_2-SALPN)$_2$Mn(III)Mn(IV), a dimanganese model complex that coordinates methanol via the Mn(III) ion. While the SALPN complex gives rise to a strong ^{13}C hyperfine interaction (HFI) indicative of direct metal-coordination, the corresponding ^{13}C HFI for PSII-bound methanol is quite small. A 3-dimensional isosurface plot drawn around the OEC using coordinates from the recent high resolution crystal structure of PSII (2) and magnetic projection factors derived from other EPR and computational results describes all possible positions that the methyl carbon of methanol can exist and give rise to the observed ^{13}C HFI. These results suggest that methanol does not bind directly to a Mn ion of the OEC, and instead displaces a water bound to calcium or occupies a second coordination sphere site. If methanol displaces a Ca-bound water, it seems likely that that water is not the source of one of the oxygens that participates in O-O bond formation.

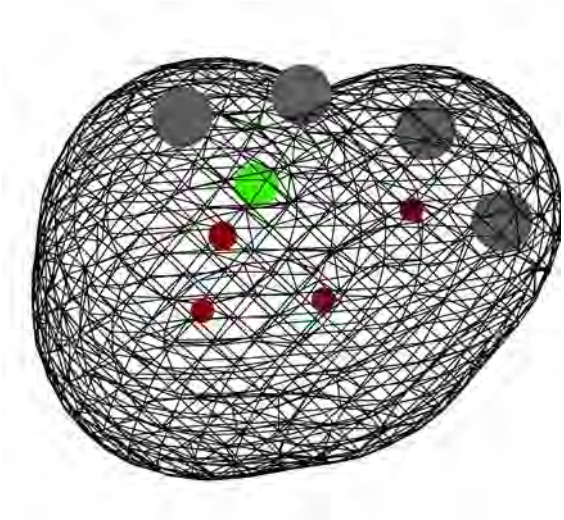

Figure 1: Three-dimensional contour map of potential ^{13}C coordinates based on observed ^{13}C couplings extracted from Q-band ^{13}C Mims electron nuclear double resonance (ENDOR) and X-band hyperfine sub-level correlation (HYSCORE) of the S_2 state of PSII in the presence of 1.2 M ^{13}C MeOH. Magenta spheres represent Mn ions, green sphere represents Ca, and grey spheres represent spherical distribution of 1.4 Å about nuclear coordinates of the oxygen atoms for bound waters from pdb file 3ARC (2).

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Investigations into the pfkB family of proteins affecting chloroplast function

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Overall Research Goals: The long-term research goals are to understand the biological function of the pfkB family of putative kinases by: 1) studying the effects of loss-of-function mutants in individual members and in multiple mutants; 2) develop an assay to test the biochemical function of a chloroplast localized family member; and to 3) focus on the role of FLN proteins.

Significant Achievements 2011-2013: John Riggs attended a workshop at the University of FL-Gainesville on using comparative genomic analyses to identify gene function. This method primarily uses homology to bacterial proteins and their operon structure to develop hypotheses for function in higher plants. pfkB proteins are amenable to this type of analyses because they have similarity to bacterial proteins. From this analysis 23 pfkB proteins in *Arabidopsis thaliana* were identified (Figure 1); 12 of these proteins are completely uncharacterized; 2 additional members are the FLN1 and FLN2 proteins under study in the laboratory (Gilkerson et al., 2012). With the knowledge that we gained from bioinformatic analyses, we generated hypotheses for one family member. One, At1g17160, shares homology with bacterial ribokinases. We can express this protein in bacteria and are setting up for activity assays to determine if it has ribokinase activity. The second approach to elucidate function is to analyze loss of function mutants. We made considerable progress, focusing on the 11/12 uncharacterized pfkB-encoding genes (one is understudy in another laboratory) for which we isolated cDNAs and T-DNA insertion mutants. Through analysis of cDNAs, we discovered that At1g50390.1 is likely not a functional gene. Of the remaining 10, only 2 do not have T-DNA insertions: creation of RNAi lines for these is in progress. All T-DNA insertion lines are viable and we have begun crosses to isolate multimers (see Table 1).

Regarding our analysis of FLN1/2 function, proteins found in transcriptional complexes, we have discovered that recombinant FLN2 protein forms homomers, FLN1 never forms heteromers under our conditions and that FLN1/FLN2 heteromers can be detected under specific conditions in *in vitro* pull-down assays (Figure 2). We are currently working to determine whether the conditional heteromer formation occurs *in vivo*. We hypothesize that these dynamic changes are important for the transcription from PEP-dependent promoters.

Objectives for 2011-2012: 1. Complete characterization of T-DNA insertions for uncharacterized pfkB to identify null alleles for subsequent studies and perform phenotypic analyses; 2) generate multiple mutants of loss of function mutants in pfkB members to determine if members have redundant functions; 3) analyze in detail At1g17160 to determine if protein has ribokinase activity. Regarding our focus on the FLN proteins our goals are to: 1) determine which FLN protein interacts with which TAC protein; 2) determine whether ATP analogs affect heteromerization; 3) further develop our model of regulated TAC transcription dependent on FLN proteins using biochemical studies.

References supported by DOE:

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Hotton SK, Castro MF, Eigenheer RA, Callis J (2012) Recovery of DDB1a (DAMAGED DNA BINDING PROTEIN1a) in a Screen to Identify Novel RUB-Modified Proteins in Arabidopsis thaliana. Molecular Plant 5: 1163-1166.

Figure 1. Phylogenetic Analysis of Uncharacterized Arabidopsis pfkB Proteins

PfkB proteins in *Arabidopsis thaliana* were identified by reiterative BLAST searches. Predicted transit peptide (ChloroP) were removed, proteins aligned with CLUSTAL and a tree made and bootstrapped in PAUP. Several subgroups can be identified, which will be analyzed with LOF mutants (see Table 1).

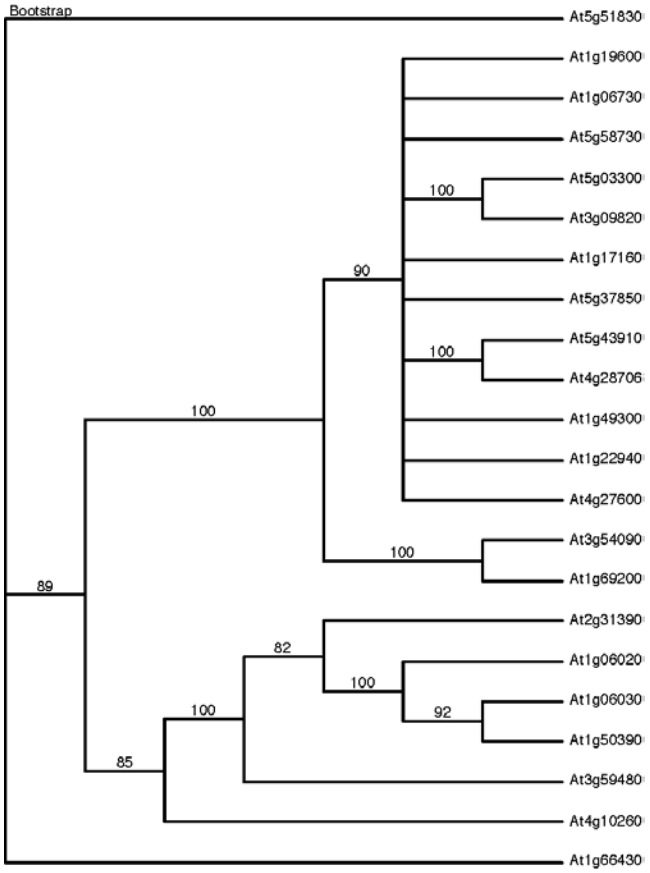

Table 1. Callis lab data of uncharacterized pfkB proteins + FLNs

gene	hmz T-DNA?	# of indep. hmz	WT mRNA in mutant?	localization	comments
At1g06020.1	yes	3	no	cytosol	
At1g06030.1	no		N/A		
At1g06730.1	yes	1, 1 IP	no	plastid	
At1g17160.1	yes	1, 1 IP	no	plastid	
At1g49350.1	yes	2		cytosol	
At1g50390.1	No	N/A		N/A	not functional
At1g69200.1	yes	3	no	plastid	FLN2
At3g54090.1	yes	1	no	plastid	FLN1
At3g59480.1	yes	1		cytosol	
At4g10260.1	no			cytosol	
At4g28706.2	yes	2			
At5g19150.1					
At5g43910.2	yes	2	no for 1.	cytosol	

IP, in progress;

N/A, not applicable

Figure 2 GST pull-down assay.

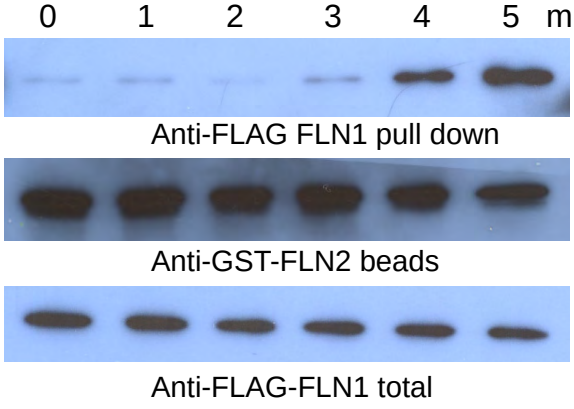

Figure 2. Bead bound GST-FLN2 was incubated with soluble FLAG-tagged FLN1 at different ATP concentrations. (top) FLAG-FLN1 interacting with GST-FLN2 in pull-down; (middle) control showing equal GST-FLN2 in reactions and; 3) (bottom) showing same amount of soluble FLAG-FLN2 in reaction prior to incubation and pull-down assay.

Protein Transport across Thylakoids: The Translocon of the Twin Arginine Transport Pathway

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Overall research goals: The research objectives are to study the mechanisms of protein transport into the lumen of thylakoids by the chloroplast Twin Arginine Transport (cpTat) pathway by: (1) identifying the cpTat component(s) that interact with the mature domain of the precursor during transport; (2) determine the organization of the cpTat translocon; and (3) compare topology of cpTat component, Tha4 in thylakoids during active transport and at rest. This particular protein transport pathway is predicted to translocate ~50% of the lumen proteins. Understanding cpTat system mechanism in chloroplasts will lead to a better understanding of the biogenesis of photosynthetic membranes potentially providing a means to engineer photosynthetic complexes into synthetic membranes for energy production.

Significant achievements 2012-20113: Proteins destined for the thylakoid lumen of chloroplasts are nuclear-encoded, synthesized in the cytoplasm, and must cross three membranes *en route* to their final destination. The chloroplast Twin Arginine Translocation (cpTat) system facilitates transport of about half of all proteins that cross the thylakoid membrane in chloroplasts. Known mechanistic features of the cpTat system are drastically different from other known translocation systems, notably in its formation of a transient complex to transport fully folded proteins utilizing only the protonmotive force for energy. However, key details such as the structure and composition of the translocation pore are still unknown. One of the three transmembrane cpTat components, Tha4, is thought to function as the pore by forming an oligomer. Yet, little is known about the basic topology of Tha4 in thylakoid, and little work has been done to detect precursor-Tha4 interactions, which are expected if Tha4 is the pore. First, we present topology studies of Tha4 via cysteine accessibility to membrane permeant and impermeant labels (Figure 1). N-terminal cysteine is not accessible to membrane impermeant labels but is accessible to membrane permeant labels. In addition, cysteine placed throughout the predicted amphipathic helix and carboxy tail region is susceptible to labelling with both kinds of labels. Taken together these data support a N_{out}-C_{in} topology for Tha4 in the thylakoid membranes. Second, we have the first evidence of interaction of the mature domain of precursor with Tha4 (Figure 2). Using disulfide exchange we demonstrate that the mature domain of the precursor interacts specifically with the amphipathic helix of Tha4, suggesting a role for Tha4 as the precursor conduit. These data provide the first evidence that Tha4 participates directly in transport of precursor on the cpTat pathway (Figure 3).

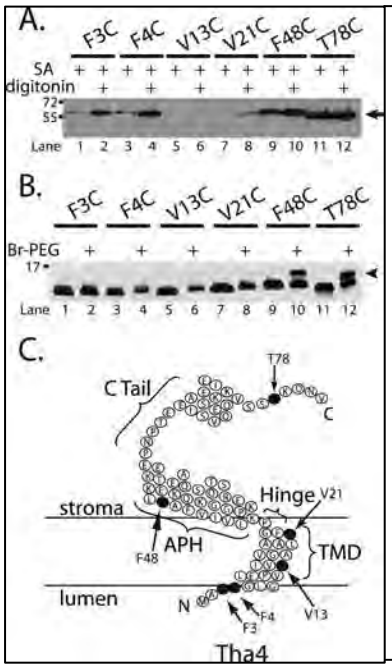

Figure 1. cpTat component, Tha4, exhibits a N_{out}-C_{in} topology in the absence of transport. Prior to detergent solubilisation (digitonin), the membrane impermeant streptavidin (+SA) cannot access cysteines near the N terminus of Tha4 (F3C, F4C), but have access to cysteines placed in the stromal domain (F48C, T78C). Neither membrane impermeant nor permeant (biocytin) molecules can bind cysteines in the apolar transmembrane region. (A) +/- streptavidin treatment of thylakoids containing [^{3}H]Tha4 after a biocytin pre-treatment and before or after membrane solubilization with the detergent digitonin. SA could not label N-terminal cysteine (F3C, F4C) prior to membrane solubilisation, whereas APH or C-terminal cysteines were labelled even prior to solubilization. The arrow indicates the presence of SA labelling of Tha4 via bound biocytin. (B) Thylakoids containing [^{3}H]Tha4 were treated with branched-PEG (br-PEG). The br-PEG contains a maleimide that can react with stromal exposed thiols. Only residues in the stroma (F48C, T78C) are labelled by br-PEG resulting in a gel shift (arrowhead). (C) location of substituted cysteines on Tha4.

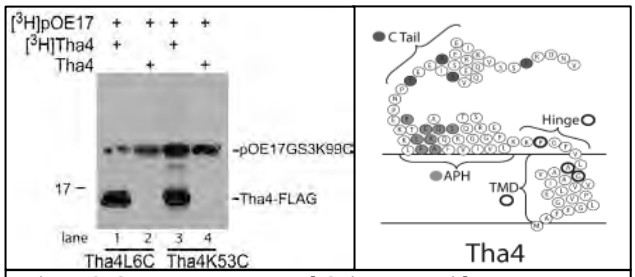

Figure 2. CpTat precursor, pPsbQ, interacts with cpTat component Tha4 in a cysteine-dependent manner. Affinity purification of PsbQ precursor (p) containing a cys-substitution at position K99 and a His-Tag linked through a $(GGGGS)_3$ polylinker. (*left panel*) Post transport for 5 min, thylakoids were subject to affinity precipitation of the FLAG-tagged Tha4 and disulfide bound partners. [^{3}H]Tha4L6C-FLAG or [^{3}H]Tha4K53C-FLAG affinity precipitated precursor. (*right panel*) Cartoon diagram of the predicted topology of Tha4 showing Cys-sustituted amino acids that interact with precursor. Open circles represent Cys placed in the TMD or Hinge; light gray circles the APH; and dark gray circles the C tail.

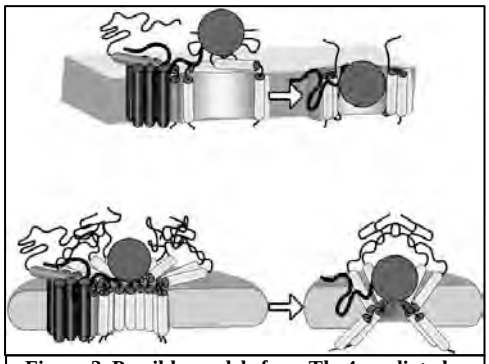

Figure 3. Possible models for a Tha4-mediated precursor translocation point of passage. Twin arginine translocation: Tha4 in light gray, Hcf106 in dark gray, and cpTatC in black. The precursor is the sphere. The top panel shows a channel model of Tha4 exhibiting conformational change such that the APH lines the channel. The bottom panel shows an alternative model whereby the overall topology of Tha4 does not change. Our data supports the latter model.

Science objectives for 2014-2015:

- While we can detect direct interactions between Tha4 and mature domain of protein, we do not know when the interaction occurs. Is the interaction prior to transport, during transport, or post transport? Further investigations with modified assays will allow us to investigate the nature and timing of the interaction.
- We are currently investigating the cpTat translocon by identifying the points of interaction between cpTatC and Hcf106 using a crosslinking.
- Structural studies on bacterial homologs of Tha4 have yielded limited functional information. Preliminary evidence in the lab suggests Tha4 responds to the presence of a protonmotive force. We are currently using EPR spectrometry to probe the environment and topology of the cpTat components, Tha4 and Hcf106, in model membranes. We are also currently investigating the membrane activity of Hcf106 using solid-state NMR.

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Resolving protein-semiquinone interactions by advanced EPR spectroscopy: The Q_A and Q_B sites of the bacterial reaction center.

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

Significant achievements 2012-2013:

The interaction of SQ_B with H-bond donors in the S(L223)A mutant of the reaction center. An interesting feature of the Q_B site in bacterial RC, suggested in crystallographic structures, is the change in configuration of S-L223 on reduction of Q_B to the SQ. In order to obtain data for independent confirmation for formation of the H-bond with S-L223 and to characterize the influence of this H-bond on the hyperfine (*hfi*) interactions with protons and nitrogens around the SQ_B we have performed a comparative study of the wild-type RC and its S(L223)A mutant. 2D ^{1}H,$^{14,15}N$ ESEEM and ^{1}H ENDOR have unambiguously provided similar *hfi* couplings for three types of H-bonded protons, and nitrogens in both proteins. These data define the H-bond network between the SQ_B and potential donors, H-L190, S-L223, I-L224 and G-L225, inferred from X-ray structure, and allow critical analysis of unpaired spin density (USD) distribution over and around the SQ_B in the $Q_AQ_B^-$ state. QM/MM calculations performed with three models of Q_B site ((1) the OH of S-L223 is H-bonded with the SQ_B O1 atom; (2) the OH of S-L223 is rotated away from the SQ_B towards other possible H-bond acceptor D-L213; (3) S(L223)A mutant) show weak variation of hfi couplings with protons and nitrogens of H-bonds and less asymmetric distribution of USD over the SQ than seen for the SQ_A. However, quantitative comparison of calculated methyl ^{1}H, and carbonyl ^{13}C and ^{17}O *hfi* couplings with available experimental data indicates significantly better agreement for the model without H-bond with S-L223. In this model N_δ of H-L190 and peptide N_p of G-L225 form H-bonds with the SQ_B, and N_p of I-L224 is coupled very weakly with the SQ_B. Ser-L223 is likely to be H-bonded to the carboxylate group of Asp-L213.

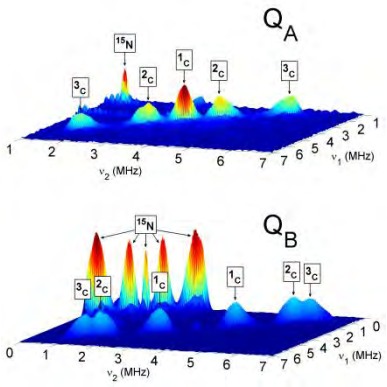
Formation of fully quinol in the Q_B site requires uptake of two H^+ ions. Results of this study indicate that both are delivered to the quinone after the second flash:

2^{nd} flash:

$$Q_A\,Q_B^- \xrightarrow{hv} Q_A^-Q_B^- \overset{H^+}{\longleftrightarrow} Q_A^-Q_BH \longrightarrow Q_A\,Q_BH^- \overset{H^+}{\longleftrightarrow} Q_A\,Q_BH_2$$

Ser-L223 functions as the donor of the first proton, to the O1 atom of Q_B^-, generating a transient neutral semiquinone, Q_BH, which accepts a second electron from Q_A^- to form Q_BH^-. The second proton is then delivered to the O4 atom, via Glu-L212.

Methoxy orientation modulates the redox potential of ubiquinone in reaction centers. The methoxy group dihedral angle has been suggested to have a strong influence on the redox midpoint potential (E_m) of ubiquinones. To address this we carried out 2D ESEEM studies of the SQ_A and SQ_B in RCs where natural UQ_{10} was extracted and was replaced with the (^{13}C-methyl, methoxy)UQ_8. ^{13}C 2D ESEEM spectra are shown in Figure. In each case three ^{13}C features attributable to CH_3 and two OCH_3 are observed, giving us ^{13}C *hfi* tensors for these groups. Comparison of experimental isotropic hfi constants, a_{iso}, of the 2-methoxy groups in Q_A (1.4 MHz) and Q_B (5.7 MHz) with QM calculated a_{iso} values as a function of the



2-methoxy dihedral angle θ defines four possible combinations for the dihedral angle θ ($C_mO_mC_2C_1$) in the two SQs. The angles determined were then compared to the computed relationship between the dihedral angle and the resulting electron affinity. X-ray structures support dihedral angle difference $\Delta\theta = -80^o$ corresponding to a redox potential gap (ΔE_m) between Q_A and Q_B of 180 mV that is significantly larger than the experimental difference in redox potentials (60−75 mV) of the two quinones. Additional confirmation of the larger contribution to the E_m gap between Q_A and Q_B comes from mutants of the Q_A site that lower the E_m of Q_A. Mutation of isoleucine M265 to threonine (mutant M265IT) decreases the E_m of Q_A by 100-120 mV, substantially increasing ΔE_m, the driving force for electron transfer from Q_A^- to Q_B. In this and similarly polar mutants 3-MeO-Q is completely inactive as Q_B. Taking into account the 60-75 mV favorable ΔE_m for ubiquinone in wild type RCs, the failure of 3-MeO-Q in M265IT mutant RCs indicates that its E_m in the Q_B site is more than 160-195 mV lower than that of ubiquinone. This is consistent with a contribution of ≥180 mV from a correctly oriented 2-methoxy group. These data clearly indicate a role for the 2-methoxy group in setting the functional redox potential gap between Q_A and Q_B, and the 2D ESEEM and computational analysis show that this effect is implemented through different dihedral angles for Q_A and Q_B. These are presumably determined by interactions with the environment of the binding sites.

Science objectives for 2013-2014:

- ^{13}C couplings provide insight into the SQ binding to the protein. We will apply a biochemical approach for selective ^{13}C labeling of ring carbons in the SQ_H. The quinones biochemically labeled in bo_3 enzyme will also be used in studies of bacterial reaction center and bc_1 complex.
- We will exploit Q-band ^1H and ^2H ENDOR to address specific questions about the *orientation* of the H-bonds around SQ in Q_B and Q_H sites based on the *hfi* (and *nqi*) tensors of exchangeable protons (deuterons).

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Epigenetic Regulation of Hormone-Dependent Plant Growth Processes

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Overall research goals:

Energy capture by plant photosynthesis is one of the most essential processes in nature. Proper function of the photosynthetic pathway and its chemical outcome in a given plant species is determined by its genome. The numbers of genes which encode for light-harvesting proteins, for example, differ among plant species. Beside this genetic pre-determination, the environment plays a fundamental role in regulating photosynthesis. Since plants are exposed to a mélange of continuously changing environmental cues throughout their entire life, a multitude of different signals have to be constantly integrated into the photosynthesis pathway to ensure its appropriate function. The underlying information of the chromatin structure serves here as a framework which equips the plant with an adequate molecular repertoire to respond to its environment.

The question of how this is mechanistically achieved on the chromatin level will be pursued in our lab. The ethylene signaling pathway will be employed as our experimental test system. The processes of ethylene perception and signal transduction resulting in an EIN3/EIL1-mediated initiation of transcription are well studied (Chang et al., 2013; Liu and Wen, 2012; Qiao et al., 2012). However, the chromatin environment which ultimately enables the appropriate transcriptional response is poorly understood. Our identification of EIN6 (ETHYLENE INSENSITIVITY6) and EEN (EIN6 ENHANCER) as chromatin-modifying enzymes strongly emphasizes the importance of chromatin dynamics in ethylene signaling and will shed light on the functions of chromatin architecture in general and also its role in plant growth and adaption to fluctuating environmental stimuli in particular.

Significant achievements 2010-2013:

The gaseous plant hormone ethylene regulates a multitude of growth and developmental processes. How the numerous growth control pathways are coordinated by the ethylene transcriptional response remains elusive.

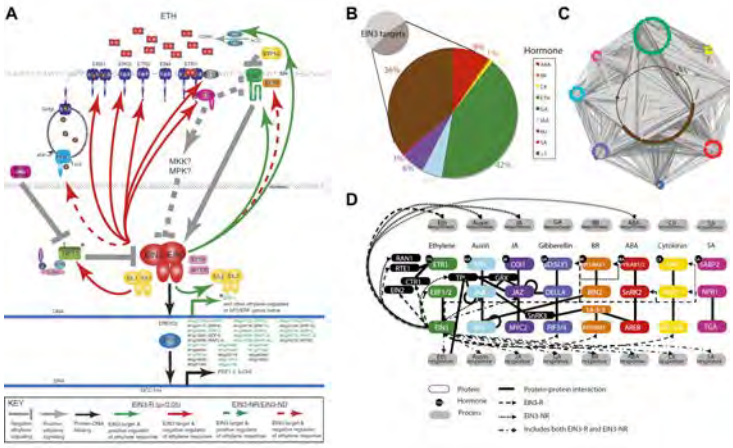

Figure 1. **Functional classification of EIN3 candidate targets reveals genes involved in hormone responses.**
(A) Feed-back (ethylene signaling components, above) of the ethylene response and feed-forward (downstream effectors, below). Downstream effectors in green are transcriptionally induced by ethylene. Known EIN3 targets are noted by asterisks; all other EIN3 candidate targets were discovered by this study. **(B, C)** EIN3 candidate targets are involved in hormone co-regulation. Node color represents hormone annotation, as

indicated in B; large nodes are EIN3 candidate targets. Dark grey edges represent protein-protein interactions (PPI) and light grey edges are protein-DNA interactions (PDI). Hormone annotation legend: abscisic acid (ABA), brassinosteroid (BR), cytokinin (CK), ethylene (ETH), gibberellin (GA), auxin (IAA), methyl jasmonate (MJ), salicylic acid (SA), >1, more than one hormone. **(D)** EIN3-mediated ethylene co-regulation occurs at many different levels. PPIs are from the Arabidopsis Interactome Mapping Consortium, and EIN3 PDIs are from this study.

We have characterized the dynamic ethylene transcriptional response by identifying targets of the master regulator of the ethylene signaling pathway, ETHYLENE INSENSITIVE3 (EIN3), using chromatin-immunoprecipitation sequencing and transcript sequencing during a high resolution time-course of ethylene treatment (Chang et al. 2013). In brief, ethylene-induced transcription occurs in temporal waves regulated by EIN3, suggesting distinct layers of transcriptional control. EIN3 binding was found to modulate a multitude of downstream transcriptional cascades, including a major feedback regulatory circuitry of the ethylene signaling pathway, as well as integrating numerous connections between most of the hormone mediated growth response pathways (Fig. 1). These findings provide direct evidence linking each of the major plant growth and development networks in novel ways.

Science objectives for 2013-2014:

We plan to carry out a detailed functional characterization of *EIN6* and *EEN* to ultimately address the question of how both regulators can shape the chromatin environment in an ethylene-dependent manner. A series of genomic, genetic and proteomic approaches will be carried out. Specifically, to gain insight into the pivotal interplay between EIN6 and EEN, we will conduct a survey of genome-wide chromatin features in response to ethylene using ChIP-seq analyses in *ein6*, *een* and *ein6een* mutants. Additional experimental approaches will be provided through mass spectrometry techniques to identify the entire protein complexes where EIN6 and EEN are part of. Our combination of genomics with proteomics will yield unprecedented insights into the functions of chromatin modifying enzymes and the mechanisms of how the plant hormone ethylene can influence their activities. It will significantly increase our understanding of how plants integrate changing environmental cues into their chromatin environment which is of fundamental importance for plant-based energy research.

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Construction and Evaluation of Well-Defined Mimics of Photosynthetic Light Harvesting Systems

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Overall research goals: This program seeks to understand and model the fundamental underpinnings of natural photosynthetic systems through the construction of well-defined and tailorable experimental systems. The central approach involves the attachment of a variety of synthetic chromophores to specific sites on self-assembled protein scaffolds through conformationally constrained linkages. Using this approach, the distances and angles between the individual chromophores in the arrays can be varied systematically. A key advantage of the synthetic approach is its ability to compare isolated chromophores on protein surfaces to those that are present in extended arrays. This can allow the effects of the protein environment to be studied separately from electronic coupling behavior. New time-resolved spectroscopy techniques are being applied to study exciton transfer in these systems, as are new computational methods to model the effects of conformational fluctuations on the systems. It is anticipated that the models and approaches developed through this program will inform the design of new artificial systems, and elucidate some of the features responsible for the high quantum efficiencies exhibited in natural photosynthesis.

Significant achievements in FY 2013: In our initial studies, we have elaborated a new self-assembling scaffold based on a circularly permuted tobacco mosaic virus capsid protein (cpTMV). This new platform affords excellent yields (>100 mg/L) of highly stable C_2-symmetric disks, and we have further developed the chemical techniques to attach virtually any synthetic chromophore to this structure (at position 123, Figure 1). In parallel, we have developed several new synthetic routes to access porphyrin, phthalocyanine, rhodamine, and coumarin chromophores with protein bioconjugation handles and rigid linking groups. We have successfully attached these pigments to the TMV assemblies, and we are currently evaluating their spectroscopic properties using fluorescence anisotropy and transient absorption measurements. We have confirmed that the fully decorated disks exhibit energy transfer among the dyes by observing a drastically decreased exciton lifetimes. We have also discovered a previously unknown ultrafast decay time component that is currently under investigation.

Science objectives for FY 2014-2015:

- One of the most important objectives during the next year is the comparison of isolated chromophores on cpTMV surfaces to fully-substituted chromophore rings that have the potential for electronic coupling. This is anticipated to provide a unique new opportunity to explore the effects of the protein environment on chromophore behavior and energy transfer. We have already noted that the dye properties change significantly when in contact with the protein, setting the stage for upcoming studies of these effects.
- We are currently preparing additional cpTMV mutants that allow chromophore attachment in different locations. This will allow the distances between the pigments to be changed systematically. In combination with our continued synthetic efforts for the preparation of new dye molecules, an unprecedented library of synthetic light harvesting systems will soon be available.

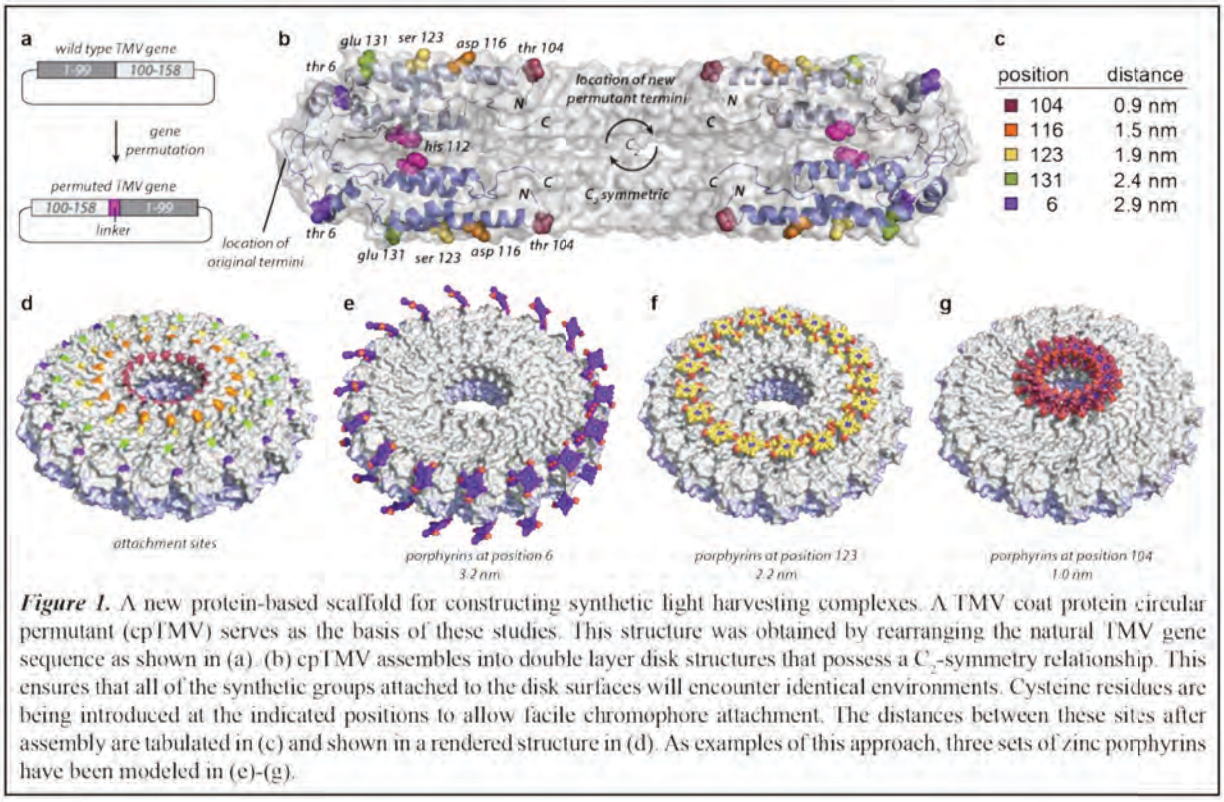

Figure 1. A new protein-based scaffold for constructing synthetic light harvesting complexes. A TMV coat protein circular permutant (cpTMV) serves as the basis of these studies. This structure was obtained by rearranging the natural TMV gene sequence as shown in (a). (b) cpTMV assembles into double layer disk structures that possess a C_2-symmetry relationship. This ensures that all of the synthetic groups attached to the disk surfaces will encounter identical environments. Cysteine residues are being introduced at the indicated positions to allow facile chromophore attachment. The distances between these sites after assembly are tabulated in (c) and shown in a rendered structure in (d). As examples of this approach, three sets of zinc porphyrins have been modeled in (e)-(g).

- A new attachment strategy is being developed for position 112 in Figure 1. If successful, this will allow the generation of chromophore arrays that are fully embedded within the protein structure. Once prepared, amino acid mutations will be used to explore the effects of the protein environment on spectroscopic and energy transfer properties.
- We will be applying new methods in excited state electronic structure calculations to understand the specific changes that occur in the chromophores when arrays are formed. The predictions from these models will be matched to experimental data, wherever possible.
- Dynamic models of the protein assemblies are being developed and evaluated to predict the level of disorder and distortion in these systems. Fluctuations on the timescale of energy transfer are being considered, as well as "static" disorder that is inherently present in the systems, but slower than the lifetimes of the excitons.

Chloroplast Dynamics and Photosynthetic Efficiency

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Overall research goals: Our project explores the roles of the actin cytoskeleton and two protein families in positioning of chloroplasts to maximize solar energy utilization and to promote efficient exchange of metabolites with other compartments within the plant cell. Chloroplasts move within leaf cells to optimize light levels, maximizing their interception of light under limiting light conditions (accumulation) and minimizing interception under excess light conditions (avoidance). We are investigating the role of the plant myosin XI and 14-3-3 protein families in chloroplast movement. We proposed to study tissue in which one or more of the genes were silenced or mutated and the localization of proteins through fluorescent protein tagging. We also proposed to determine whether myosin XIs and 14-3-3 proteins interact with each other and with the blue-light receptors PHOT1 and PHOT2.

The current project was developed from prior DOE-funded research in which we investigated the role of stromules in chloroplast dynamics and protein flow. In April 2012, a paper appeared in *Plant Cell* which challenged the conclusions of our previous work. Another goal for this year was to verify our prior conclusions about stromules and seek an explanation for the contradictory data that was presented.

Significant achievements 2011-2013: Previously we had published that a fusion of a 42-amino acid region within Arabidopsis myosin XI-F with YFP decorated the periphery of chloroplasts and stromules. This 42 amino-acid region, which is homologous to the yeast myo2p tail region known to be essential for vacuole and mitochondrial inheritance, was named the "PAL" domain and is present in additional myosin XI proteins. We have found that seven YFP::PAL sub-domain fusions decorate Golgi and six localize to mitochondria. Simultaneous transient expression of the PAL sub-domains of myosin XI-H, XI-I, and XI-K resulted in inhibition of movement of mitochondria and Golgi, likely due to a dominant negative effect. Because none of these domains except XI-F PAL labeled chloroplast envelopes, we will not be studying them further under the auspices of the current project. A manuscript describing this study has been accepted for publication in *Frontiers in Plant Cell Biology* (Sattarzadeh et al., in press).

A paper appeared from a Canadian group (Schattat et al. 2012, *Plant Cell* 24:1465-77) that claimed our prior DOE-funded work demonstrating flow of proteins from one plastid to another was incorrect. Furthermore, text in this paper stated incorrectly that there was a "dogma" that plastids form an interconnected network, although we had demonstrated in a paper published in 2000 that no such network exists. The authors used a green-to-red photoconvertible GFP while our prior studies had been performed by photobleaching of standard GFP. We obtained the Arabidopsis line containing the photoconvertible GFP from the Canadian group to determine why they failed to observe transfer of protein from one plastid to another. We could demonstrate movement of both the photoconvertible GFP and standard GFP between plastids, provided that low laser power was used to perform the photoconversion. We believe that the use of high levels of irradiation for photoconversion by the Canadian group resulted in photodamage that prevented

them from observing movement of fluorescent protein. Our paper has recently appeared in Plant *Cell* (Hanson and Sattarzadeh, 2013).

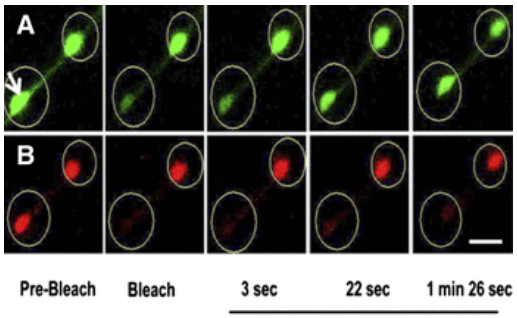
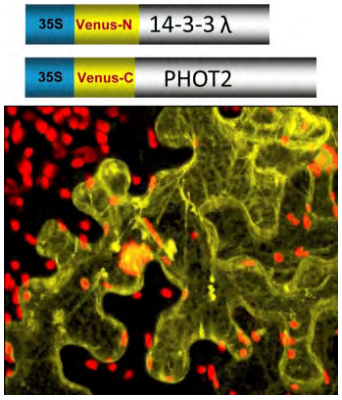


Figure 1. Following laser irradiation of a chloroplast (arrow), GFP fluorescence is reduced and chlorophyll autofluorescence is not detectable. Over time, GFP signal recovers due to flow from unbleached plastid, while thylakoid-bound chlorophyll does not move through the stromule.

From stock centers we have obtained two to four insertional mutants for each of 10 of the 13 Arabidopsis genes encoding 14-3-3 proteins, and we have one mutant in each of two additional genes. We presently have homozygous mutants in nine different 14-3-3 genes and have observed that mutants in four of the nine genes have obvious growth phenotypes. Co-PI Owens assembled a dual source system (variable intensity actinic blue light to induce chloroplast movements, low intensity modulated red light) to measure changes in %T as small as 0.01%. When this system is used on single 14-3-3 mutants or single myosin mutants, we do not detect any effect on chloroplast movement, likely due to redundancy of function. We have detected that Arabidopsis plants undergoing virus-induced silencing of certain combinations of myosin XI genes exhibit altered chloroplast movement.

By biomolecular fluorescence complementation (BIFC), we have observed an interaction of PHOT2 with 14-3-3 lambda and with one other 14-3-3 protein (Figure 2). Interaction of two 14-3-3 proteins with the blue-light receptors for chloroplast responses to light indicates that further study of the role of this protein family in chloroplast movement is warranted.



Science objectives 2013-2014

Use virus-induced gene silencing of specific combinations of genes to examine the role of particular myosin XIs and 14-3-3 proteins in chloroplast accumulation and avoidance responses. Continue to examine protein-protein interactions through BIFC. Observe localization of 14-3-3 proteins, actin, PHOT1, PHOT2, and myosin XI during chloroplast movement in response to light.

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Significance of Protein Maturation for Thylakoid Biogenesis and Its Possible Regulation

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Overall research goals: The research objective is to further our mechanistic understanding of photosynthetic membrane development. To this end, our DOE-funded research has been focusing on studying thylakoidal processing peptidase (TPP) in the model plant Arabidopsis. TPP catalyzes removal of a thylakoid-transfer signal (TTS) from a variety of thylakoid lumenal proteins including components as well as assembly and quality control factors of the photosynthetic electron transport chain. Our previous work had shown that i) one of TPP homologs called plastidic type I signal peptidase 1 (Plsp1) is necessary for proper assembly and maintenance of thylakoids; ii) Plsp1 is required for complete maturation of the protein import channel Toc75 in the envelope and two oxygen-evolving complex (OEC) subunits and plastocyanin in the thylakoid lumen; iii) the complete maturation of Toc75 is dispensable for proper thylakoid development. Our current goals are to elucidate the underlying mechanism for Plsp1-dependent thylakoid development and to define the pathway by which Plsp1 is targeted to the chloroplast membranes.

Significant achievements 2011-2013: We have used genetic and biochemical assays to make the following four significant achievements.

1. Multiple fates of non-mature lumenal proteins: TTS-dependent protein transport is mediated mainly by two energy-consuming pathways, called the cpSEC and cpTAT pathways. The cpSEC pathway utilizes ATP hydrolysis in the stroma. The cpTAT pathway requires proton motive force (PMF) across the thylakoid membrane. Our analyses revealed that *plsp1*-null plastids contain a sufficient amount of ATP to support the cpSEC pathway but are deficient in active cpTAT pathway apparently due to the lack of PMF (Fig. 1A). We further used *in vitro* import assays to show that inhibition of TTS removal by amino acid mutations around the processing site did not disrupt cpSEC- and cpTAT-dependent targeting, but caused accumulation of non-mature (nm) proteins in the membrane, seemingly away from the translocon. These membrane-associated proteins were found to take multiple fates: nmPetE (plastocyanin) was quickly degraded under light, nmPsbO (the 33-kD subunit of OEC) was assembled into an oligomeric complex of about 400 kD via a disulphide bridge or more, and nmPsbP (the 23-kD subunit of OEC) was found as a monomer (Fig. 1B). Formation of the 400-kD nmPsbO complex was also found in the transgenic Arabidopsis that produced un-cleavable PsbO. These results provide a molecular insight into the mechanism of Plsp1-dependent assembly of photosynthetic systems. The findings also revealed the presence of multiple mechanisms to control unprocessed lumenal proteins in thylakoids.

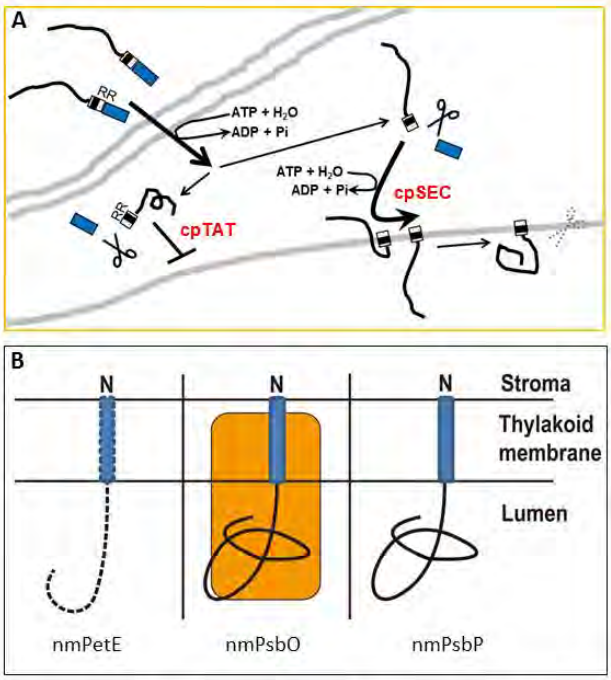

Figure 1. **A**. A schematic showing the protein transport in the chloroplast that lacks Plsp1. **B**. Three fates of nonmature lumenal proteins. PetE is plastocyanin. PsbO and PsbP are the 33-kD and 23-kD subunits of OEC, respectively.

2. Stable complex formation of Plsp1 and PGRL1 – a novel link between energy use and generation?: Blue native-polyacrylamide gel electrophoresis analysis revealed that Plsp1 is present mainly in two oligomeric complexes. By using the mutant Arabidopsis that accumulated tagged Plsp1 instead of endogenous Plsp1, we identified a protein called PGRL1 as a component of the larger, but not the smaller, Plsp1 complex. PGRL1 is known to be necessary for induction of non-photochemical quenching and protection of photosystem I from photoinhibition *in vivo*, and could catalyse electron transfer from ferredoxin to plastoquinone *in vitro* as reviewed in Leister and Shikanai, Front Plant Sci **4**:161 (2013). Formation of the larger Plsp1 complex is dispensable for the function of Plsp1 because the *pgrl1*-null mutant, which accumulates only a smaller Plsp1 complex, did not show any obvious defects in thylakoid development and maturation of Plsp1 substrates under the normal growth condition. Our results also showed that the larger Plsp1 complex contains less than 3% of the entire pool of PGRL1 and more than 50% of Plsp1. Taken together, available results suggest that PGRL1 may act as a non-essential regulator of Plsp1, thus may provide a potential link between energy use and generation at the photosynthetic membrane.

3. Catalytic activities of recombinant Plsp1: We produced full-length mature Plsp1, which includes a transmembrane domain, in *E. coli*, solubilized it with a detergent, and showed its catalytic activity against a series of TPP substrates and Toc75. The results provide a direct support for Plsp1 being TPP. We are currently comparing Plsp1 and a well-studied type I signal peptidase from *E. coli* (LepB) in kinetic properties and sensitivity to various factors.

4. The mechanism by which Plsp1 is sorted to the thylakoid membrane: By using *in vitro* import and transport assays, we found that Plsp1 is sorted to the thylakoid membrane via a stromal intermediate. We are currently testing molecular factors that are required for the integration of Plsp1 into the membrane.

Science objectives for 2013-2014:

We will use genetic and biochemical assays to address the following four questions.

- Which proteins are in the 400-kD nmPsbO complex?
- How similar is Plsp1 to LepB in catalytic properties?
- How is Plsp1 sorted to the thylakoid membrane?
- What is the biological function of envelope-localized Plsp1 other than maturation of Toc75?

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The Energy Budget of Steady-State Photosynthesis

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To achieve and maintain efficiency, photosynthesis must be finely regulated to balance the competing needs for efficient energy conversion and the avoidance of deleterious side reactions. The project goals focus on understanding how photosynthesis balances its energy budget, matching the output of energy in NADPH and ATP to precisely meet biochemical demands. If this balancing does not occur, the system will fail, leading to photodamage and loss of energy storage. Efforts to improve the efficiency of photosynthesis by introducing CO_2 concentrating mechanisms, altering metabolism or biosynthetic pathways to shunt energy to alternative products, will likely exacerbate these imbalances. Our specific aims concern the mechanisms of 1) cyclic electron flow (CEF) which acts to generate ATP to balance the chloroplast energy budget, and 2) the transport and sharing of ATP among the chloroplast, cytoplasm and mitochondria.

Significant achievements 2012-2013:

CEF can power CO_2 concentrating mechanism (CCM) of algae (1, 2). The chloroplast must rapidly and precisely adjust photosynthetic ATP and NADPH output to meet changing metabolic demands imposed by fluctuating environmental and metabolic conditions. In particular, we are interested in how the chloroplast balances its energy budget to accommodate the activation of the CCM. This question is especially critical in light of recent efforts to introduce CCM into higher plants to overcome kinetic limitations of photosynthesis. We used the in vivo spectroscopic techniques developed in this project to show that CEF can be rapidly activated in response to changing CO2 and organic carbon availability and the energy stored by CEF is ufficient to power CCM. Thus, CEF can act to balance even large changes in the cellular ATP/NADPH budget under fluctuating environmental or metabolic conditions.

CEF is not regulated by state transitions (1, 2). We also assesses major models about the regulation of CEF. A series of previous papers concluded that CEF is regulated by antenna state transitions. However, we showed that CEF regulation under fluctuating CO_2 occurs much more rapidly than can be accounted for by state transitions. Furthermore, CEF is equally activated in the state transition mutant *stt7-9*, implying that CEF is regulated by processes other than state transitions. In parallel work on Arabidopsis, we demonstrated that state transitions also do not control CEF in higher plants. We proposed that CEF is rapidly regulated by allosteric, redox or reactive oxygen species.

Hydrogen Peroxide Activates CEF *in vivo (3, 4).* We observed a strong correlation between activation of CEF and accumulation of H_2O_2 in high CEF mutants. In this work, we tested whether H_2O_2 could regulate CEF. Using *in vivo* spectroscopy, we observed a concentration dependent increase in CEF1 after application of exogenous H_2O_2. These results were confirmed using a line of mutants with glycolate oxidase targeted to the chloroplast, therefore accumulating H_2O_2 specifically at the chloroplast (Fahnenstich et al., 2008). The CEF1 activity was insensitive to antimycin A, suggesting the involvement of the NDH complex. H_2O_2 activation could act at the level of a redox sensor or indirectly via effects on metabolic enzymes. In either case, we propose that

H_2O_2 formed under reducing conditions, e.g. during an imbalance in ATP/NADPH production and consumption rates, may be a missing link in the activation of CEF1.

The Fd/NADPH-dependent fluorescence rise phenomenon: It doesn't mean what we thought it meant. A large fraction of the current literature on CEF, including all results on the PGR5 pathway, are based on interpretation of the antimycin A-sensitive "Mills-Munekage"(M-M) fluorescence signal upon addition of NADPH and ferredoxin. We show that, contrary to previous interpretations, the M-M signal is not related to plastoquinone pool reduction and therefore does not reflect the activity of CEF. Instead the signal is related to the reduction of a low potential (E_M~-340 mV) electron carrier associated with PSII or conformational change within the antenna, and is probably related to the "low potential Q_A" signal seen in earlier work on PSII. These results lead us to reassess a large fraction of past literature, especially those on the PGR5 pathway. In particular, we have additional evidence that the major effect of pgr5 is on regulation of the chloroplast ATP synthase rather than on CEF. They also suggest that it is possible to transfer electrons from low potential Q_A to soluble electron carriers like ferredoxin.

Isolation of novel mutants that activate CEF *in vivo*. In order to understand the mechanism and control of CEF, we isolated, mapped and/or characterized a series of mutants that display high CEF, i.e the *hcef* mutants. In the last year, we mapped *hcef6* to a nonsense mutation Q334 (889C>T) in a protein of unknown function (AT3G29185) that is localized to the chloroplast. The *hcef6* mutation is recessive mutant and results in a mild growth phenoptype under laboratory conditions, but strong effects on photo-synthesis and growth under fluctuating light suggesting a role for CEF in dynamic responses to environmental conditions. We are also submitting a manuscript on *hcef2*, which we mapped to TADA1, a chloroplast tRNA editing enzyme. We show evidence that loss of TADA1 results in uncoordinated protein expression in the chloroplast, leading to partial assembly of chloroplast's energy transducing complexes (photosystem I and II, cytochrome b_6f complex, ATP synthase etc.).

Science objectives for 2013-2014

- Determine the mechanism of hcef6 and other hcef mutants
- Determine if CEF involved a proton pumping NDH complex (preliminary data is highly promising)
- Determine if the chloroplast nucleotide transporter acts to balance ATP/NADPH budget during fluctuations in metabolic demands
- Test the hypothesis that a thylakoid proton release valve is activated under conditions of excess photosynthetic proton pumping capacity

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Extending the Bacteriorhodopsin Paradigm for Proton Pumps

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Overall research goals: The research objectives are to study eubacterial light-driven retinal-based proton pumps, with the expectation that new information will come to light to extend, and perhaps even contradict, the rules established from many years of work with bacteriorhodopsin. Along these lines, in past funding periods we reported on a dual-chromophore proton pump, in which a carotenoid functions as light-harvesting antenna to the retinal, increasing the cross-section for light absorption. This year, we report on two findings with a rhodopsin from *Exiguobacterium sibiricum*: the role of a histidine-aspartate complex as the proton acceptor to the retinal Schiff base after photoexcitation (unlike a simple aspartate in bacteriorhodopsin), and the role of a lysine as proton donor to the retinal Schiff base (unlike an aspartate in bacteriorhodopsin).

Significant achievements 2011-2013:

In *E. sibiricum* rhodopsin, as in other eubacterial rhodopsins, but not in bacteriorhodopsin, there is a histidine tightly hydrogen-bonded to the counter-ion aspartate. We found, from a novel approach to investigating the protonation state of the counter-ion, that there are two pK values, at ca. 3 where ca. 90% of the counter-ion ionizes, and ca. 9 where the remaining 10% does. This indicates coupling with another ionizable group, which we identified as the histidine. It explains many unusual findings, including the very small pH dependent shift of the chromophore maximum at pH 6, thought earlier to be the single pK of the aspartate. The appearance of deprotonated Schiff base in the photocycle only at pH as high as 9 and above, is traced back to the ionization of the histidine: below this pH it is protonated and the counter-ion accepts protons so slowly that reprotonation is the faster process.

In the same protein, lysine is found in the place of the acidic group that normally functions as the proton donor to the Schiff base in the photocycle. A basic residue has never been identified as proton donor in a bacterial rhodopsin. Yet, its replacement with any other residue results in very slow Schiff base reprotonation. We found, uniquely in this protein, proton uptake during the photocycle prior to Schiff base reprotonation, indicating that there is an internal proton donor but it needs to be protonated before it can pass a proton to the Schiff base. Thus, the same conformational change that lowers the pK of an aspartate (or glutamate) in other rhodopsins will raise, in this case, the pK of the donor.

Science objectives for 2014-2015:

- The kinetics of the appearance and disappearance of M (deprotonated Schiff base) after photoexcitation will be further investigated. It is very unusual not to observe this intermediate even though it must be formed.
- We know that in various eubacterial rhodopsins the ionization states of the histidine and the aspartate are coupled to different extents. We will generalize our findings over a range of this large family of proteins.
- Although the essential role of the lysine in proton transfer is established, it is unclear whether the origin of the proton is the lysine NH_2 that deprotonates or water that is coordinated by the lysine.

We will attempt to decide this question with FTIR spectroscopy, utilizing isotope labeled (^{2}H, ^{15}N) protein.

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MSH1 is a plastid-localized protein that influences epigenetic changes in the plant

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The *MSH1* gene encodes a plant-specific protein that targets to both mitochondria and plastids. The gene was cloned by our group several years ago as part of a DOE-sponsored project to understand the role of MSH1 in maintaining organellar genome stability. More recently we showed that depletion of MSH1 results in developmental reprogramming of the plant, a condition that is maintained epigenetically and associated with genome-wide cytosine methylation changes. In the present project, we have taken these studies further to investigate the influence of MSH1 on this plant reprogramming process. Researching this question has resulted in several important recent observations:

- MSH1 localizes to a specialized plastid type in the epidermal and vascular parenchyma cells of a plant. This localization is the consequence of spatial expression by the MSH1 promoter, and the specialized plastids, about 1/3 the size of a mesophyll chloroplast, have been termed "sensory plastids" to reflect their apparent role in plant signaling.
- Plastid MSH1 appears to associate with the thylakoid membrane together with components of PSII oxygen evolving complex and the cyt b_6f complex.
- Depletion of MSH1 results in changes in non-photochemical quenching, plastoquinone redox, and plastochromanol-8 levels within these plastids. These changes imply a novel redox state within the plastids.
- The localization of MSH1 to the vascular tissue implies that these plastids might be involved in plant signaling. In support of this concept, grafting experiments involving msh1 and Col-0 wildtype produce dramatic and heritable growth changes in the progeny of the graft.

  Our goals for the future will be to understand the nature and composition of sensory plastids in plants, and to identify the signaling molecule that originates from MSH1 depletion.

Relevant publications:

Xu, Y-Z, de la Rosa, R., Virdi KS, Arrieta-Montiel MP, Razvi F, Li S, Ren G, Yu B, Alexander D, Guo L, Feng X, Dweikat IM, Clemente TE and Mackenzie SA. 2012. The chloroplast triggers developmental reprogramming when MUTS HOMOLOG1 is suppressed in plants. Plant Physiol. 159(2):710-20.

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Virdi, KS, Wamboldt, Y., Basset, G, Xu Y-Z, Elowsky, C. Bricker, T, Kundariya, H, Mackenzie SA. A MutS homolog in plants localizes to sensory plastids and influences retrograde signaling. In preparation.

RNA Quality Control in the Chloroplast

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Overall research goals:
RNA molecules serve either as templates for protein production, or function directly in the form of RNA. These so-called non-coding RNAs may influence gene expression by binding to chromosomes, activate RNA interference pathways, bind metabolites to regulate related operons, or bind to messenger RNAs to activate or repress their translation into proteins. Our project focuses on the chloroplast, where photosynthesis occurs, and how these various RNA populations are created and managed. Functional RNAs must have the correct length and sequence composition. For example, intron-containing RNAs cannot be translated, and long antisense RNAs can have undesirable impacts on gene expression. *RNA quality control* is a term that describes the mechanisms by which organisms differentiate between functional and unwanted RNAs, and dispose selectively of the latter.

Our approach is to analyze chloroplast RNA populations, and the enzymes that act on them, in different fractions of the chloroplast. Our hypothesis is that the spatial separation of ribonucleases within the chloroplast underpins quality control. Thus, quality control is progressively exerted as an RNA progresses along a pipeline of gene expression from the site of transcription to the site of gene expression. To test this, we are using high-throughput RNA sequencing (RNA-seq) which identifies quantitatively and separately sense and antisense RNAs. We are also using mutants defective in particular RNases to verify their roles in quality control.

Significant achievements 2011-2013:
- We discovered that RNase J, which had only been studied in bacteria, has two critical and very different roles in chloroplasts. Indeed, RNase J mutants are embryo-lethal. First, it has an essential role in RNA quality control because it is needed to remove long antisense RNAs that otherwise impede gene expression (publication 1). Second, it is required to correctly mature the 5' ends of protein-coding RNAs (publication 5).
- We used RNA-seq to describe for the first time a comprehensive list of noncoding RNAs found in chloroplasts (publications 2 and 7). Their abundance and diversity were both surprisingly great, and suggest that they may have unrecognized regulatory functions. Similar approaches have since been pursued by other groups.
- We showed that RNA-seq can be used quantitatively and qualitatively to study RNA editing, splicing or maturation of the termini of chloroplast RNAs (publications 4 and 6).

Science objectives for 2013-2014
- Using RNA-seq we have now obtained strand-specific results from several chloroplast compartments: the nucleoid, where DNA is transcribed, and where

certain RNases and other RNA-binding proteins are localized; the stroma, the soluble phase where we posit that RNA maturation is completed; and polysomes, where RNA is translated into protein. These subfractions have not been previously analyzed comprehensively for their RNA populations. As the RNA-seq data are analyzed, we will be validating them and testing new hypotheses that result from those observations.

- Also using RNA-seq we have analyzed two mutants deficient in RNases that have not been previously studied. Like RNase J, these analyses may reveal new functions. The mutants under study are *rnc3rnc4*, and a mutant lacking an RNase called L-PSP. L-PSP has been extensively studied in animal cells, but never in plants.
- RNase III is an enzyme that uses double-stranded RNA as a template, for example paired sense and antisense RNAs, or RNAs that fold internally. RNase III has not be studied in chloroplasts, and we have now shown that this activity is conferred in Arabidopsis by the *RNC3* and *RNC4* gene products. The RNA-seq data is meant to reveal whether these mainly redundant enzymes act on antisense RNAs, whose abundance should increase in the double mutant background. Using conventional techniques, we have already found that RNC3/4 are required for correct ribosomal RNA maturation in the chloroplast.

References to work supported by this project (DOE-supported in **bold**):

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Photosystem I – Molecular Catalyst Hybrids for Solar Hydrogen Production

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Overall research goals: Natural photosynthetic energy research is aimed at resolving fundamental mechanisms of photochemical energy conversion in photosynthetic proteins and using this information in the design of bio-inspired materials for the generation of solar fuels. Our group has initiated efforts to exploit Nature and make use of the light-chemistry of photosynthetic reaction center (RC) proteins to drive non-native chemical reactions from abiotic catalysts. We are developing new energy conversion strategies that couple the photon energy, efficiently captured as a stabilized charge separation across the RC, to the direct synthesis of the environmentally clean fuel, hydrogen, using synthetic first-row transition metal catalysts. Specifically, our goals include (1) the design and optimization of new H_2-producing RC-catalyst complexes and (2) development of experimental biochemical and spectroscopic methods to interrogate structure/function relationships and fundamental mechanisms of photochemical proton-coupled electron transfer reactions in RC-catalyst hybrids. The combined effort in forefront biochemical and spectroscopic experimental approaches gives the opportunity for breakthroughs in the resolution of fundamental mechanisms for coupling photons to fuels in photosynthetic hybrid systems, thereby advancing our development of molecular systems for efficient solar fuel production.

Significant achievements 2011-2013:

PSI-Cobaloxime H_2 Production. We have developed an exciting new approach for solar fuel production that links Nature's finely tuned photosynthetic machinery with a synthetic *molecular* catalyst to create a hybrid complex that uses visible light to rapidly produce hydrogen from water. This novel hybrid architecture was realized by simple self-assembly of the photosynthetic reaction center protein Photosystem I (PSI) with a well-known molecular cobaloxime hydrogen electrocatalyst. The resultant complex provides the first example of light-driven hydrogen production from a synthetic molecular catalyst linked to PSI. The maximum rate for the photoreduction of water by this hybrid was measured to be >10,000 mol H_2 (mol PSI)$^{-1}$ h^{-1}, with a total turnover number of 5200 mol H_2 (mol PSI)$^{-1}$, which compares favorably to related synthetic photosensitizers and nears that of our PSI/Pt nanoparticle hybrid. Importantly, the PSI-cobaloxime biohybrid accomplishes solar photocatalysis using inexpensive, earth abundant elements to make a clean fuel and creates new opportunities for solar fuel production that merges synthetic inorganic and biochemical capabilities.

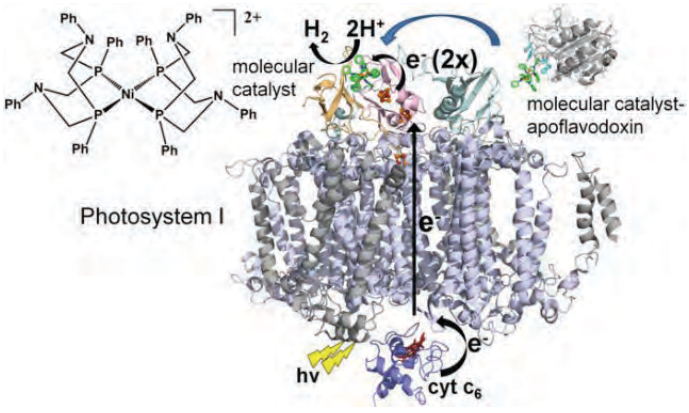

Figure 1. Photocatalytic scheme of H_2 production from a PSI-Ni molecular catalyst hybrid complex and Ni-ApoFlavodoxin.

Protein Delivery of a Ni Catalyst to PSI. We have prepared the first solar fuel hybrid that couples PSI light-driven chemistry to H_2 production from a nickel diphosphine molecular catalyst $[Ni(P_2^{Ph}N_2^{Ph})_2](BF_4)_2$. The protein environment enables photocatalysis at pH 6.3 in completely aqueous condtions. Additionally, we have developed a strategy for incorporating the Ni molecular catalyst with the native acceptor protein of PSI, flavodoxin. Photocatalysis experiments with this modified flavodoxin demonstrate a new mechanism for biohybrid creation that involves protein-directed delivery of a molecular catalyst to the reducing side of PSI for light-driven catalysis. This approach provides the potential for self-repair of the biohybrid system with a mechanism for introducing fresh catalyst to the acceptor end of PSI.

Science objectives for 2013-2014:

- PSI has proven to be an excellent photochemical module, and we will continue our studies of PSI-molecular catalyst constructs. Our intent is to explore new catalysts, understand how and where the catalysts bind to PSI, and develop methodologies to spectroscopically probe the molecular catalyst electronic structure in the protein environment.
- We are exploring new bioinorganic and electron carrier protein-based strategies to connect PSI photochemistry to molecular catalyst hydrogen production. Our goals include: using ferredoxin and flavodoxin to site-specifically deliver catalysts to the acceptor protein docking site of PSI, opportunities for protein-encapsulated stabilization of molecular catalysts and self-repair of the photocatalytic system, and spectroscopic characterization of interprotein-catalyst ET pathways.

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Towards a Comprehensive Molecular Model of Rubisco Regulation by Rubisco Activase

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Overall research goals: In higher plants, the P-loop ATPase Rubisco activase (Rca) helps coordinate the light and dark reactions of photosynthesis by catalyzing the release of inhibitors from Rubisco. When ATP levels drop or the temperature rises, Rca activity falls off and carbon fixation ceases. The prediction of net photosynthesis under different environmental conditions requires a quantitative understanding of the parameters controlling Rca activity in response to light-dark adaptation, heat stress, etc. The overarching goal of this work is to gain a full understanding of the mechanism of Rca-mediated Rubisco remodelling as a function of stromal conditions. The research objectives are (1) to determine X-ray structures of higher plant Rcas relevant to the functional ATPase cycle, and (2) to elucidate the structural and thermodynamic features of the physical interaction between Rubisco and Rca.

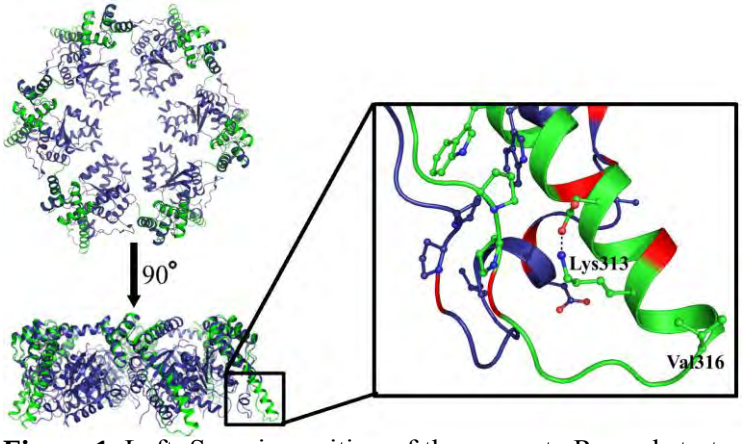

Figure 1. Left: Superimposition of the creosote Rca substrate recognition domain (green, PDB ID 3HTG) and the tobacco Rca AAA+ closed ring model (blue, PDB ID 3ZW6). Right: Conformational differences within the substrate recognition domain.

Significant achievements in 2012-2013:

1. Protein crystallography. We are continuing to use a variety of biophysical and biochemical methods to study full-length and truncated Rca constructs from multiple plant species. Using Thermofluor stability assays, we have defined a minimal construct capable of nucleotide binding. We have demonstrated that Mg^{2+} stabilizes the ATP-bound state, but destabilizes the ADP-bound state. The AAA+ C-terminal substrate recognition domain from creosote Rca was crystallized and its structure solved to 1.9 Å resolution. Comparison to the structure of the tobacco apo-AAA+ module [Stotz et al. (2011), Nat. Struct. Mol. Biol. 18, 1366-1378] reveals substantial conformational changes within a helical insert that contains residues required for species-specific reactivation of Rubisco (Figure 1).

2. Rca subunit assembly. To-date, the detailed mechanism by which Rca self-association controls Rubisco reactivation remains poorly understood. We are using fluorescence correlation spectroscopy (FCS) to characterize the assembly process of cotton β-Rca labeled with an AlexaFluor dye. We find that in the presence of Mg·ADP, Rca self-associates in a step-wise fashion to form oligomeric and higher order forms. The most plausible model supports dissociation constants of 3.5 μM for the monomer − dimer, 1 μM for the dimer − tetramer, and 1 μM for the tetramer − hexamer equilibrium. Large supramolecular assemblies become dominant above 40 μM (Figure 2). To examine the effect of Mg·ATP on oligomerization, we have generated the D173N mutant of Rca, which binds but does not hydrolyze ATP. The FCS data for ATP-dependent

assembly suggest that under these conditions, the hexamer becomes the dominant form over a much broader concentration range. We propose that the physiologically relevant assembly state may involve the formation of closed hexameric rings starting at 50 μM subunit concentration.

3. Rca steady-state turnover kinetics. To characterize the regulation of Rca ATPase activity as a function of physiologically relevant factors, we have developed an enzyme-coupled continuous assay to monitor P_i production during ATP hydrolysis. For tobacco Rca, steady-state turnover kinetics analyzed according to Briggs-Haldane models provide a k_{cat} value of 19 min^{-1} and a K_m value of 17 μM. The Hill coefficient supports positive cooperativity of two catalytic sites. The addition of small amounts of ADP pushes the apparent K_m for ATP hydrolysis into the low-millimolar range. The K_i value for competitive ADP binding is calculated to be about 4 μM, consistent with moderate product inhibition. We find that the ATPase turnover rate reaches a maximum at 1.5 μM Rca, suggesting that assembly to (at least) the dimeric state is a prerequisite for ATP hydrolysis. In addition, we find that with increasing free Mg^{2+} concentrations, the catalytic efficiency (k_{cat}/K_m) increases sharply, consistent with a K_d value of 3.5 mM for a second magnesium binding site. In combination, the data are consistent with an allosteric regulatory mechanism that is controlled by the ADP/ATP ratio and the Mg^{2+} concentration in the stroma.

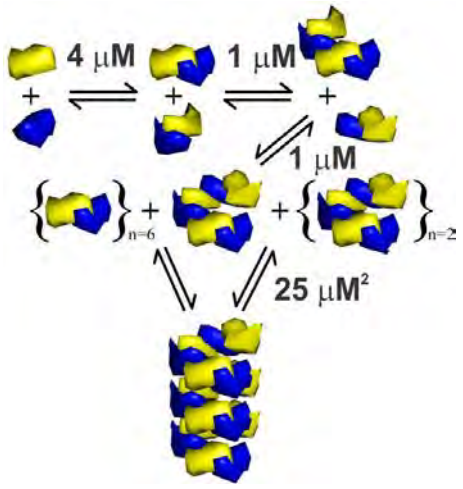

Figure 2. ADP-mediated Rca assembly mechanism consistent with FCS data.

Science objectives for 2013-2014:

- We aim to better understand the origin of the conformational differences observed in the Rca tobacco and creosote structures (Figure 1). Although this region contains residues required for species-specific reactivation of Rubisco, the functional significance of these structural adjustments remains unclear.
- We aim to generate a complete thermodynamic description of Rca assembly as a function of nucleotide, magnesium and Rubisco. This will aid in understanding the molecular mechanism of Rubisco reactivation.
- We aim to provide a full description of Rca steady-state turnover kinetics. In oligomeric assemblies such as hexameric rings, different classes of sites may coexist that provide a means for coordinated positive and negative subunit interactions, in line with a binding-change mechanism for ATP hydrolysis.

References to work supported by this project 2011-2013:

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2. Henderson, J. N., Hazra, S., Dunkle, A. M., Salvucci, M. E., Wachter, R. M. (2013) Biophysical Characterization of Higher Plant Rubisco Activase. *Biophys. Biochim. Acta 1834, 87-97.*
3. Chakraborty, M., Kuriata, A. M., Henderson, J. N., Salvucci, M. E., Wachter, R. M., Levitus, M. (2012) Protein Oligomerization Monitored by Fluorescence Fluctuation Spectroscopy: Self-Assembly of Rubisco Activase. *Biophys. J. 103, 949-958.*
4. Henderson, J. N., Kuriata, A. M., Fromme, R., Salvucci, M. E., Wachter, R. M. (2011) Atomic Resolution X-ray Structure of the Substrate Recognition Domain of Higher Plant Rubisco Activase. *J. Biol. Chem. 286, 35683-35688.*

Water Oxidizing Efficiency in the Thylakoid-less Cyanobacterium *Gloeobacter violaceous*.

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The ancient cyanobacterium *Gloeobacter violaceus* PCC 7421 is unique among oxygenic phototrophs in that it lacks separate cytoplasmic and inner cytoplasmic (thylakoid) membranes. Instead it performs both respiration and photosynthetic electron transport reactions within a single intracellur membrane that must sustain adequate energy production via a *pmf*. Here we show that *G. violaceus* grows photoautotrophically in two distinct physiological states that are distinguished by pigmentation and the degree of coupling of photosynthetic and respiratory electron fluxes; violet cells are produced at low light intensity < 10 μE $m^{-2}s^{-1}$, while brown cells occur at 50 μE $m^{-2}s^{-1}$. These states can be interconverted. We know of no other oxygenic phototroph with this trait. Using Fast Repetition Rate Fluorometry (FRRF), and flash O_2 yield, we have characterized charge separation from water to PQ pool via PSII in both states. We provide the first evidence for period-four oscillations of O_2 yield and variable fluorescence (Fv/Fm) establishing catalysis by a typical WOC in violet cells that exhibit a Fv/Fm ratio (0.24 – 0.30), proportional to the PSII quantum efficiency of charge separation. That is twice lower than in other cyanobacteria, while Fv/Fm in brown cells is 0.13 – 0.15. The reoxidation time of Q_A^- and the Kok parameters ("alpha" and "beta") and period of oscillations (P) reflecting the efficiency of S-state transitions in the WOC of violet cells are comparable to *A. maxima* and *Synechococcus elongatus* PCC 7942. Photoactivation studies show relatively fast assembly of Mn_4Ca cluster with typical $t_{1/2}$ = 6-8 min. The WOC PSII expressed period four oscillations in Fv/Fm and flash O_2 after >1 month complete dark incubation (remaining activity 20% recovering up to 60% in 6 hours). O_2 light curve results are consistent with high efficiency of light utilization by *G. violaceus* compared to *Synechocystis* PCC 6803 at low light. These results suggest that while *G. violaceus* has weak charge separation efficiency compared to other cyanobacteria, its PSII-WOC cycles with comparable efficiency. Also, we will present new data analysis of flash oxygen evolution by *Thermosynechococcus elongatus*, His-tag CP-47 using VZAD model (Vinyard et al., 2013, BBA Bioenergetics, 1827, 861-868). This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy Grant DE-FG02-10ER16195.

Session IV

Analyses of hyperosmotic-induced Ca^{2+}responses in *Arabidopsis thaliana* to understand early osmo-sensory mechanisms in plants

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Overall research goals:
Plants undergo a multitude of responses to conserve water and preserve a viable state when challenged with water limitation by drought or increased soil salinity; included in these responses are a rapid rise in intracellular Ca^{2+}, synthesis of the stress hormone abscisic acid, subsequent closure of stomata, alteration of transcriptional profiles, and substantial changes in growth. However, the early mechanisms of drought- or osmotic- sensing leading to induction of responses to water limitation remain poorly understood. Toward the goal of identifying sensors and early effectors of osmotic stress in plants, the goals of this project are 1) Develop a highly-quantitative, high-throughput pipeline for measuring rapid Ca^{2+} responses in hyperosmotic-stimulated *Arabidopsis* seedlings. 2) Apply this pipeline to assess how environmental factors influence the hyperosmotic-induced Ca^{2+} response. 3) Perform a forward-genetic screen and map mutants that display abnormal hyperosmotic-induced Ca^{2+} responses.

Significant achievements 2011-present:
I developed an assay using a 96-well plate reader that is capable of stimulating *Arabidopsis* seedlings with hyperosmotic NaCl stress and measuring Ca^{2+} from an aequorin reporter in an automated fashion. In parallel, I developed a data analysis pipeline that extracts over 40 features of the NaCl-induced Ca^{2+} responses. I found a strong influence by environmental factors and significant heterogeneity between individuals, suggesting that even at this early stage a complex regulatory network feeds into this response. I found that inhibitors of a distinct family of Ca^{2+} channels dampen the salt-induced Ca^{2+} signal sharply. Using a complementary approach, I performed a forward genetic screen and identified >20 mutant lines that show alterations in rapid salt-induced responses. One mutant, 9.3C09K, has been characterized and reveals interesting and unexpected links to stress-associated growth and development, which will be presented.

Science objectives for 2014:
I will map the causative mutations in several of the mutants isolated from the genetic screen. To this end, I have established in-house the method of mapping by next-generation sequencing of bulked segregants. I have validated the method using published data sets, and one mutant line has already been sequenced. The causative mutation will be verified by genetic complementation and isolation of additional alleles by T-DNA insertion or application of CRISPR/CAS9 modification.

Publications resulting from the DOE-funded project:
Brandt, B., Brodsky, D.E., Xue, S., Negi, J., Iba, K., Kangasjärvi, J., Ghassemian, M., Stephan, A.B., Hu, H., and Schroeder, J.I. (2012). Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. PNAS *109*, 10593–10598.

*This paper describes earlier work establishing the sufficiency of a core set of proteins in re-creating the "Ca^{2+}-priming" phenomenon in abscisic acid signal transduction, a signaling pathway downstream of the rapid osmotic-induced Ca^{2+} responses described above.

Two-Dimensional Electronic Spectroscopies for Probing Electronic Structure and Charge Transfer: Applications to Photosystem II

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Overall research goals: This research program focuses on key deficits in our current understanding of the PSII reaction center (PSII RC). Understanding the PSII RC's design principles has importance for both fundamental and applied sciences wishing to mimic its remarkable properties. The program aims to address the following open questions: 1) What is the electronic structure of the PSII RC? 2) What is the charge transfer mechanism in the PSII RC? 3) Does electronic coherence facilitate energy transfer in the PSII RC? To address these questions we are utilizing two-dimensional electronic spectroscopy (2DES). We are also developing new variations on this method to more clearly separate the spectral signatures of energy transfer and charge separation.

Significant achievements 2012-2013: This year we achieved a number of goals. We tested current exciton models for the PSII RC against our 2DES data, finding significant discrepancies between the simulations and data[1]. We believe many of these discrepancies arise from the ad-hoc inclusion of charge transfer states within the exciton models. To address this problem we have used a tight-binding formalism to describe charge transfer in the PSII RC and have succeeded in using it to simulate our 2DES data, finding good agreement[2]. On the experimental front, we have exciting new data that shows clear coherent dynamics as illustrated in Figure 1. We have also extended the detection window of 2DES to probe outside the Qy band[3], and have written an invited review on the utility of 2DES for studying photosynthetic systems[4].

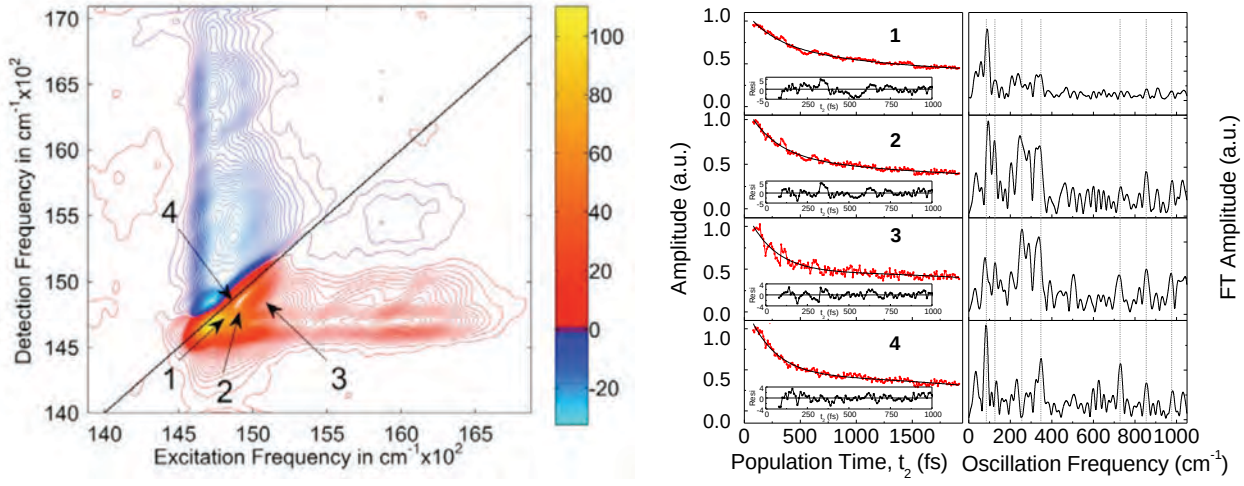

Figure 1. Left: 2D spectrum of the PSII RC at 77K at a population time of 170 fs. Right: Representative population time (t_2) traces and corresponding Fourier transforms taken at locations marked with arrows on the 2D spectrum shown on the left. Faint lines indicate frequencies observed throughout the 2D spectrum.

Science objectives for 2009-2010:

- We aim to further investigate the role of coherence in charge separation in the PSII RC. We will pursue a number of different directions. To better understand the signatures of coherence in 2DES data we are currently studying chlorophyll a in solution as a control for learning about vibrational coherence. We will also perform two color experiments to probe the pheophytin anion band to determine whether coherence is maintained throughout the charge separation process.
- To understand the signatures of coherence in 2DES data we are simulating 2DES data of chlorophyll a monomers for comparison with our 2DES data.
- We aim to combine 2DES measurements with Stark spectroscopy on model systems to highlight signatures of charge separation. We will then apply the method to the PSII RC.
- We will use 2DES to study PSII core complexes for comparison with D1D2-cyt b559 complexes to determine 1) the existence or absence of coherence in PSII cores, and 2) whether the excitonic structure is consistent in these two preparations.

References to work supported by this project 2012-2013:

1. Lewis, K. L.; Fuller, F. D.; Myers, J. A.; Yocum, C. F.; Mukamel, S.; Abramavicius, D.; Ogilvie, J. P., Simulations of the two-dimensional electronic spectroscopy of the photosystem II reaction center. *Journal of Physical Chemistry A* **2013,** *117*, 34-41.

2. Gelzinis, A.; Fuller, F. D.; Valkunas, L.; Ogilvie, J. P.; Mukamel, S.; Abramavicius, D., Tight-binding model of the photosystem II reaction center: application to two-dimensional spectroscopy. *New Journal of Physics* **2013,** *15*, 075013.

3. Fuller, F. D.; Ogilvie, J. P. In *Continuum probe two dimensional electronic spectroscopy of the photosystem II reaction center*, International Conference on Ultrafast Phenomena XVIII, Lausanne, Chergui, M.; Taylor, A.; Cundiff, S.; de Vivie-Riedle, R.; Yamagouchi, K., Eds. EDP Sciences: Lausanne, 2012; p 08018.

4. Lewis, K. L. M.; Ogilvie, J. P., Probing photosynthetic energy and charge transfer with two-dimensional electronic spectroscopy. *Journal of Physical Chemistry Letters* **2012,** *3*, 503-510.

Session V

The Structure and Function of Photosystem II

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Overall research goals: The principal research objective is to study the structure and function of Photosystem II (PS II). These studies include: (1) the identification of natively oxidized amino acid residues in the vicinity of the redox centers of the photosystem, (2) the use of synchrotron radiolysis to identify buried oxidizable residues which are adjacent to water and/or oxygen channels, and, (3) the use of protein crosslinking to examine the interactions of PsbO, PsbP and PsbQ with other PS II components.

Significant achievements 2011-2013: Significant progress has been made: (1) Natively oxidized amino acid residues on both the oxidizing and reducing sides of Photosystem II were identified. These define probable ROS production sites within the photosystem. (2) We have used the synchrotron radiolysis of water to oxidatively modify buried amino acid residues in the photosystem. We have proposed that these residues are in contact with putative water and dioxygen channels. (3) We have examined RNAi mutants of the lumenally localized PsbP domain proteins. PPD5 is involved in strigolactone biosynthesis and PPD1 is an essential PS I assembly factor.

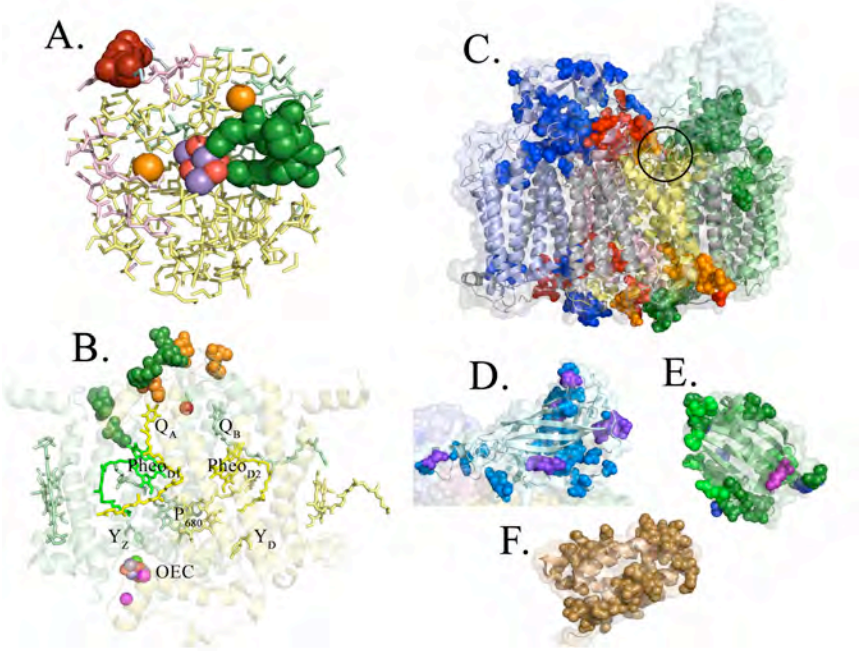

Figure 1. Oxidized Amino Acid Residues in Photosystem II. A. and B., Natively oxidized residues in PS II. In A., CP43 residues shown as green spheres are in close proximity (15 Å) to the Mn_4CaO_5 cluster and may be associated with a dioxygen egress pathway, being modified by ROS produced at the manganese cluster. In B., oxidized residues in the vicinity of $Pheo_{D1}$ (green spheres) and Q_A (orange spheres) indicate that these cofactors may be sites which produce reducing-side ROS. C.-F. Oxidized residues produced by synchrotron radiolysis. In C., CP47 (blue), D2 (red) and D1 (orange) spheres identify putative water channels leading to the Mn_4CaO_5 cluster (circled). CP43 residues (green) may represent either another water channel or a putative dioxygen egress pathway. In D., E. and F., domains on PsbO, PsbP and PsbQ, respectively, are resistant to oxidative modification. These may represent domains on these three extrinsic components which interact with other components of PS II.

Science objectives for 2013-2014:

- Ongoing experiments are designed to establish the kinetics of oxidative modifications which occur during the photoinactivation of PS II, using both $H_2{}^{18}O$ to probe oxidizing-side modifications and $^{18}O_2$ to probe both reducing-side modifications and modifications produced by singlet oxygen.
- Under moderate heat stress, $OH^{\bullet}$ is produced at the Mn_4O_5Ca cluster upon illumination (reviewed in Pospíšil, P. (2009) *Biochim Biophys Acta* 1787,1151). If performed in the presence of $H_2{}^{18}O$, $^{18}OH^{\bullet}$ will be produced. If one assumes that $^{18}OH^{\bullet}$ exits PS II by the same pathway as dioxygen, the identification of ^{18}O-modified residues will directly identify the dioxygen egress pathway.
- We have recently obtained direct evidence from protein crosslinking experiments that large conformational changes occur in the PsbP protein upon its association with PS II. In the crystal structure of isolated PsbP, ^{40}Lys is 16.2 Å from ^{155}Lys. However, when PsbP is bound to PS II these two residues are crosslinked with the homobifunctional reagent BS3, which croslinks lysyl residues within 11.4 Å. This result indicates that a conformational change of at least 4.8 Å has occurred within the PsbP subunit. These observations will be confirmed and extended.

References to work supported by this project 2011-2013:

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6. Frankel, L.K., Sallans, L., Limbach, P.A., and Bricker, T.M. "Oxidized Amino Acid Residues in the Vicinity of Q_A and $Pheo_{D1}$ of the Photosystem II Reaction Center: Putative Generation Sites of Reducing-Side Reactive Oxygen Species". *PLoS One* 8,e58042 (2013).

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9. Roose, J.L., Frankel, L.K. and Bricker, T.M. "Functioning of PPD1 in the Assembly of Photosystem I in Arabidopsis". Submitted, *Journal of Biological Chemistry*.

10. Bricker, T.M., Bell, A.J., Tran, L., Frankel, L.K. and Theg, S.M. "Photoheterotrophic Growth of *Physcomitrella patens*". Submitted, *Plant Physiology*

Watching Photosystem II at Work: Taking Snapshots of Photosynthetic Water Oxidation with an X-ray Laser

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Overall research goals: The specific questions that are the focus of our studies are: 1) What is the geometric and electronic structure and the changes of the Mn_4Ca cluster as it traverses the enzymatic cycle driven by the absorption of four photons? 2) What is the mechanism of the water-oxidation reaction that is catalyzed by the Mn_4Ca cluster? We are using steady state and time-resolved X-ray spectroscopy and crystallography methodologies at synchrotron and X-ray free electron laser (XFEL) sources in pursuit of these goals.

Significant achievements 2011-2013:

1) We developed methodologies for collecting simultaneous X-ray diffraction (XRD)/X-ray emission spectroscopy (XES) data from PS II using femtosecond pulses from an XFEL. These include: a) An electrofocusing nanoflow system that introduces very small volumes of PS II into the X-ray laser interaction region. With this system we were able to conserve the amounts of PS II used. b) We designed and optimized a dispersive XES method for collecting shot-by-shot spectra using the X-ray laser. We demonstrated that XES of high-valent Mn complexes relevant to PS II is possible at room temperature using an XFEL, without any damage to the Mn complexes. c) We then collected simultaneous XRD and XES data of PS II at room temperature in the dark S_1 and illuminated S_2 states. We showed that the 'probe before destroy' approach works for PS II and that we can collect undamaged XRD/XES data from the Mn_4Ca cluster at room temperature. We identified the electron-density from the Mn_4Ca cluster from both the S_1 and S_2 states. At the present resolution, no significant changes were observed between the S_1 and S_2 states. The setup we developed for these simultaneous measurements is shown in Fig. 1.

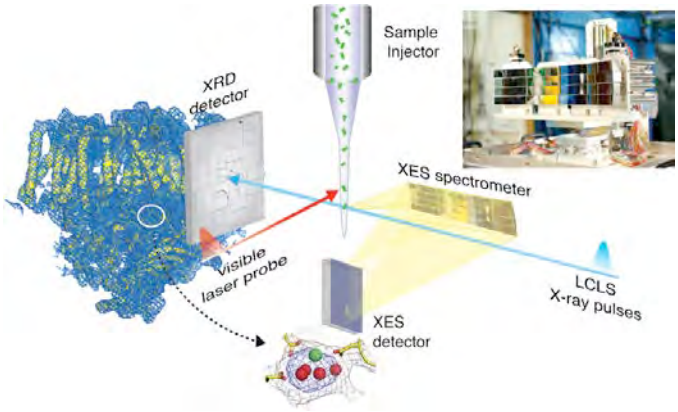

Fig. 1. Schematic of the simultaneous detection of XRD and XES of PS II crystals. PS II crystals are injected using an electrofocusing nanoflow system. Downstream of the X-ray laser is a multi-pixel detector that collects the X-ray diffraction from the crystals. A dispersive X-ray emission spectrometer with 16-cylindrically bent analyzer crystals focus the Mn Kβ XES on a position sensitive detector.

2) Using the XFEL, we have collected undamaged Mn L-edge spectra from dilute aqueous solutions of Mn using a newly designed zone-plate spectrometer that can discriminate the Mn Lα signal from the overwhelming signal from the O Kα. This study demonstrated that studies with PS II are feasible.
3) We further developed methodologies for collecting simultaneous XRD/XES data from the S-state intermediates of PS II using the XFEL pulses by triggering the S-state transitions with

multiple visible laser excitations. Three visible laser fiber optic cables, that are coupled to the capillary, illuminate the samples with 0.5 s intervals between flashes to achieve S-state turnover. We used a similar system to measure O_2 evolution yields using $H_2{}^{18}O$ labelled water and optimized the flow rate, laser power and time interval required for S-state turnover.

4) Structure determination by XRD depends on the accurate extraction of Bragg reflection intensities from the diffraction pattern and this proves to be challenging for XFEL data. Firstly, experiments involve the sequential exposure of micron-sized crystals from a heterogeneous pool. Secondly, pulses from the XFEL source vary greatly in intensity and have a wide spectrum (bandwidth~0.5%) that can affect the size and shape of the Bragg reflections. Finally, currently available area detectors consist of tiled imaging sensors whose mutual alignment requires calibration. We are developing new computational methods designed to account for all of these phenomena. Protocols for analysing both XRD and XES data from the XFEL in real time were developed, so we could assess the quality of the data to decide on the course of the experiment. We used thermolysin as a test case to validate all our XFEL analysis protocols and experimental conditions. We were able to identify the Zn and Ca atoms in thermolysin using the anomalous signals from the metals. The same protocols were used to identify anomalous signals from Mn in PS II in the S-states.

Science objectives for 2013-2014:

- We have two regular and two screening X-ray beamtimes at the X-ray laser over the next year. We plan to conduct simultaneous XRD and XES of all the S-states at room temperature, and particularly follow the fast steps between the S_3 to S_0 transition in a time-resolved manner.

  The S_3 to S_0 transition, and the time-points between these transitions will be the focus of these studies. Anomalous scattering analysis will be pursued.

- Mn L-edge spectroscopy of all the S-states and the intermediate states between the S_3 and S_0 states will be studied using the X-ray laser to determine the electronic structural changes that are involved in the O-O bond formation.

- We will extend our studies at the XFEL to XANES/EXAFS and $K\beta_{2,5}$ XES.

- The quality of PS II crystals will be improved by exploring new detergent extraction protocols. We will explore new methodologies for injecting the PS II samples into the intersection region.

References to work supported by this project 2011-2013:

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Elucidating the Principles that Control Proton-Coupled Electron Transfer Reactions in the Photosynthetic Protein, Photosystem II.

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Program Scope. The solar water-splitting protein complex, photosystem II (PSII), catalyzes one of the most energetically demanding reactions in Nature by using light energy to drive a catalyst capable of oxidizing water.[1] Proton-coupled electron transfer (PCET) reactions, which are exquisitely tuned by smart protein matrix effects, are central to the water-splitting chemistry of PSII. Elucidating the water-splitting chemistry of PSII is of major importance in designing bio-inspired catalytic systems for solar fuels production. Proton motion coupled to electron transfer is the basic mechanism of biological energy conversion. However, the details of PCET processes are not yet understood because of the inability of conventional methods to directly probe the reactions. A major challenge is to develop methods to directly probe PCET reactions to understand the structural requirements for minimizing the energetic penalty for multiple charge transfers. *The objective of our research program is to understand the tuning and regulation of PCET reactions of PSII and to elucidate their role in the early charge-transfer steps of photosynthesis. We are determining the factors that control the coupling of proton (PT) and electron transfer (ET) pathways in PSII by the development of new state-of-the-art multi-dimensional and time-resolved nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy methods.*

Significant Achievements 2011-13. (A) The binding and activation of substrate water molecules in the oxygen-evolving complex of photosystem II. The light-driven four-electron water oxidation reaction occurs at the tetranuclear manganese-calcium-oxo (Mn_4Ca-oxo) catalytic cluster in the oxygen-evolving complex (OEC) of PSII. The mechanism of the water oxidation reaction has been the subject of intense interest and the OEC has been studied extensively by structural, spectroscopic, biochemical and computational methods. The recent 3.8-1.9 Å resolution X-ray crystal structures and single-crystal EXAFS studies provide a model for the catalytic Mn_4Ca-oxo cluster. However, the structure of the OEC, the participation of the protein environment in substrate activation and the mechanism of charge transfer at the bound water molecules have been elusive. (a) In a ground-breaking development, we have unambiguously resolved the individual spectroscopic signatures of the substrate water molecules that are directly ligated to the Mn_4Ca-oxo cluster in the S_2 state of the OEC of PSII. These experiments provide a direct handle to monitor the activation and catalysis of the substrate water molecules in the solar water oxidation reaction.[2] (b) We have used pulsed EPR spectroscopy to investigate the role of the Ca^{2+} ion in the solar water oxidation reaction of PSII. The Mn_4Ca-oxo cluster of PSII consists of three manganese ions and a calcium ion that form a distorted cubane and fourth manganese ion that is a dangler. The structural and functional differences that are induced by metal ion substitution provide valuable insight on the role of the Ca^{2+} ion in water oxidation. By exploiting the power of 2D ^{1}H HYSCORE spectroscopy, for the first time, we detect the protons that are weakly hyperfine coupled to the Mn_4Ca-oxo cluster in the S_2 state of Sr^{2+}-substituted PSII.[3] (c) The oxo-manganese dimer, $[H_2O(terpy)Mn^{III}(\mu\text{-}O)_2Mn^{IV}(terpy)OH_2](NO_3)_3$ (terpy = 2,2':6',2''-terpyridine) (1) is an excellent biomimetic model of the Mn_4Ca-oxo cluster. Recently, we have quantitatively characterized the weak magnetic interactions between protons of two terminal water ligands and the paramagnetic dimanganese 'di-μ-oxo' core of 1 using 2D ^{1}H and ^{14}N HYSCORE spectroscopy. The hyperfine parameters provide important insight on the mechanism of water oxidation by 1 and serve as a valuable resource to interpret the 2D ^{1}H HYSCORE spectroscopy of the bound substrate waters in the OEC of PSII.[4,5] **(B) The tuning and control of quinone cofactors in photosynthetic reaction centers**. Quinones are widely used as electron transport cofactors in photosynthetic reaction centers (RC). It is thought that the structure and substituent groups of the quinone, the location of the quinone cofactor, the geometry of its binding site, redox potential and 'smart' matrix effects from the surrounding protein environment

greatly influence the functional properties of quinones in photosynthesis. (a) We have conducted a systematic study of the principal components of the g-tensors of a library of model benzosemiquinone anion radicals in both protic and aprotic solvents using high-frequency 130 GHz EPR spectroscopy. The increased electron Zeeman interaction at 130 GHz EPR frequency leads to a complete resolution of spectral features arising from three canonical orientations of the g-tensor. This study significantly extends the experimental basis needed for elucidating the functional tuning of quinone cofactors in solar energy conversion.[6] (b) We have examined an extensive library of 1, 4 naphthoquinone models using cyclic voltammetry, *cw* and pulsed EPR spectroscopy to directly determine the electronic properties of naphthoquinones in aprotic and protic environments. We find that there is a direct correlation between the redox potential and the change in electronic properties of the naphthosemiquinone anion radicals. A comparison of naphthosemiquinones in both aprotic and protic solvents indicates that H-bonds consistently accentuate the effects of the substituent groups on the naphthoquinone.[7] (c) PSII offers a unique system to investigate the factors that influence quinone function in photosynthesis. It contains identical plastoquinones in the primary and secondary quinone acceptor sites, Q_A and Q_B, which exhibit very different functional properties. We have utilized 2D ^{1}H and ^{14}N HYSCORE spectroscopy to directly probe the strength and orientation of the H-bonds of the Q_A^- state with the surrounding protein environment of PSII.[8] (d) The phylloquinones of PSI, A_{1A} and A_{1B}, exist in near-equivalent protein environments but possess distinct thermodynamic and kinetic properties. The strength and geometry of hydrogen bond interactions are considered significant factors in tuning and control of function. The 2D ^{1}H HYSCORE experiments indicate that A_{1A}^- forms a single out-of-plane hydrogen bond and the 2D ^{14}N HYSCORE spectroscopy experiments support a backbone amide nitrogen as the hydrogen bond donor.[9] **(C) The functional specificity of the tyrosine residues of PSII: Mechanism of proton-coupled electron transfer at Y_Z and Y_D.** The D1 and D2 proteins that form the core of PSII each contain a redox-active tyrosine residue, Y_Z and Y_D, respectively. Recent models invoke Y_Z directly in the O_2 evolution reaction as an abstractor of protons and/or hydrogen atoms from substrate water molecules bound to the Mn_4Ca-oxo cluster. In contrast, Y_D does not participate in rapid electron transfer in the O_2 evolution process. We have developed pulsed HF ENDOR spectroscopy methods to structurally characterize the $Y_D^\bullet$ PCET intermediates of PSII. This study provides direct 'snapshots' of functional PCET intermediates and, for the first time, makes it possible to detail the mechanism of PCET in biological solar energy transduction.[10]

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Immunophilins and their functions in the assembly of photosynthetic complexes

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Overall research goals:

The research objectives are to study the function of immunophilins in the assembly and maintenance of photosynthetic complexes, the photosynthetic machines that convert light energy into chemical forms. The specific objectives are: (1) To dissect the mechanism of action of FKBP20-2 and its homologues in the assembly of photosystem II (PSII); (2) To identify the targets of Cyclophilin 38 (CYP38) and its mode of action by structural analysis; (3) To identify the function of other immunophilins in the thylakoid lumen of Arabidopsis chloroplasts.

Immunophilins are chaperones and/or foldase of proteins and represent the largest family of thylakoid proteome in the photosynthetic organelles. Illustrating function and mechanism of action of immunophilins should provide a critical path towards greater control and enhancement of enzymatic reactions. Extending study of these reactions to the level of single molecules should reveal novel insights into reaction trajectories and dynamics, perhaps eventually yielding higher reaction rates with enhanced stabilities.

Significant achievements 2011-2013:

- Function of FKBP20-2 and its homologues: Single mutant containing FKBP20-2 knockout showed defects in photosystem II (PSII) assembly. Several approaches hare been taken to explore the mechanism of action of this critical immunophilin in the thylakoid lumen. First, we identified FKBP20-2 interacting proteins and are testing the functional relevance of these partner proteins to the assembly of PSII. Secondly, we started to test the functional redundancy of FKBP20-2 with other FKBPs by double mutant analysis. In particular, we isolated the double mutant *fkbp20-2/fkbp16-3* that showed more severe defects in photosynthesis as compared to either single mutant.
- Structure of CYP38 and its mode of action: To identify mechanism of action by CYP38, a key cyclophilin that function in PSII assembly, we have solved the crystal structure of this protein and found some very interesting features of this protein. The structure reveals two distinct domains: an N-terminal helical bundle and a C-terminal cyclophilin β-barrel, connected by an acidic loop. Two N-terminal β-strands become part of the C-terminal cyclophilin β-barrel, thereby making it a novel domain organization. The study shows that CYP38 does not possess a PPIase function and identified a possible interaction of CYP38 with the E-loop of CP47, a component of photosystem II. The interaction of CYP38 with the E-loop of CP47 is mediated through its cyclophilin domain. The N-terminal helical domain is closely packed together with the putative C-terminal cyclophilin domain and establishes a strong intramolecular interaction, thereby preventing the access of the cyclophilin domain to other proteins. This study describes the first structure of a plant cyclophilin and explains a possible mechanism of auto-inhibition of its function through an intramolecular interaction.
- Characterization of D1 C-terminal processing enzyme: CtpA was identified by a yeast two hybrid assay as partner for an immununophilin member. Functional analysis of this enzyme

by genetic mutant in Arabidopsis showed that CtpA is required for the function and assembly of PSII, a central function of immunohilins.

- Other immunophilins and their function in photosynthesis: There are 16 immunophilins thylakoid lumen of Arabidopsis, making this family of proteins the largest of the proteome in the thylakoid lumen. FKBP20-2 and CYP38 are the only members of this family that have been genetically analyzed, which were done first in our group. In the process of analyzing other members, we identified all the mutants of the corresponding genes and are in the process of phenotyping the mutants.

Science objectives for 2013-2014:

- Characterization of partners of FKBP20-2 and FKBP16-3: Double mutant of the two genes caused more severe defects in photosynthesis, suggesting that FKBP20-2 and FKBP16-3 functionally overlap. The two proteins each has a disulfide bond and appears to be regulated by redox potential. We will determine whether the two pairs of cysteins are required for the function of the proteins by complementation of the mutant plants.
- Identification of CYP38 partners and their function in PSII assembly: CYP38 has two domains one of which is catalytic and should interact with substrates or targets. Several candidate proteins have been identified by yeast two hybrid assay. Further study will confirm interaction of these proteins with CYP38 and make link between CYP38, their targets, and PSII assembly.
- Continue to dissect the function of other immunophilins in the thylakoid lumen: CYP37 is a unique member of the thylakoid immunophilin. This member may be related to the retrograde signaling between chloroplast and nucleus. We will further determine the function and the mechanism of action of CYP37 using the genetic and biochemical approaches.

Publications:

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- Vasudevan D, Fu A, Luan S, Swaminathan K. (2012) Crystal structure of Arabidopsis cyclophilin38 reveals a previously uncharacterized immunophilin fold and a possible autoinhibitory mechanism. **Plant Cell** 24, 2666-74.
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Session VI

Chloroplast and Thylakoid Biogenesis

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Overall research goals: The overall research goals of the lab are to understand the various factors that play a role in chloroplast biogenesis. Much of the lab's effort is directed toward studying the accumulation of nuclear-encoded proteins in the plastid, especially the mechanisms and energetics of protein transport across chloroplast membranes. Other avenues that shed light on chloroplast biogenesis can be explored, and we have made significant inroads in the past year in one of these, namely, the mechanism by which thylakoids reproduce in concert with chloroplast division.

Significant achievements 2011-2013: One of the major goals of the lab is the quantitation of the amount of energy required for the import of proteins into chloroplasts. We took advantage of the fact that this import process has been shown to depend only on exogenously added ATP to monitor ATP hydrolysis during protein import and then normalize it to the amount of protein imported. We found that approximately 650 ATP molecules are hydrolyzed during the import of the RuBisCO small subunit, from which we estimate the $\Delta G_{protein\ import}$ to be about 27,300 kJ/mol protein imported. This represents only the second complete accounting of energy required to transport a protein across a biological membrane.

We also explored the question of whether thylakoids possess a replication machinery independent of that governing plastid division. To this end we made two size-indicating measurements of thylakoids present in the Arabidopsis chloroplast division mutant Arc6. This mutant displays a giant chloroplast phenotype in which the normally 60 – 100 chloroplasts found in wild-type cells is replaced by one giant plastid with little loss in total chlorophyll. That is, the chloroplasts in the Arc6 mutant appear to grow and accumulate chlorophyll as do wild-type cells, but they do not divide, so that the photosynthetic content of 60 – 100 plastids is contained in one giant chloroplast. Since it is known that each wild-type chloroplast possesses only one multiply folded thylakoid, we reasoned that if thylakoids possess their own division machinery, the giant Arc6 chloroplast should contain 60- 100 normal-sized thylakoids. If, on the other hand, thylakoids divide only by being pinched in two during plastid division, then giant Arc6 plastids would be expected to contain one giant thylakoid.

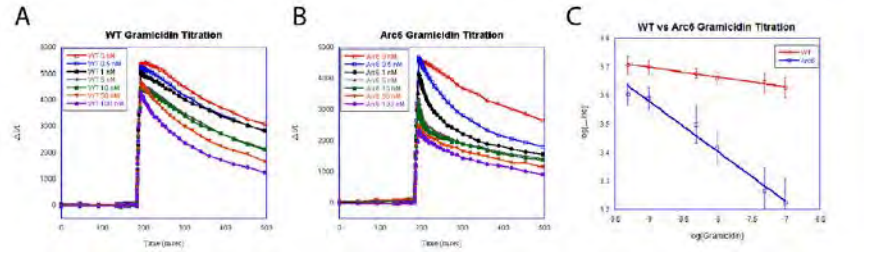

Figure 1. Titration of the electric field-indicating carotenoid electrochromic shift with gramicidin in wild-type (A) and Arc6 thylakoids (B). Signals were illuminated for 9 ms and were fitted to a falling double exponential equation. C, height of signal at 9 ms (start of dark period) as a function of gramicidin. The slope is an indication of the sensitivity of this amplitude to the ionophore.

These two possibilities were distinguished by measuring the size of the Arc6 thylakoids by two independent methods. In the first, we determined the relative size of the thylakoid electrical unit by titration with the pore-forming ionophore gramicidin.

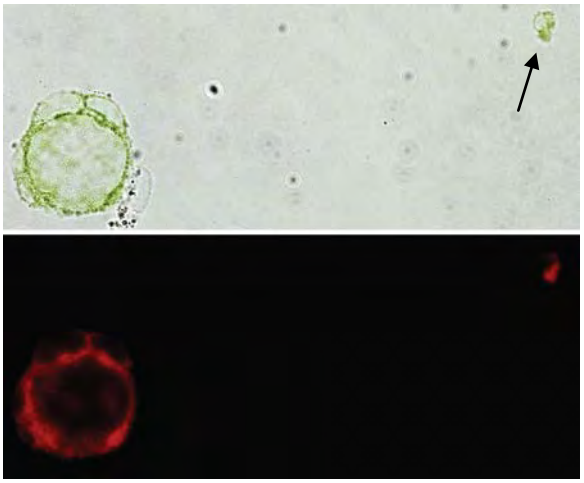

Figure 2. Blebs formed from Arc6 (left) and wild-type (right) chloroplasts. The upper and lower panels show bright field and auto-fluorescence images, respectively. The arrow in the upper panel marks the wild-type bleb.

The results, shown in Fig. 1, indicate that Arc6 thylakoids are more sensitive to membrane potential collapse by gramicidin than are those from wild-type chloroplasts, indicating that the target membranes into which gramicidin is incorporated are considerably larger in the mutant thylakoids.

The second method to determine the relative sizes of wild-type and Arc6 thylakoids is to form blebs by placing the isolated chloroplasts in distilled water. Figure 2 shows a microscopic image with both Arc6 and wild-type blebs in the same field. This once again indicates that the giant chloroplasts contain giant thylakoids. We interpret these results to indicate that the thylakoids do not replicate independently from the plastids, and are likely pinched in two during the chloroplast division process.

Science objectives for 2014 – 2015: In the time remaining before the project's termination we will have the following objectives:

- We will attempt to determine the factors that control the energetics of protein import, e.g., the size or stability of the precursor. Success with this objective requires us to further decrease the amount of ATP hydrolyzed by intact chloroplasts that is unrelated to the import process.
- We will examine the size of blebs formed by other chloroplast division mutants, e.g., Arc3 and Arc5. The lesions in these mutants produce different chloroplasts numbers than Arc6, and are targeted to different proteins in the division machinery.

References to work supported by this project 2011 – 2013:

Shi, L-X and Theg, SM (2010) Measurement of the energetic cost of protein import across the envelope membranes of chloroplasts. Proc. Natl. Acad. Sci. USA 110:930-935.

Photobiological Solar Fuels

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Overall research goals: The goal of the Photobiological Solar Fuels Program is to investigate the regulation of photosynthetic reductant partitioning between H_2 evolution, biomass and other storage molecules that accumulate under different stress conditions. The objective is to understand the interplay between stress sensing, signal transduction, and transcriptional regulation of specific genes that determine the fate of photosynthetic reductant. This, in turn, will allow one to manipulate and change the metabolic state of the cells under controlled conditions. We are particularly interested in the mechanism of the interaction between different signal/sensor, and sensor/transducer molecules, and the complex networks between different signal and the expression of specific genes at the mechanistic level. This project consists of two subtasks that address, respectively, (a) factors required for the regulation of photosynthetic reductant partitioning between CO_2 fixation and H_2 production under anaerobic conditions; and (b) factors required for regulation of reductant and carbon partitioning between biomass and carbon storage molecules under N and other stresses.

Significant Achievements FY 2012-13:

Subtask A: A *Chlamydomonas reinhardtii* mutant identified from an insertional mutagenesis library as attenuated in hydrogen photoproduction contains an interruption in a prolyl 4-hydroxylase (P4H) gene. Interestingly, P4Hs were shown to be up-regulated upon anaerobic induction in Chlamydomonas and, in other organisms, these proteins are known to regulate a number of targets, including those involved in cell wall integrity and hypoxic response. Characterization of this P4H mutant indicates (a) altered aerobic and anaerobic metabolism, and (b) no significant effect on the transcription of hydrogenase and other genes involved in anaerobic metabolism (see Figure 1). These results suggest a role for this P4H in post-trancriptional regulation of selected enzymes. We cloned the P4H gene into an expression vector, expressed the protein in *E. coli*, and purified the recombinant protein. Future research efforts will focus on elucidating its specific role in *Chlamydomonas a*naerobic metabolism.

Subtask B: Synechocystis 6803 was engineered to be (1) unable to synthesize glycogen, a major carbon storage compound, and (2) able to grow on xylose, a previously unusable carbon source for this cyanobacterium. Under either N starvation and/or mixotrophic growth with xylose, carbon flux was redirected to the synthesis of pyruvate and alpha ketoglutarate (AKG), which were secreted into the medium. This represents a more general physiological state termed “metabolic overflow” that was also found in other microbes but lacks a clear mechanistic understanding. We hypothesize that the driving force of metabolic overflow in our cyanobacterium is the generation of ATP and NAD(P)H by the combination of photosynthesis and sugar metabolism. More specifically, we suggest that the ratio of ATP:NADPH regulates

carbon flux between the two new sinks, as a means to maintain proper cofactor balance in the cells. Using red, yellow, and white lights that are known to differentially excite PSI and PSII and thus enhance production of ATP versus NADPH, we found changes in the ratio of pyruvate:AKG that are consistent with this hypothesis.

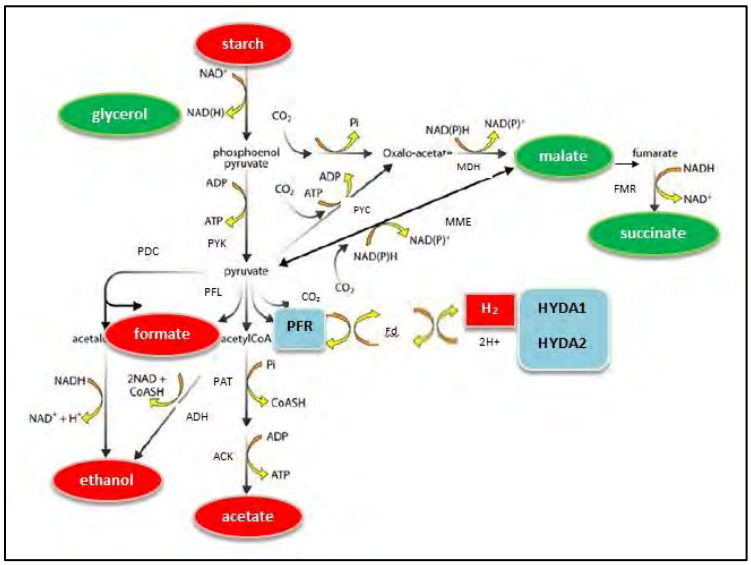

Figure 1. Under aerobic growth conditions, the P4H mutant shows decreased accumulation of starch (red). Following anaerobic induction, it shows increased concentrations of glycerol, malate, succinate (green), and decreased concentrations of acetate, ethanol, and formate, as well as decreased rates of H_2 photoproduction (red). Transcriptional abundance of hydrogenase genes and pyruvate formate lyase (PFR) (blue) are not significantly increased.

Science objectives for FY 2014-2015:

- Subtask A: The purified P4H protein is being used in pull-down assays to identify its protein targets in *Chlamydomonas* and will be assayed in vitro for its enzymatic properties. Concomitantly, analyses of transcription abundance will be completed for several other metabolic genes. These results will guide subsequent studies in order to ascertain the role of this P4H in the regulation of H_2 photoproduction.
- Subtask A: A transcriptional factor homologous to the Whirly factor in plants was previously identified as interacting with the HYDA1 and HYDA2 promoter regions. This interaction will be confirmed through gel-shift assays, followed by investigation of the protein intracellular localization and interacting partners.
- Subtask B: The hypothesis that ATP and NAD(P)H regulate metabolic overflow in our cyanobacterium will be further tested by direct measurement of these metabolites, and by additional physiological manipulations of their relative levels that may include inhibition of PSII activity or of cyclic electron flow around PSI.

References to work supported by this project in FY 2013:

Damian Carrieri, Troy Paddock, Pin-Ching Maness, Michael Seibert and Jianping Yu. Photo-catalytic conversion of carbon dioxide to organic acids by a recombinant cyanobacterium incapable of glycogen storage. (2012) Energy and Environmental Science 5: 9457-9461.

Biochemical Integration of Metabolic Networks Critical for Energy Transformation in *Chlamydomonas reinhardtii*

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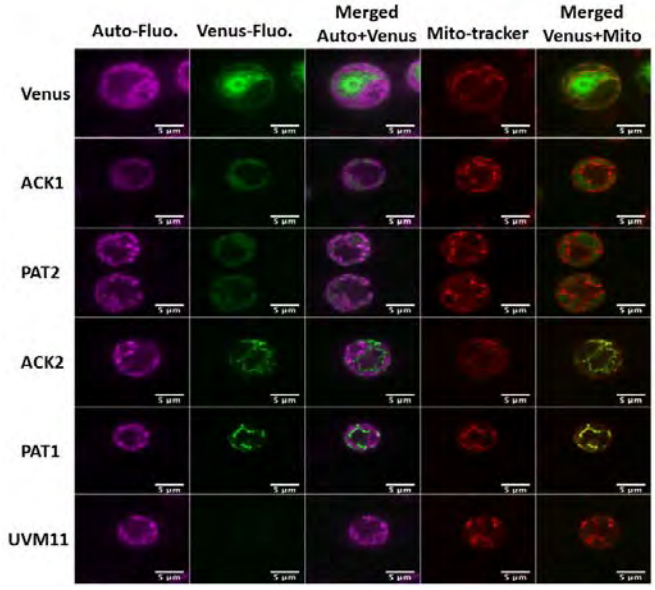
Overall Research Goals: Genome sequencing combined with 'omics' techniques have provided important biological insights regarding gene content and arrangement, splicing variants, promoter sequences, putative protein activities, expression patterns and metabolite levels (especially during environmental fluctuation). This in turn has allowed the rudimentary construction of metabolic pathway maps in several model photosynthetic microorganisms (PSMs). To further refine these models, it is now necessary to probe how metabolic pathways are activated, regulated and prioritized, and how specific enzymes are spatially organized within cellular compartments and integrated into metabolic protein complexes. This understanding requires biochemical characterizations that include defining the precise site(s) to which proteins are targeted within a cell, protein-protein interactions (and the dynamics of these interactions), metabolite channeling, and the analysis of mutants that impact specific metabolic processes to verify/refute hypotheses. To elucidate how PSM metabolism is organized and regulated in *Chlamydomonas reinhardtii* we are (**a**) determining the spatial localization of key metabolic enzymes within specific subcellular compartments, (**b**) establishing the network of protein-protein interactions that define metabolic complexes and metabolite routing, (**c**) exploring the various ways in which enzyme activities are modulated at the posttranslational level, (**d**) assessing the impact of deleting key metabolic activities, and (**e**) determining how specific enzyme activities are integrated into a global biochemical palette that strongly impacts growth, development and primary productivity.

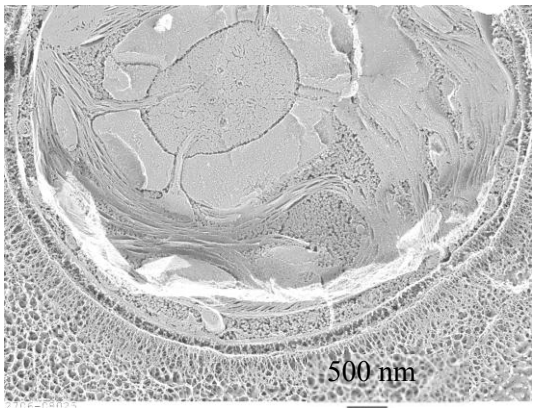
Significant achievements 2012-2013:
A suite of mutants with deletions in key *C. reinhardtii* fermentative enzymes were constructed or acquired from other efforts) and are being complemented with tagged versions for protein purification and localization. Additionally, metabolic impacts are being assessed under a variety of culturing conditions and in multi-gene deletion backgrounds. Genetic disruptions include genes encoding acetate kinase, phosphate acetyltransferase, pyruvate formate lyase, alcohol dehydrogenase, [FeFe]-hydrogenases, NAD(H)/NADP(H) transhydrogenase, and a ferredoxin isoform (FDX5). Some of these deletions (e.g. ferredoxin and NAD(H)/NADP(H) transhydrogenase) also impact oxic metabolism. In the last year, efforts have focused primarily on the characterization of mutants with disruptions in acetate kinase and phosphate acetyltransferase. *Chlamydomonas reinhardtii* encodes two distinct isoforms of each enzyme, and in the case of acetate kinase, we have demonstrated that distinct plastid and mitochondrial isoforms exist. Surprisingly, disruption of both acetate kinase genes resulted in cells that are still able to secrete high levels of acetate, which was not predicted from metabolic models and indicates that alternative routes to acetate are present. This is in stark contrast to deletions in pyruvate formate lyase and alcohol dehydrogenase activity, mutants that exhibit profound metabolic reorganizations resulting in the secretion of products that are typically below detection limits in control strains.

Starch is a primary product of photosynthetic activity and a substrate for a myriad of downstream metabolic processes. We have manipulated the expression of the isoamylase gene to dramatically impact starch accumulation, which is eliminated in the deletion strain and hyperaccumulated (~4x in nutrient replete medium) in overexpressing cell lines. Both mutants have a profound effect on overall

carbon metabolism. We have shown that isoamylase transformants that hyperaccumulate starch have a significantly altered pyrenoid starch structure, and that thylakoid membrane spans gaps in the pyrenoid starch sheath to make substantial contact with the RuBisCO rich protein complexes within the pyrenoid. This has implications in the localization of starch biosynthetic/catabolic enzymes and the localization of Calvin cycle enzymes.



Left: localization of acetate kinases (ACK1/2) and phosphate acetyltransferase (PAT1/2) in *C. reinhardtii*. Below: pyrenoid starch in *C. reinhardtii* isoamylase transformant. The pyrenoid sheath is ~2 fold thicker than in control cells and thylakoid membrane spanning gaps in the pyrenoid starch can be clearly visualized.



Science objectives for 2013-2014:

- Conclude detailing the localization and role of acetate kinase and phosphate acetyltransferase in fermentation metabolism.
- Characterize and spatially localize the NAD(H)/NADP(H) transhydrogenase, and study the metabolic role of this enzyme in *C. reinhardtii*.
- Characterize and localize the FDX5 protein and assess its role in *C. reinhardtii* metabolism.
- Localize the position of isoamylase within the *C. reinhardtii* plastid and further characterize its role in starch metabolism.

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Biochemical Integration of Metabolic Networks Critical for Energy Transformation in *Chlamydomonas reinhardtii*

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Research objectives: *Chlamydomonas reinhardtii* (Chlamydomonas throughout) is a unicellular soil-dwelling green alga. Sequencing of the Chlamydomonas genome has provided us with important biological insights concerning metabolic pathways in this alga. We are now using experimental approaches to examine the roles of these pathways in the overall metabolism of Chlamydomonas; these approaches include determining the subcellular localization of proteins/pathways, exploring interactions of proteins both within and among pathways, understanding how the proteins are modified (and what conditions cause modification) and how metals like iron modulate energetic and stress pathways (Hoehner et al., 2013), and determining how deletions of specific enzymes in key pathways of energy metabolism impact anoxic and oxic metabolism (Yang et al., 2013a; Catalanotti et al., 2013). The integration of chloroplast and mitochondrial metabolism will be absolutely critical to elucidate if we are to understand the dynamics of energy metabolism, so over the last year we have focused on determining the subcellular localizations of proteins important for energy metabolism (see below). However, we have also begun to examine the roles of enzymes and regulatory proteins critical for energy metabolism.

Specific Achievements.

Phosphate acetyltransferase (PAT)/acetate kinase (ACK): Previous work (proteomic work) with Chlamydomonas has suggested that PAT2/ACK1 are localized to chloroplasts and PAT1/ACK2 to mitochondria (Atteia et al., 2009; Terashima et al., 2011; Yang et al., 2013a). Wenqiang Wen used various experimental procedures to determine the subcellular localizations of these proteins in Chlamydomonas; this data is presented in the abstract submitted by Matthew Posewitz.

We also recently isolated Chlamydomonas insertion mutants disrupted for the *ACK1* and *ACK2* genes, as well as for the *PAT2* (but not *PAT1*) gene; the pathway encoded by these genes use acetyl-CoA for the synthesis of acetate coupled to ATP production (Yang et al., 2013b). External metabolites synthesized in the *pat ack* mutants under anoxic conditions suggest that there is more acetate production from the chloroplast than from the mitochondrion and that the rate of glycolysis is signficiantly diminished in the mutants releative to wild-type cells. Interestingly, although we were unable to detect acetate kinase activity in the *ack1ack2* double mutant, this strain was still able to accumulate extracellular acetate. These results suggest that other enzymes in the cell can be recruited to convert pyruvate, acetaldehyde and/or acetyl-CoA to acetate in the absense of the ACK proteins (and we have ideas concerning the enzymes involved in the converstion). Furthermore, while the *ack1* and *ack2* single mutants and the *ack1ack2* double mutant exhibited essentially no lactate production, lactate production in the *pat2-1* and *pat2-1ack2* mutants was elevated, suggesting differences in the regulation of fermentation metabolism among the strains, with the *pat2* lesion eliciting a marked difference in accumulation of extracellular fermentation products. Much of this work will be discussed by Matthew Posewitz.

Fdx5 and its role under dark anoxic conditions: Ferredoxins (FDXs) play a crucial role in delivering reducing equivalents to various reactions in the cell. Chlamydomonas has 6 plant type FDXs. We do not know the functions/specificities of many of these redox proteins in cellular metabolism. Interestingly, the *FDX5* gene seemed to be activated under anoxic conditions as well as during various stresses. We isolated a *fdx5* mutant and found that it showed some very pronounced phenotypes (Yang et al., 2013c).

1. It was unable to grow in the dark.
2. Photosynthetic electron transport and mitochondrial respiration are compromised in the dark.
3. It has a strongly altered ratio of DGDG to MGDG; the DGDG in the mutant is much higher than the MGDG (relative to wild-type cells), but only in the dark. It looks normal in the light.

4. Additionally, the mutant doesn't make much C16 fatty acid with four double bonds (unusual fatty acid made by Chlamydomonas); when this fatty acid is present in MGDG, it prevents its conversion to DGDG.
5. Within 48 h of transfer of the cells from light oxic to dark oxic conditions, the membranes of the thylakoid become severely deformed, which might explain the lack of growth in the dark and altered photosynthetic and respiratory activities of the mutant in the dark. OUR HYPOTHESIS IS THAT FDX5 IS NEEDED FOR THE DESATURATION OF FATTY ACIDS TO MAKE THE C16 WITH THE FOUR DOUBLE BONDS. WHEN THIS FATTY ACID CAN'T BE MADE, THE MGDG IS CONVERTED TO DGDG, WHICH CAUSES PROBLEMS WITH THE MEMBRANE STRUCTURE, DISRUPTING PHOTOSYNTHESIS AND RESPIRATION, AND CRIPPLES THE CELLS VERY RAPIDLY IN THE DARK. Membranes from the mutant grown in light and dark oxic conditions are shown in **Figure 1**.

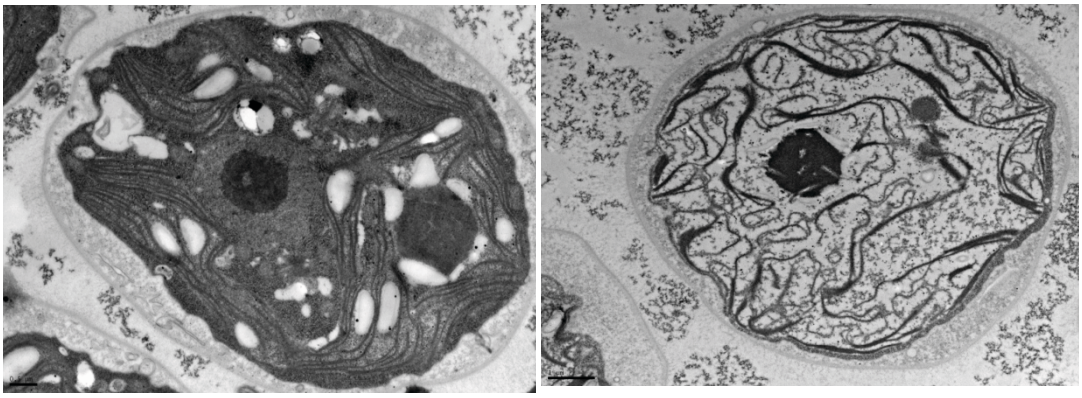

Figure 1. TEM of Chlamydomonas *fdx5* mutant grown in light (left) and in the dark (right). Note the disorder of the membranes in the dark. This is dramatically different from wild-type cells.

There are other aspects of the project that are being explored:
1. The function of prolylhydroxylases in controlling anoxic metabolism (we have deletion mutants in the four prolyl hydroxylase that are upregulated during anoxia.
2. Further characterization of the *fdx* mutant and detemination of its role in dark metabolism.
3. Examination of the *pfr* (also call *pfo*) mutants (we have 5 point mutants), determine which are null for activity, determine if the PFR works in the reverse direction (making pyruvate), determine whether other reactions (other than PFR) allow the reduction of FDX for the generation of H_2.
4. Explore the interactions between PFR and PFL.

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Enhancement of Source-Sink Relationships by Manipulation of Two Starch Regulatory Enzymes, ADPglucose Pyrophosphorylase and Phosphorylase

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Overall research goals: The research objectives are to elucidate the roles of two regulatory enzymes, starch phosphorylase (Pho1) and ADPglucose pyrophosphorylase (AGPase) in controlling starch synthesis. Although Pho is responsible for α-glucan degradation in animals and yeasts, in higher plants it plays an undefined role in starch biosynthesis. Although much more biochemical information has been obtained over the years on the enzymatic properties of the starch regulatory AGPase, significant gaps in our knowledge remain. The overall goals of this project are to understand the biochemical structure-function relationships of these two starch regulatory enzymes and to define their physiological roles in governing source-sink relationships during plant growth and development.

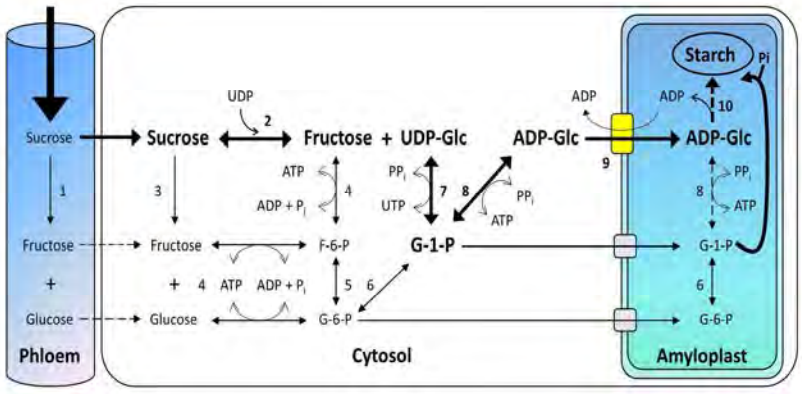

Figure 1. The pathway of carbon flow into starch in cereal endosperm. Sucrose, transported from leaves, is metabolized to UDPglc and then to Glc 1-P, the latter reacting with ATP to form ADPglc (reaction 8). This sugar nucleotide is then transported to the amyloplast where it is utilized by starch synthases. The amyloplast also contains a second AGPase activity (reaction 10) but appears to play a minor role in starch synthesis. Pho1, which uses Glc 1-P for starch synthesis, is also located in the amyloplast and plays some undefined role in starch synthesis.

Significant achievements 2011-2013:

- Detailed kinetic studies of Pho1, even under conditions that favor degradation, support its role in starch biosynthesis. Pho1 is also inhibited by ADPglucose by a mixed type inhibition process, suggesting that this substrate for starch synthesis binds to the Pho1 substrate binding site. The biological significance of this interaction of ADPglc to Pho1 remains unclear.
- The higher plant Pho1 is structurally similar to the animal and yeast phosphorylases except that it contains an extra 80 amino acid peptide (L80) located at about the middle of the primary sequence. Deletion of this peptide had no significant effect on the catalytic properties of the deleted enzyme, suggesting that L80 has a non-enzymatic role.
- Affinity chromatography studies showed that α-1,4-glucantransferase (also known as D-enzyme or Dpe1) interacted with Pho1 and Pho1ΔL80 as baits. D-enzyme catalyzes the transfer of oligosaccharide from one oligosaccharide to another. Gel permeation chromatography and native PAGE analyses showed that Dpe1 forms a homodimer and physically interacts with Pho1 at 1:1 molar ratio to form a multi-subunit complex.
- TILLING studies identified two rice lines containing missense mutations in the cytoplasmic AGPase large (L) subunit. Interestingly, these rice lines have lower starch content than plants

lacking L subunit. Kinetic studies showed that the mutant heterotetramer enzyme, $S^{WT}L^{mut}$ and homotetramer S^{WT} were both defective in 3-PGA activation but that the latter enzyme was more resistant to Pi inhibition than the former. These results provide direct evidence that the large subunit is essential for specifying the allosteric regulatory properties of the endosperm AGPase and that the major route of carbon flux to starch is through the cytoplasmic enzyme form.

- Studies have suggested the regulation of the major endosperm (cytoplasmic) AGPase by redox potential similar to the plastidial enzyme. The cytoplasmic enzyme, however, lacks the conserved small subunit's Cys12, which forms a disulfide bond between the small subunits. Kinetic studies have demonstrated that the rice cytoplasmic AGPase is redox regulated and that the Cys residues required for this control are located on N-terminal region of the large subunit.

Science objectives for 2013-2014:

- We will continue efforts to obtain the 3-D structures of AGPase and Pho1 by identifying conditions to obtain highly diffractive crystals for these enzymes. Ongoing studies have resulted in formation of AGPase crystals, albeit of poor quality. In addition, the wild type AGPase and its large and small subunit homotetramer forms are currently being analyzed by Isothermal Titration Calorimetry (ITC) to determine the substrate and effector binding properties of the large and small subunits.

- The rice *pho1* mutant line has been successfully complemented by wild type Pho1 and Pho1ΔL80 gene sequences. Comparison of the two transgenic lines will provide insights on the possible role of the L80 peptide in starch biosynthesis.

- Transcriptome profiling analysis of transgenic rice over-expressing and under-expressing AGPase have been initiated to assess how rice endosperm copes with elevated and reduced ADPglc and starch levels, respectively. Studies are also being planned to conduct transcriptome profiling of Arabidopsis lines that accumulate elevated leaf starch and lower amounts of leaf starch than wild type. These studies are likely to provide new insights on the relationship between starch metabolism and photosynthetic feedback.

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Poster Session II

Regulation of Thylakoid Lipid Biosynthesis in Plants

Christoph Benning (Principal Investigator)
Jenny Wang, Kun Wang (Graduate Students); Rebecca Roston (Postdoctoral Research Associate); Bagya Muthan (Research Associate).

Overall Research Goals: The overall goal is to gain a mechanistic understanding of the assembly and maintenance of the photosynthetic membranes in plants with a strong focus on its lipid constituents. Establishment and maintenance of the photosynthetic membrane is a dynamic process throughout the life cycle of the plant and involves finely tuned mechanisms to adjust synthesis and breakdown of polar lipids. Currently, the focus is on the turnover of the principal membrane lipid of the photosynthetic membrane, monogalactosyldiacylglycerol (MGDG). Two different processes are investigated that involve recently discovered enzymes SENSITIVE TO FREEZING2 (SFR2) and PLASTID GALACTOLIPID DEGRADATION 1 (PGD1), which catalyze the recycling of MGDG derived diacylglycerol or acyl moieties, respectively, into triacylglycerol following the exposure to abiotic stresses. The specific aims are (1) to determine the activation mechanism of SFR2, (2) to determine the function of SFR2 in lipid remodeling in response to different abiotic stresses, and (3) to determine the function of plant PGD1-like proteins in lipid remodeling and abiotic stress responses.

Significant Achievements (2012-2013):

1. Diacylglycerol (DAG) pools: A detailed characterization of DAG pools in each leaflet of the chloroplast envelope membranes was completed and published. A set of Arabidopsis transgenic lines were produced and are now available to address how DAG pools are affected by remodeling enzymes such as SFR2 and PGD1.
2. SFR2 reaction mechanism: SFR2 was modeled against multiple family 1 glycosyl hydrolases, yielding a well organized structure (Figure 1A, B). Hydrophobic residues conserved among SFR2-like proteins but not among other family 1 glycosyl hydrolases were identified as necessary. Individual mutation of hydrophobic residues near the active site had little effect, while mutation of multiple residues abolished activity, presumably disrupting substrate binding (Figure 1 C).

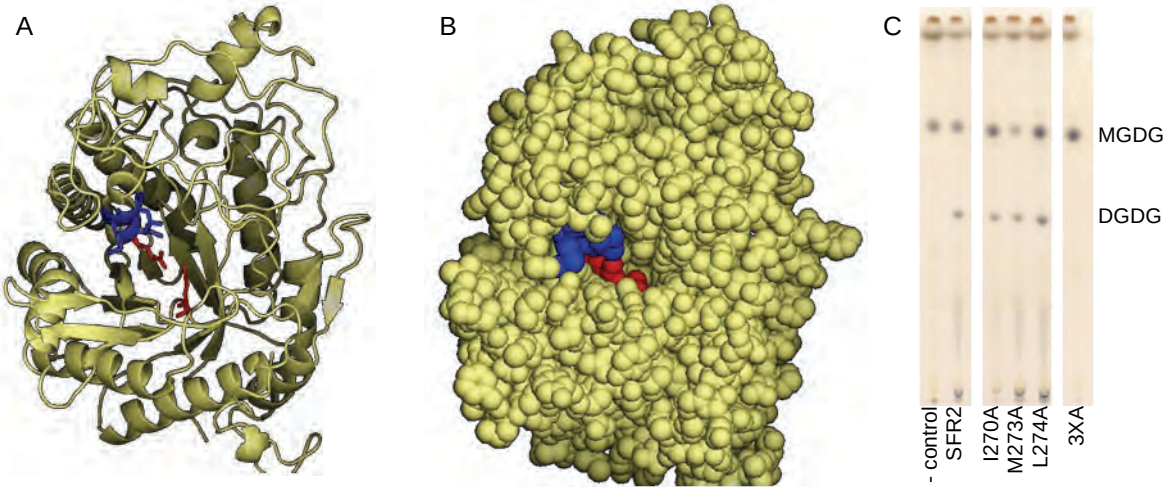

Figure 1. Structure and function of SFR2. (A) Cartoon or (B) Space-filling representation of sophisticated SFR2 model. Catalytic residues E267 and E463 are shown in red, required hydrophobic residues I270, M273 and L274 are shown in blue. They line one side of the substrate binding pocket of SFR2. (C) A thin-layer chromatogram stained for sugars after an SFR2 assay shows the requirement of hydrophobic residues for the conversion of MGDG to DGDG and TGDG. Microsomes isolated from yeast expressing SFR2 constructs or negative control LacZ were assayed *in vitro*. Construct 3XA is a triple mutant of I270A, M273A and L274A.

3. SFR2 activation mechanism: Assays of SFR2 activity in isolated chloroplasts of freezing tolerant Arabidopsis (*A. thaliana*) and freezing sensitive pea (*P. sativum*) have shown that SFR2 is highly

sensitive to external pH within physiological ranges. This observation was confirmed with excised leaves, and is now being extended to study of cellular pH under freezing conditions.

4. Function of SFR2 in different physiological contexts: It is hypothesized that SFR2 plays roles in drought and salt stress tolerance in non-freezing tolerant plants. To study SFR2 in a different physiological context, we generated tomato *SFR2* RNAi lines as an example of a cold/salt stress sensitive plant. Detailed analysis of the transgenic lines is currently underway.
5. Phenotype of *pgd1-like* (*pgdl*) mutants of Arabidopsis and activity of recombinant proteins: The growth of *pgdl* mutants of Arabidopsis was found to be sensitive to abscisic acid present in agar-solidified medium and to cold temperatures. Recombinant PGDL3 was shown to have lipase activity in *E. coli.*

Science Objectives for (2014-2015):

1. Probe the function of Mg^{2+} in SFR2. Deletion and point mutants of SFR2 will be used in determining the precise location of Mg^{2+} to test hypotheses about its role in the SFR2 reaction mechanism.
2. Test the pH activation hypothesis for SFR2 *in vivo*. SFR2 is sensitive to pH, but the role of pH in freezing stress has not been investigated. Arabidopsis expressing pH-sensitive GFP (*Pt*GFP) is being used to probe the cellular pH change during freezing. In parallel, *in organello* tests of SFR2 activation by alteration of membrane fluidity will be pursued. It seems likely that SFR2 is being activated by more than one cellular signal.
3. *Eutrema* (aka *Thelungiella*) *salsugineum SFR2* RNAi lines will be generated to study the function of SFR2 in a freezing and salt tolerant species. A comparative analysis with the tomato *SFR2* RNAi lines will allow us to determine the physiological role of SFR2 in different physiological contexts. In addition, we will produce the recombinant SFR2 proteins from different sources and probe their activity and mode of activation in comparison to the Arabidopsis enzyme. Differences in substrate specificity, processivity, or activation may be exploited in the engineering of plants with elevated abiotic stress tolerance.
4. Test substrate specificity of PGDL proteins in *in vitro* assays. In order to find the potential substrate(s) of PGDL proteins, recombinant proteins will be produced in either *E. coli* or yeast and purified. Lipase *in vitro* assays will be developed and various lipid molecular species will be tested as substrates.
5. Completing the analysis of Arabidopsis *pdgl* mutants. The *pdgl* mutants will be exposed to additional abiotic stress conditions such as osmotic and high salt. Moreover, we will analyze these mutants (as well as the *sfr2* mutant) under fluctuating environmental conditions using the MSU phenomics facility built by David Kramer. D. Kramer has recently shown that environmental perturbations can reveal phenotypes that are otherwise not detectable under constant laboratory conditions.

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Role of Chloride in Photosynthetic Water Oxidation

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Overall research goals: The recent 1.9 Å X-ray crystal structure of photosystem II (PSII) provides a detailed architecture of the O_2-evolving complex (OEC) and the surrounding amino acids. Building on the new structural information, the objective of this project is to characterize the function of PSII by using biophysical studies in conjunction with manganese model chemistry. These experimental studies will guide and test computational analyses of PSII carried out in collaboration with Victor Batista (Yale Univ.), Marilyn Gunner (City College) and Doug Bruce/Sergej Vassiliev (Brock Univ.). The specific aims are: (1) to characterize the redox functions of carotenoids and the secondary electron-transfer reactions in PSII by (a) studying site-directed mutants of PSII in which amino-acid residues predicted to perturb cofactors in the secondary electron-transfer pathways are changed, and (b) redirecting electron transfer on the acceptor side of PSII in order to utilize the electrons liberated from water oxidation by PSII for photoelectrochemical fuel formation; and (2) to carry out structure-based experiments on PSII to characterize the function of specific residues and the mechanism of the OEC by (a) investigating the function of chloride in oxygen evolution by studies of anion-substituted PSII and site-directed mutants of D2-Lys317 and (b) using oxygen isotope studies to gain insight into how the substrate waters are bound and activated for reaction in the OEC.

Significant achievements 2012-2013: X-ray crystallography has revealed that chloride binds as an ion-pair with D2-K317 6-7 Å from the Mn_4Ca cluster in the OEC. Using the structural information for PSII and a comparison with other chloride-activated enzymes, we proposed a role for chloride at the D2-K317 site in PSII [Pokhrel (2011) *et al. Biochemistry* 50, 2725-2734]. To probe the role of chloride at this site, D2-K317R, D2-K317A, D2-K317Q, and D2-K317E mutations were created in the cyanobacterium *Synechocystis* sp. PCC 6803. Purified PSII from the mutants was probed with FTIR difference spectroscopy, EPR spectroscopy, steady-state O_2-evolution assays, and time-resolved O_2-evolution measurements following single-turnover flashes. We found that O_2-evolution is independent of chloride for the D2-K317A mutant. This is the first time a chloride-independent water-oxidizing PSII has been engineered. In contrast, the O_2-evolution activity of D2-K317R is sensitive to the chloride concentration in the assay buffer, but the effective K_D for chloride binding is higher, possibly due to a less optimal binding site in the mutant. Measurements of flash-dependent O_2 yields showed that D2-K317A and D2-K317R PSII have a higher miss factor than wild-type PSII. The O_2-release kinetics for D2-K317A and D2-K317R PSII were slower compared to wild-type PSII, in the order of D2-K317A < D2-K317R < wild type. These results collectively suggest that proton transfer is inefficient in D2-K317A and D2-K317R PSII.

Science objectives for 2013-2014:

- Our studies of PSII in which D2-Lys317 has been mutated highlights the important role played by the hydrogen-bonding network around the OEC. In the coming year, other residues that participate in this hydrogen-bonding network will be investigated including D1-Asn181.
- A focus during the coming year will be to use isotope ratio mass spectrometry to investigate $^{18}O/^{16}O$ kinetic isotope effects for the O_2-evolving reactions of both PSII and inorganic

oxomanganese model complexes. These experimental data will be used to guide computational models of the reaction mechanisms carried out in collaboration with the Batista group.

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11. "S_0 State of the Oxygen-Evolving Complex of Photosystem II", Rhitankar Pal, Christian F. A. Negre, Leslie Vogt, Ravi Pokhrel, Mehmed Z. Ertem, Gary W. Brudvig and Victor S. Batista (2013) *Biochemistry 52*, submitted.

Disruption of the CO_2 concentrating mechanism in C_4 plants: Implications for CO_2 fixation and photosynthetic efficiency during C_4 photosynthesis.

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Overall research goals

- The *overall objective* of this proposal is to determine how changes in the capacity of the C_3 and C_4 cycles and bundle sheath cell wall properties determine the photosynthetic efficiency of the CO_2 concentrating mechanism in C_4 plants in response to changes in light and temperatures.
- Our *central hypothesis* is that the efficiency of the CO_2 concentrating mechanism in C_4 plants is optimized through balancing the activity of the C_3 and C_4 cycles with bundle sheath properties.
- Our *specific aims* are 1) Determine the mechanisms that disrupt the metabolic coordination of C_4 photosynthesis in response to changes in leaf temperature and 2) Determine how changes in the capacity of the C_3 and C_4 cycle control the photosynthetic efficiency of C_4 photosynthesis in response to changes in leaf temperature and light availability.

Significant achievements 2012-2013

Transient changes in light quality Unequal light distribution within leaves and an imbalance in the absorption of photons between photosystems I and II are likely to affect the efficiency of the CO_2 concentrating mechanism in C_4 plants (Ubierna et al., 2013). To test these hypotheses, we measured leaf gas exchange, photosynthetic discrimination, chlorophyll fluorescence, electrochromatic shift, photosynthetic metabolite pools and chloroplast movement in *Zea mays* and *Miscanthus* × *giganteus* following transitional changes in light quality. The rate of net CO_2 assimilation responded quickly to changes in light quality; however, the coordination of C_3 and C_4 cycles in *M. giganteus* was more affected than in *Z. mays*. The species differences appears to be related to differences in the response of cyclic-electron flux around photosystem I and rearrange of chloroplast position in response to changes in light quality (Sun et al., *In press*).

***Temperature response in Z.* mays** We compared measurements of leaf gas exchange and CO_2 discrimination with theoretical models of C_4 photosynthesis and CO_2 isotope exchange to constrain the rate limiting steps of C_4 photosynthesis and the CO_2 concentrating mechanism in *Z. mays* between 10 and 40 °C. Combining modeled and measured gas exchange and CO_2 discrimination provided additional constrains to more accurately determine the *in vivo* temperature response of photosynthesis and the CO_2 concentrating. This analysis demonstrated that carbonic anhydrase (CA) limits C_4 photosynthesis in *Z. mays* under low CO_2 availability across all measurement temperatures (Ubierna and Cousins, *In prep*).

***Biochemical temperature response in* Setaria viridis** Phosphoenolpyruvate carboxylase (PEPC), CA and Rubisco potentially catalyze rate-limiting steps of C_4 photosynthesis in response to temperature. However, there are few reports of the temperature response of these enzymes from C_4 plants. Therefore, we've measured with a membrane inlet mass spectrometer the *in vitro* temperature responses of CA, PEPC, and Rubisco in the C_4 model plant *S. viridis* (Boyd et al., *In prep*). These parameters were incorporated into theoretical models of leaf gas exchange and CO_2 discrimination models of C_4 photosynthesis to constrain the rate limiting steps of photosynthesis and the CO_2 concentrating mechanism to accurately determine the temperature response of C_4 photosynthesis (Gandin et al., *In prep*).

Carbonic anhydrase and C_4 photosynthesis The importance of CA for C_4 photosynthesis is unclear but potentially limits rates of photosynthesis. Therefore, measurements of leaf gas exchange were made in *Z. mays* mutants with <2% of wild-type CA activity. Net CO_2 assimilation under sub-ambient CO_2 concen-

tration was lower in the CA mutant; however, at ambient and above CO_2 there was no effect of on photosynthesis. This suggests that CA in *Z. mays* is essential for maximum rates of CO_2 assimilation under limited CO_2 availability but not under ambient or higher concentrations of CO_2 (Studer et al., *In review*).

Growth light and leaf structure The goal of this research was to determine how growth under limited light affects leaf structure and the form of C_4 photosynthesis. We have grown *M. giganthus* under two light treatments and three nitrogen levels to determine how these growth conditions influence leaf development and photosynthetic efficiency (Ma et al., *In prep*). Additionally, we submitted a paper on the influence of growth light conditions on the efficiency of the single-cell C_4 system (Stutz et a., *In review*).

Antisense reduction in PEPC and Rubisco We have developed eight RNAi constructs using Gateway technology to target genetic reduction in PEPC and Rubisco content in *S. viridis*. These constructs have been sent to our collaborator Dr. Tom Brutnell at the Donald Danforth Plant Science Center for transformation into *S. viridis*.

Science objectives for 2013-2014

Antisense reduction in PEPC and Rubisco The plants with low PEPC or Rubisco activity will be used to determine how changes in the relative capacity of the C_3 and C_4 cycles influence the efficiency of C_4 photosynthesis under low light or high temperature.

Kinetic properties of PEPC We have initiated analysis of recombinant PEPC from C_3 and C_4 plants expressed in *E. coli* to determine the temperature response of PEPC kinetics and to identify which amino acid substitutions confer the change in affinity for bicarbonate.

Complete temperature response of Rubisco kinetics The thermal dependency of Rubisco kinetics between 10 and 40 °C will be measured from the changes in the rate of CO_2 and O_2 consumption over a range of substrate availability (CO_2 and O_2). This will provide important information on how Rubisco kinetic properties change with temperature providing a more accurate means to model the temperature response of C_4 photosynthesis.

References to work supported by this project 2012-2013:

Ubierna N., Sun W., Cousins A.B. (2013) The efficiency of C_4 photosynthesis under low light conditions in *Zea mays*, *Miscanthus x giganteus* and *Flaveria bidentis*. *Plant Cell and Environment* 36:365-381

Sun W., Ubierna N., Ma J-Y., Cousins A.B. The effect of light quality on C_4 photosynthetic efficiency in *Zea mays* and *Miscanthus giganteus*. *Plant Physiology* In press

In review

Studer A., Gandin A., Kolbe A., Cousins A.B., Brutnell, T. The limitation of maize (*Zea mays*) C_4 photo synthesis by carbonic anhydrase under limited CO_2 availability. *Nature*

Stutz S, Edwards, G.E., Cousins A.B. Efficiency of the CO_2 concentrating mechanism in the single-cell C_4 plant *Bienertia sinuspersici* in response to light and oxygen: implications of leaf anatomy and bio chemistry. *New Phytologist*

In preparation

Ubierna N., Cousins A.B. The CO_2 concentrating mechanism in maize (*Zea mays*) is limited by rates of carbonic anhydrase under low temperature and CO_2 availability.

Ma J-Y, Sun W., Ubierna N., Cousins A.B. Influence of growth light conditions and nitrogen availability on the efficiency of the CO_2 concentrating mechanisms in *Miscanthus* × *giganteus*.

Boyd R., Gandin, A., Cousins A.B. The temperature response of Rubisco and PEP-carboxylase kinetics from *Setaria viridis*.

Gandin A., Serrano E., Cousins A.B. The temperature response of C_4 photosynthesis in *Setaria viridis.*

Membrane-attached Electron Carriers in Photosynthesis and Respiration:
Cytochrome *c* maturation (Ccm-System I) in a facultative photosynthetic bacterium

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Overall research goals: The long term aim of our work is to understand the function and biogenesis of bacterial cytochromes (cyt) at a molecular level. Cyts are ubiquitous hemoproteins that are key elements in many cellular energy-producing processes such as **photosynthesis** and **respiration** as well as other important biological pathways, like metal reduction and signal transduction (apoptosis). Our goals are to elucidate the mechanisms of maturation of cyts *c* and ultimately gain the ability to manipulate at will, their assembly into mature electron transfer complexes, using phototrophic bacteria (*Rhodobacter*) as an experimental model. Cyt *c* maturation (**Ccm**) is a complex process that involves several membrane-associated proteins, which are responsible for the ligation of the *b* heme cofactor to the conserved heme-binding site (CXXCH motif) of apocyts. Current focus of our work is on the molecular recognition of competent apocyt *c* substrates by specific components of the Ccm machinery to yield mature holocyts *c*. The study of Ccm is essential for understanding the energy transduction pathways.

Significant achievement 2011-2013: We are dissecting the specific interactions between the isolated Ccm components and the apocyt *c* substrates: **a-** We developed a reliable protein-protein interaction assay that demonstrated that *R. capsulatus* CcmI (a bipartite component of the heme ligation complex) is an apocyt *c* chaperone, and binds to the C-terminus of the apocyt *c* substrates; **b-** Using a similar assay we established that the heme chaperone CcmE recognizes the apocyt *c* substrates in the absence of heme. Moreover, we showed that apoCcmE and CcmI interact together to form a ternary complex with apocyt *c*. Thus, CcmI as a part of the CcmFHI

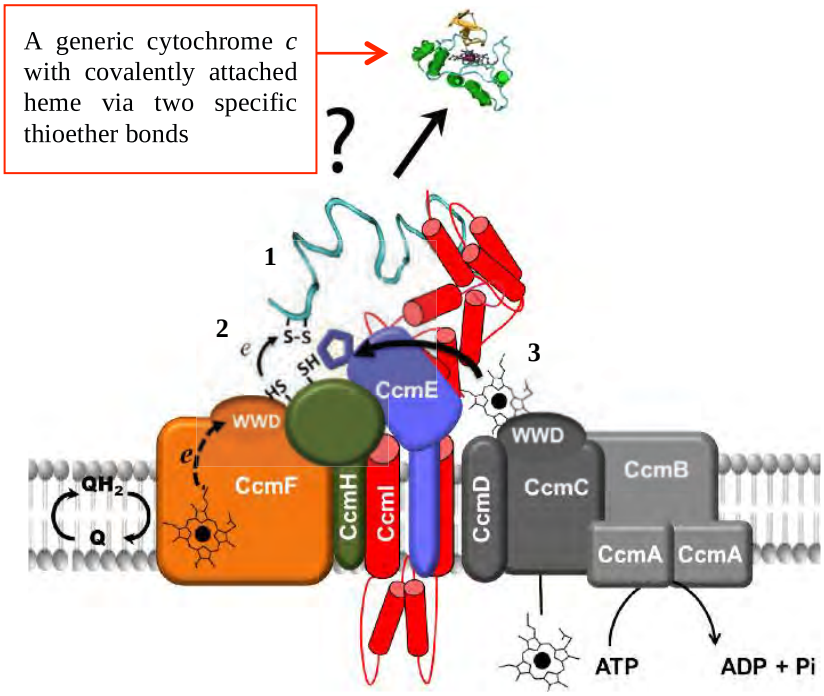

Figure. The figure depicts our hypothetical model of a large molecular entity composed of the CcmFHI-CcmE complex associated with the heme translocation complex CcmABCD. The C-terminal portion of the apocyts *c* is captured via the periplasmic domain of CcmI of the CcmFHI core complex (**1**). Upon interaction with apoCcmE, its heme-binding site CXXCH is positioned near CcmH so that the Cys thiols are reduced, if needed (**2**). When heme is translocated from the cytoplasm and becomes available at CcmC, apoCcmE is converted to holoCcmE by interacting directly with CcmC and CcmD (**3**). Following ATP hydrolysis by CcmA, holoCcmE becomes available to transfer heme to the apocyt *c*, which remains trapped at the CcmFHI complex.

core complex that we had evidenced earlier, forms together with CcmE and apocyt *c* a multi subunit complex. Since apoCcmE interacts with the CcmABCD components to receive heme (*i.e.*, to become holoCcmE), our current working model hypothesizes physical interactions of the CcmABCD with the CcmFHI-CcmE-apocyt *c* complex to produce a large molecular weight Ccm complex (see **Figure**). **c-** In parallel to the Ccm-System I studies, we also contributed to the issue of apocyts *c* recognition by the Ccm-System III, found in mitochondria of fungi, metazoans and some protozoa. Our work allowed the identification of the amino acid sequence within the apocyt *c* N-terminus that is recognized by the cyt *c* heme lyase (CCHL) in Ccm-system III.

Science objectives for 2014-2016: The following specific aims are being pursued:

- Determine how the Ccm components recognize and interact with different types of apocyt *c* substrates, such as the C-terminally anchored apocyt c_1 and the four-helical bundle class II apocyt *c*', and compare these interactions with those seen with apocyt c_2.
- Expand our studies to the maturation of multiheme *c*-type cyts, such as a pentaheme cyt *c* (*e.g., R. capsulatus* DorC) and eventually a decaheme cyt *c* (*Shewanella* MtrA/MtrC).
- Explore the interactions of the heme ligation components CcmH and CcmF with the two Ccm substrates apocyt *c* and heme; in particular, examine the role of CcmH in thioreduction as a partner of CcmG and apocyt *c*.
- Investigate the role of the heme chaperone CcmE as the link between the heme ligation core complex CcmFHI and the heme handling CcmABCD complex in the formation of a large macromolecular entity as the "Ccm machine".
- Further pursue our ultimate goal towards an *in vitro* Ccm system able to carry out apocyt *c*-heme ligation, not only with different apocyts *c* but also with differently metallated porphyrin rings.

References to the works directly related to Ccm studies supported by this project 2010-2013

Andreia F. Verissimo, Hongui Yang, Xiaomin Wu, Carsten Sanders and Fevzi Daldal. (2011) CcmI subunit of CcmFHI heme Ligation Complex functions as an Apocytochrome *c* Chaperone during *c*-type Cytochrome Maturation. *JBC* **286**, 40452-40463.

Andreia F. Verissimo, Joohee Sanders, Fevzi Daldal and Carsten Sanders. (2012). Engineering a prokaryotic apocytochrome *c* substrate as an efficient substrate for *Saccharomyces cerevisiae* cytochrome *c* heme lyase. *BBRC* **424**, 130-135.

Andreia F. Verissimo, Mohamad A. Mohtar and Fevzi Daldal. (2013). The heme chaperone apoCcmE forms a ternary complex with CcmI and apocytochrome *c*. *JBC* **288**, 6272-6283.

Our other works related generally to the biogenesis of cytochrome *c* complexes are not listed.

Maximizing Photosystem II Water Oxidizing Efficiency Through the Identification of Optimal Protein Coordination Environments

DE-FG02-10ER16195

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Overall research goals: This proposal sought to reveal the range of catalytic activities of water oxidizing complexes from a phylogenetically diverse range of plants, algae and cyanobacteria. The approach aimed to reveal the catalytic determinants of natural photosynthetic water oxidizing enzymes by examining the "desirable design motifs" among PSII‐WOC enzymes having different protein scaffolds that deviate from native amino acid sequences.

Significant achievements 10/01/12 – 09/30/2013: This grant provided primary support for one graduate student and fractional support for another. It has produced five refereed publications to date. We report here significant progress appearing in the past year that has been published in three manuscripts and one manuscript currently under review.

1) "Natural variants of Photosystem II subunit D1 tune photochemical fitness to solar intensity" Vinyard, D. J., Gimpel, J., Ananyev, G. M., Cornejo, M. A. Golden, S. S., Mayfield, S. P., Dismukes, G. C. *The Journal of Biological Chemistry* 2013, 288, Issue 8, 5451-62. http://www.jbc.org/content/288/8/5451.full **Research Gate 667 downloads since March 2013.**

This manuscript describes an important attribute of microbial photosynthesis at low light intensity that allows cyanobacteria to outcompete algae for solar energy capture and results in greater biomass accumulation. Photosynthetic organisms use several strategies to cope with vast solar flux intensity differences based on time of day and weather conditions. Given too much light, the photosynthetic apparatus produces damaging radical species, while not enough light leaves the organism starved for chemical energy and stunts growth. Photosynthesis at very low light flux is particularly challenging, as the usual coping mechanism – increasing the pigment antenna to reaction center ratio by synthesizing more antenna pigment or protein complexes – requires *more* chemical energy to be spent, not *less*. This paradox has been resolved in some cyanobacteria that have developed an alternative strategy. Under low light conditions many cyanobacteria use a standard version of the D1 reaction center protein subunit (D1:1) of Photosystem II (PSII), the complex that uses sunlight to convert solar into chemical energy by making its own fuel. It does so by transferring hydrogen atoms from water to plastoquinone molecules, while producing a proton gradient and oxygen as byproduct. However, when the cells are exposed to a stress such as even moderate light intensity, a more robust D1 isoform (D1:2) is produced and preferentially incorporated into PSII. D1:2 protects cell from the consequences of high light flux: both radical damage and aberrant photochemistry. The D1:2 isoform is so useful that eukaryotic phototrophs (all algae and higher plants on earth) only contain this version in their genomes. At low light, they must divert resources to increase their relative antenna size, which may result in survival, but severely stunts growth. Why then have cyanobacteria maintained the seemingly inferior D1:1 isoform over billions of years of evolution and, equally enigmatic, why is it the dominant version expressed under normal light conditions? Researchers at Rutgers University and the University of California, San Diego have shown that D1:1-PSII is not only more efficient at converting solar to chemical energy at very low light intensities compared to D1:2-PSII, but also grows faster. Their research shows that D1:1-PSII extends the lifetime of the chemical intermediates that form the charge separated state in the reaction center, the first "electrical battery" of photosynthesis. The authors show that transgenic algal cells containing only the cyanobacterial D1:1-PSII accumulate more biomass than cells containing only cyanobacterial D1:2-PSII

or the native algal D1 isoform, at very low incident light flux or in dense cultures where cells self-shade. This discovery not only answers an important question in the evolution of photosynthesis, but may open the door for applications in the commercial growth of high density biofuels and agronomic crops

2) "Photosystem II: The reaction center of oxygenic photosynthesis" Vinyard, D.J., G. Ananyev, and G.C. Dismukes, *Annual Review of Biochemistry*, 2013. 82(1): p. 577-606.
http://www.annualreviews.org/toc/biochem/82/1. **Research Gate 183 downloads since May 2013.**
This article is part review and part new insights. We provide an overview of the kinetics and thermodynamics of water oxidation that highlights the conserved performance of PSIIs across species. We discuss recent advances in our understanding of the site of water oxidation based upon the improved (1.9 Å resolution) atomic structure of the Mn_4CaO_5 water-oxidizing complex (WOC) within cyanobacterial PSII. We combine these insights with recent knowledge gained from studies of the biogenesis and assembly of the WOC (called photo-assembly) to arrive at a proposed chemical mechanism for water oxidation.

3) "A mathematical solution to asymmetric Markov chains: Application to the catalytic cycle of photosynthetic water oxidation" Vinyard, D. J., Zachary, C. E., Ananyev, G., Dismukes, G. C. *Biochim Biophys Acta*, 2013. 1827(7): 861-8.
http://www.sciencedirect.com/science/article/pii/S0005272813000856 **Research Gate 8 downloads since July 2013.**
Multiple "Kok" models have appeared that aim to account for the damped yield of O_2 produced by short flashes from dark adapted PSII enzymes and whole cells. To date, analytical solutions have been found only for unrealistic (symmetrical) Kok models (inefficiencies are equally probable for all intermediate S-states). However, it is widely accepted that WOC turnover steps are not symmetrical as they violate fundamental thermodynamic principles. Through the application of established linear algebra theorems to these asymmetric systems several new criteria of merit have been identified and significant improvements to simulations of flash O2 yield and variable fluorescence provide significantly improved models for PSII operation that do not violate thermodynamics.

4) "Engineered Photosystem II reaction centers optimized for different light intensities" by D. J. Vinyard, J. Gimpel, G. M. Ananyev, S. P. Mayfield, and G. C. Dismukes. Under review.
This manuscript reveals the physico-chemical basis for how cyanobacteria (and other oxygenic photosynthetic organisms) maximize solar-to-chemical energy conversion within the reaction center at both high and low light intensities. Here, we expanded our previous studies of natural D1 isoforms to include numerous genetically engineered unnatural D1 isoforms. This manuscript provides two significant findings. For the first time, *in vivo* (whole cell) measurements could be made that enabled statistically significant correlations to be drawn between photochemical quantum yield, water oxidation kinetics, charge recombination kinetics, light induced damage, and biomass yield. These correlations reveal a trade-off in performance between photochemical yield and tolerance to photo-inhibition that are shown to have consequences for cellular growth and biomass yield. Significantly, these results provide the fundamental design principles needed to engineer PSII reaction centers that achieve optimal photosynthetic efficiencies for growth in either low or high light intensities. Second, the specific amino acid residues in the D1 protein that confer low light or high light fitness to PS II activity are revealed for the first time.

Science objectives 2013-2014:
Aim B1. The absolute quantum requirement for water oxidation – the product of PSII primary charge separation yield and WOC cycling yield – provides a measure of functional performance *in vivo* that is needed for comparison across species.
Aim B2. To what extent do electron transfer steps downstream of PSII control flux and lower efficiency?
Aim C1. "STEAMM" analysis will be extended to model the period-four oscillations in the yield and rise time of variable chlorophyll fluorescence.

Generation of a tyrosyl radical during catalytic turnover by the *E. coli* cytochrome bo_3 ubiquinol oxidase

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Overall research goals: The general goal of our research is to understand the mechanism by which the redox reaction catalyzed by the *E. coli* cytochrome bo_3 ubiquinol:dioxygen oxidoreductase conserves the free energy from this reaction in the form of a proton motive force. The enzyme is one of three respiratory oxygen reductases that are encoded in the genome of *E. coli*, and it is maximally expressed at high aeration growth conditions. Cytochrome bo_3 is a member of the heme-copper superfamily and pumps 4 protons across the membrane per O_2 (reduced to two water molecules).

$$2\ UQH_2 + O_2 + 8\ H^+_{in} \rightleftarrows 2\ UQ + 2H_2O + 8\ H^+_{out}$$

We are focusing our efforts on two aspects of the mechanism.

1) During the reduction of the enzyme, a quinol bound at the "high affinity" site of the enzyme is oxidized to a semiquinone species. We are examining the interactions between the stabilized ubisemiquione and the enzyme by pulsed EPR techniques to determine how the hydrogen bond network favors the reaction mechanism and formation of this species.
2) During the oxidation of fully reduced cytochrome c oxidases by O_2, it is it is generally assumed that a free radical is formed at the active-site tyrosine. We are examining this in the cytochrome bo_3 ubiquinol oxidase. Recent results concerning this specific aim will be discussed.

Background: When any heme-copper oxygen reductase is reduced by two-electrons, it reacts rapidly with O_2, and many studies have demonstrated that the product is an oxygenated form of the enzyme, called the P_M state, in which four electrons have been transferred to the oxygen species which are bound to the Fe and Cu at the active site. This reaction requires the two electrons added to the enzyme as well as two additional electrons. One "extra" electron comes from forming a hypervalent heme iron (Fe^{4+}) and the second is proposed to come from a nearby amino acid, forming an amino acid radical. The most likely immediate source of this electron is the cross linked tyrosine-histidine, which is present at the active site of all heme-copper oxygen reductases. A nearby tryptophan is also a reasonable candidate (*1*). Efforts to trap and identify the radical have taken two different approaches. One approach is to use ultra-fast rapid quench devices to view radicals formed upon reaction of the fully reduced (4-electron) enzyme with O_2. Transient radicals have been observed that disappear after a milliseconds. One group identifies the source as tryptophan (*2, 3*) and another group claims there are no protein-based radicals but only those from the breakdown products of ascorbate or dithionite, reductants that are present (*4, 5*). The second approach is to use the reaction of hydrogen peroxide with the enzyme, which forms a state (P_H) that closely mimics the P_M state. At least one tyrosyl radical is clearly associated with the P_H state (*6*) and it has been shown that in the steady state reaction with H_2O_2, the radical observed in the cytochrome oxidase from *Paracoccus denitrificans* is at position Y167 in subunit I, which is near the active site (*7*). However, site-directed mutagenesis shows that the Y167F mutant is fully active although the radical is no longer observed.

Our results with cytochrome bo_3

1. A tyrosine radical is observed during the steady state reaction of H_2O_2 with cytochrome bo_3. Selective labeling with deutero-tyrosine confirms the assignment.

2. The same radical is observed even after replacement of the active site, cross linked Y288 by phenylalanine, although the Y288F mutant exhibits no oxidase activity.
3. The equivalent of the mutation which abolished the radical in the *P. denitrificans* oxygen reductase (Y167F) was made in cytochrome bo_3 (Y173F). The enzyme is fully active as a quinol oxidase. However, the reaction with H_2O_2 is blocked after forming the P_H intermediate, as determined by stopped flow optical spectroscopy.
4. In contrast to the report on the *P. denitrificans* mutant (*7*), the Y173F mutant of cytochrome bo_3 still exhibits a tyrosine radical but from a different site in the enzyme.
5. By making a series of double mutants, Y173F/YxxxF, the apparent site of this second radical has been determined to be $Y184^{II}$, located in subunit II at the interface with subunit I.
6. The Y173F/$Y184^{II}$F double mutant is fully active as a quinol oxidase but exhibits no free radical in the steady state reaction with H_2O_2.
7. The $Y184^{II}$F mutant (with wild type Y173) is fully active, as expected.
8. Unexpectedly, the $Y184^{II}$F cytochrome bo_3 mutant exhibits a stable tyrosine radical at position Y173 without the addition of H_2O_2 . Oxidation of the reduced enzyme by O_2 is sufficient to generate the radical, and the enzyme, as isolated, has this radicalAddition of reductant eliminates the radical, and the subsequent addition of O_2 restores the radical. . This is the first report of an EPR-detectable tyrosyl radical generated in a heme-copper enzyme as a result of the reaction with O_2.
9. It is concluded that $Y184^{II}$ plays an essential role for quenching the radical at Y173. However, Y173 is not directly oxidized by the chemistry at the active site, nor required for oxygen reductase function.

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Studies of Photosynthetic Reaction Centers and Biomimetic Systems

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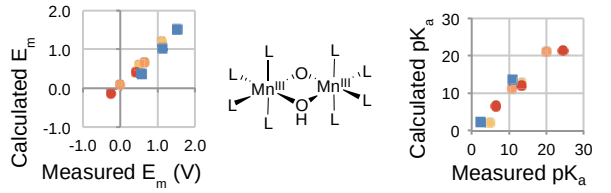
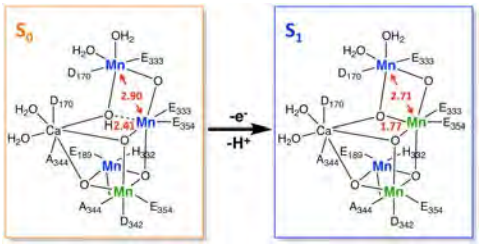
Overall research goals. The goal is to establish a research program for fundamental studies of natural photosynthetic proteins and biomemetic systems, incorporating Monte Carlo simulations for calculations of pK_as and electrochemical midpoints (Gunner, CCNY); quantum mechanics and QM/MM analysis (Batista, Yale Univ, USA); and large-scale molecular dynamics of photosynthetic reaction centers (Bruce, Brock Univ, Canada). Our efforts have been focused on the analysis of the structure and thermodynamics of the Oxygen Evolving Complex (OEC) of Photosystem II (PSII) through the S state cycle.

Significant achievements 2011-2013

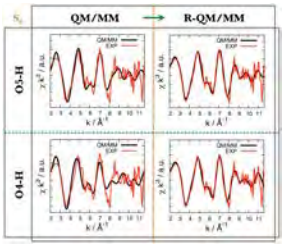


Comparison between experimental (a) E_ms for transition from Mn(III) to Mn(IV) and (b) pK_as for terminal and bridging oxygens in different oxomanganese complexes.[1]

Novel Continuum Electrostatic/DFT/Monte Carlo method to investigate the thermodynamics of proton and electron transfers in oxomanganees complexes.[1] The influence of electrostatic interactions on the free energy of proton-coupled-electron-transfer (PCET) in biomimetic oxomanganese complexes inspired by the oxygen-evolving complex (OEC) of photosystem II (PSII) were investigated. An enhanced Multi-Conformer Continuum Electrostatics (MCCE) model, parameterized at the density functional theory (DFT) level with a classical valence model for the oxomanganese core has been used to calculate pK_as and oxidation midpoint potentials (E_ms). The calculated pK_as and E_ms were shown to match experimental values for eight complexes indicating that purely electrostatic contributions account for most of the observed connection between deprotonation and oxidation state transitions. The analysis highlights the strong coupling between electron and proton transfers, with any Mn oxidation lowering the pK_a of an oxo bridge by 10.5±0.9 pH units. The model also accounts for changes in the E_ms due to ligand substituents, including electron withdrawing effects of -Cl or $-NO_2$. The reported study provides the foundation for analysis of electrostatic effects in other oxomanganese complexes and metalloenzymes, where PCET plays a fundamental role in redox-leveling mechanisms.



The structural changes that take place as a result of the proton coupled electron transfer during the S_0 to S_1 transition in the OEC. The distance between Mn3 and Mn4 is shortened by ~0.2 Å and the Mn3-O5 covalent bond is formed during this step. Mn^{IV} atoms are shown in green and the Mn^{III} atoms in blue.[2]

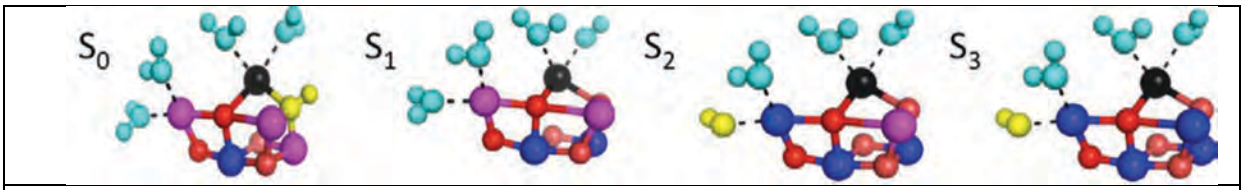


Comparison of experimental EXAFS spectrum (red) to the calculated spectra (black) obtained with QM/MM S_0 state models with protonated O4 of O5 oxo-bridges.[2]

QM/MM calculations of the $S_0 \rightarrow S_1$ transition of the oxygen-evolving complex (OEC) of photosystem II (PSII).[2] The $S_0 \rightarrow S_1$ transition in the OEC is one of the least understood steps in the Kok cycle of water-splitting. We obtained a quantum mechanics/molecular mechanics (QM/MM) model of the

S_0 state that is consistent with extended X-ray absorption fine structure spectroscopy. In conjunction with the previously reported QM/MM model of the S_1-state [*Biochemistry* (2011) **50**: 6308-6311], we have addressed the proton-coupled electron transfer (PCET) process that occurs during the $S_0 \rightarrow S_1$ transition where oxidation of a Mn center and deprotonation of a μ-hydroxo bridge leads to a significant rearrangement of the Mn cluster. We propose that a hydrogen bond network linking the D1-D61 residue to a Mn-bound water molecule facilitates the PCET mechanism. Current work focuses on the development of analogous models for the S_3 intermediate and the $S_2 \rightarrow S_3$ transition.

Analysis of the OEC S state cycle within the context of PSII.[3] A computational model that combines DFT, MD and MC techniques has been used to study the thermodynamics of the OEC S-state cycle in the PSII protein. All 20 subunits of PSII and a low dielectric membrane region are included. The order of oxidation of the Mn in the cluster and deprotonation of bridging oxygens are determined through the S state cycle. The S_0 to S_1 includes a deprotonation of μ-oxo, the S_1 to S_2 transition includes deprotonation of terminal water and the S_2 to S_3 transition involves deprotonation of amino acid residue (D61 or H337). However, within the protein the S_1 to S_2 transition transfers the proton to the adjacent D61 so no proton release is observed. This pattern of proton loss is consistent with experiment. In addition, when the predicted OEC redox and protonation states are used in DFT optimization, the calculated structural changes through the S state cycle are consistent with changes seen by EXAFs spectroscopy. The OEC E_m's range from ≈0.9 – 1.6 V vs SHE for the transition from S_0 to S_3. The role of His 337, Asp 61 and Cl are explored.



Calaculated Mn oxidation states and bridging oxygen and terminal water protonation states through the S-state cycle. Magents:Mn(III); Blue: Mn(IV); yellow: OH^- oxygen bridge or terminal water; red: μ-O^{-2} bridge; cyan: terminal water.[3]

Science objectives for 2013-2014

The plans for the next grant period. (1) We will continue to build and analyze models of the OEC. This will be carried out at the QM/MM level where the system can be studied in detail and at the coupled DFT/MD/MC level, which allows equilibration of OEC and amino acid protonation states as well as ion binding through the S state cycle. (2) The OEC will be studied in a series of mutated proteins that are expected to perturb the function; (3) A network analysis will be carried out to determine the lowest energy proton exit channels in PSII. This relies on software being developed in the Gunner and the Batista laboratories. The pathways will be determined in conjunction with MD analysis carried out in the Bruce laboratory. (4) The valance model for the analysis of oxomanganse complexes will be extended to other metallocomplexes.

References to work supported by this project 2011-2013

1. Amin, M.; Vogt, L.; Vassiliev, S.; Rivalta, I.; Sultan, M.; Bruce, D.; Brudvig, G.W.; Batista,V.S.; and Gunner, M.R. (2013_ Electrostatic effects on proton-coupled electron transfer in oxomanganes complexes inspired by the oxygen-evolving complex of photosystem II, *J. Phys. Chem B* **117**: 6217-6226.
2. Pal, R.; Negre, C. F.A.; Vogt, L.; Pokhrel, R.; Ertem, M. Z.; Brudvig, G. W.; Batista, V. S. S_0-State Model of the Oxygen Evolving Complex of Photosystem II, (2013) *Biochemistry* submitted.
3. Amin, M.; Vassiliev, S.; Vogt, L.; Brudvig, G.W.; Batista, V.S.; Doug Bruce, D. and Gunner, M.R. A model for the Kok cycle of the oxygen-evolving complex of photosystem II inspired by electrostatic energy (in prep)
4. Vassiliev, S.; Zaraiskaya, T. and Bruce, D. *Biochim et Biophysica Acta* (2013) Molecular Dynamics simulations reveal highly permeable oxygen exit channels shared with water uptake channels in photosystem II. 1827, 1148-1155.
5. Vassiliev, S.; Zaraiskaya, T and Bruce, D. *Biochim et Biophysica Acta* (2012) Exploring the energetics of water permeation in photosystem II by multiple steered molecular dynamics simulations. 1817, 1671-1678.

Structure and Function of a Chloroplast Signal Recognition Particle in Thylakoid Targeting and Insertion of Light Harvesting Chlorophyll-binding proteins (the LHCs) by the Albino3 Insertase.

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Overall research goals: The goal of our research is to understand how interaction between cpSRP and the Albino3 insertase serves to coordinate the order and timing of targeting events at the thylakoid membrane: The research objectives are (1) Identify mutations in the Alb3 C-terminal peptide (Alb3 Cterm) that result in loss of cpSRP43 binding; (2) Use NMR to determine the structure of Alb3 Cterm alone and bound to the Alb3-binding domain of cpSRP43; (3) determine how interaction between cpSRP43 and Alb3 insertase acts to promote unidirectional transfer of the LHC targeting substrate from cpSRP to Alb3. Completion of the proposed studies will establish the order and timing of membrane events needed for LHC targeting to Alb3. Additionally, our studies will provide structural details to understand how Alb3 Cterm binding to cpSRP43 leads to LHC release from cpSRP43 and how LHC polypeptides may initially interact with Alb3 following their release from cpSRP.

Significant achievements 2010-2013: We demonstrated that a recombinant protein corresponding to the stroma-exposed Alb3 Cterm binds to the 43 kDa subunit of cpSRP (cpSRP43), causes the release of LHCP targeting substrate from cpSRP, and stimulates GTP hydrolysis by cpSRP and its receptor using a mechanism that requires cpSRP43.

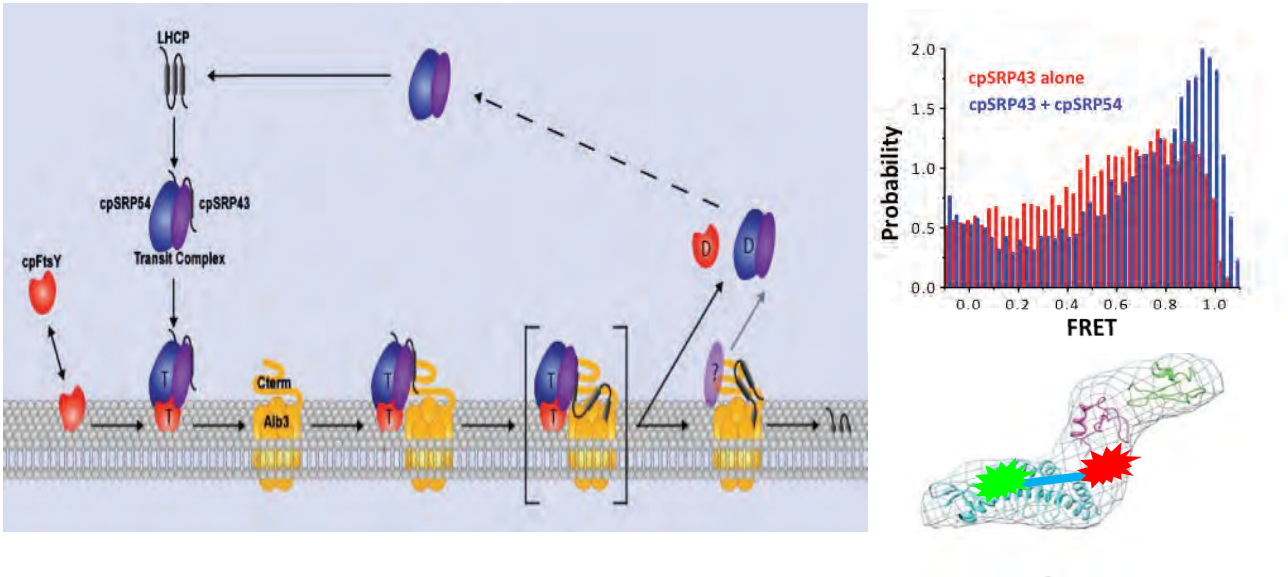

Figure 1. *Left Panel:* Hypothesized order of membrane events mediated by cpSRP43 interaction with Alb3 Cterm (adapted from Lewis et al., 2010); *Right Panel:* Structural dynamics studies of cpSRP43 using site-directed fluorescence labelling and single-molecule FRET are being used to examine structural changes in cpSRP43 upon interaction with the targeting substrate and with targeting components. Results shown indicate that the structure of cpSRP43 alone is extremely heterogeneous (red bars, indicating that single molecules show a wide range of FRET efficiencies), and that it becomes less so upon binding cpSRP54 (blue bars, showing a narrower distribution of FRET efficiencies).

Science objectives for 2013-2014:

1. We have designed an LHCP construct with affinity tags at the N-terminus, which is active in forming a cpSRP-LHCP targeting complex and can be separated from individual components of the targeting complex. As such, we have used the tag on LHCP to tether intact targeting complex to a surface suitable for smFRET. Using position specific labels in cpSRP43, we will compare the structural changes in cpSRP43 that take place as a result of LCHP binding and examine changes in cpSRP43 that result from addition of Alb3 Cterm peptide to the targeting complex in order to understand structural changes in cpSRP43 that result in release of LHCP targeting substrates. We will also begin to place fluorescent tags at specific positions in LHCP to identify FRET pairs using position-specific labels in cpSRP43 (and cpSRP54). Identification of FRET pairs between LHCP and cpSRP43 or LHCP and cpSRP54 can be used to understand dynamics of LHCP release from cpSRP upon Alb3 Cterm binding by cpSRP43 and understand how the three transmembrane domains of LHCP are shielded from water through interaction with cpSRP components.

2. We have developed methods to make mg quantities of purified cpSRP. We will use this material to obtain SAXS data, which can be used to generate a solution structure of cpSRP. Important here is that the resulting structure can be compared to the published SAXS structure of cpSRP43 alone to understand how cpSRP43 and cpSRP54 are positioned to bind LHCP. The SAXS structure of cpSRP, along with NMR and crystial structures of cpSRP domains, will also enable computer models of cpSRP to be verified and used to model LHC binding to cpSRP.

References to work supported by this project 2010-2013:

1. Lewis, NE, NJ Marty, KM Kathir, D Rajalingam, AD Kight, A Daily, TKS Kumar, RL Henry, RL Goforth (2010). A Dynamic CpSRP43-Albino3 Interaction Mediates Translocase Regulation of CpSRP Targeting Components. **J Biol Chem** 285(44): 34220-34230. PMID: 20729200

2. Henry, RL (2010) SRP: Adapting To Life In The Chloroplast. **Nature Structural & Molecular Biology** 17(6): 676-677. PMID: 20520661

Resonant and Non-Resonant Hole-Burning and Delta Fluorescence Line-Narrowing Study of BChls in Excitonically Coupled Photosynthetic Systems

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Overall research goals:

The research objectives are to study excitonically coupled bacteriochlorophylls (BChls) that are ubiquitous in bacterial photosynthetic complexes. Hole-burning (HB) and delta fluorescence line-narrowing (ΔFLNS) spectroscopies and modeling studies are used to provide additional insight into the excitonic structure, electron-phonon and vibronic couplings, and excitation energy transfer (EET)/electron transfer (ET) processes in: i) various model protein systems; ii) the Zn-reaction center (RC) and its mutants; iii) *Rb. sphaeroides* RC and its mutants; and iv) various FMO complexes, found in anoxygenic green sulfur bacteria. Deeper insight into the origin of hole-burned (HB) spectra will provide a better framework for probing the electronic structure of complex biological systems via HB/ΔFLNS spectroscopies. Our approach will provide a more complete picture of the EET/ET processes in various photosynthetic systems.

Significant achievements 2012-2013:

- We provided a critical assessment of typical phonon spectral densities, $J(\omega)$, used to describe linear/nonlinear optical spectra in photosynthetic complexes [3]. We showed that many densities (especially the Drude-Lorentz/constant damping Brownian oscillator) display qualitatively wrong behavior when compared to experiment. We proposed that a lognormal distribution can be used to fit experimental data and exhibits desired attributes for a physically meaningful phonon $J(\omega)$, in contrast to several commonly used spectral densities which exhibit low frequency behavior in qualitative disagreement with experiment.
- We developed analytical formulas to describe the FLN spectra of weakly coupled dimers in the presence of EET. This model can be extended to multiple chromophores. Modeling studies show that the FLN spectra (including absorption and emission spectra) calculated for various model systems are in good agreement with spectra calculated by: *i)* the simple convolution method and *ii)* the more rigorous treatment using the Redfield approach [5].
- We showed that the widely used reorganization energy (E_λ) of 35 cm^{-1} in various modeling studies of the FMO two-dimensional electronic spectra from *C. tepidum* is overestimated by a factor of ~3, while its value has important implications for the contributions to the coherence rate. We showed, using both nonresonant and resonant HB spectra, and modeling studies, that the nature of the so-called 825nm absorption band of the FMO trimer, contrary to the presently accepted consensus, cannot be explained by a single transition. To explain the shape of emission and HB spectra, a downward uncorrelated EET between trimer subunits should be taken into account. That is, after light induced coherences vanish within each monomer, the uncorrelated EET between the lowest exciton levels of each monomer takes place due to static structural inhomogeneities in the trimer [6,7].

Science objectives for 2013-2014:

- Complete the theoretical description of various optical spectra (including HB spectra) of Chl *a* WSCP from cauliflower and *Lepidium*. Prove that both WSCP from *Lepidium virginicum* and cauliflower contain four Chl *a* and demonstrate that a slow protein relaxation between energetically inequivalent conformational substates within the lowest exciton state proposed for WSCP-C in Refs [Pieper et al. JPC B 2011, 115, 4053; Schmitt et al., JPC B 2008, 112, 13951] is not applicable to WSCP complexes.

- Although there have been many studies regarding the excitonic structure and ET dynamics in bacterial RCs, some issues related to electrochromic effects and the position of the upper excitonic component of P (i.e., P_+) are yet to be fully understood. Preliminary results for the wild-type (WT) RC from *Rb. sphaeroides* and its mutants indicate that the large bleach near 810 nm in the absorption spectrum of the WT RC upon formation of the $P^+Q_A^-$ state is caused primarily by an electrochromic shift of the absorption band(s) of the monomeric BChls (B_A and B_B), rather than bleaching of the P_+ exciton band. Demonstrate that the P_+ transition in the WT RC from *Rb. sphaeroides* contributes to the absorption near 800 nm, while the P_+ excitonic component in the M214G mutant is blue shifted by about 10 nm.

- Complete the work on the optical lineshape function formulas involving multi-phonon transitions in HB spectra. Apply the models to analyze data obtained for various photosynthetic complexes.

Publications supported by this project 2012-2013:

1. R. Jankowiak, *"Probing Electron Transfer Times in Photosynthetic Reaction Centers by Hole-Burning Spectroscopy"* J. Phys. Chem. Lett., 3, 1684−1694 (**2012**).
2. B. Neupane et al., *"Electron Transfer in the Rb. Sphaeroides RC Containing Zn-Bacteriochlorophylls and its β-Zn-RC Mutant: HB Study"*, J. Phys. Chem. B 116, 3457 **(2012).**
3. A. Kell, X. Feng, M. Reppert, and R. Jankowiak, *"On the Shape of the Phonon Spectral Density in Photosynthetic Complexes"*, J. Phys. Chem. B 117(24):7317-23 (**2013**).
4. C. Lin et al., *"Fluorescence Line-Narrowing Difference Spectra: Dependence of Huang-Rhys Factor on Excitation Wavelength"*, Chem. Phys. Lett., 576:15-20 (**2013**).
5. C. Lin et al., *"Modeling of Fluorescence Line-Narrowed Spectra in Weakly Coupled Dimers in the Presence of Excitation Energy Transfer"*, J. Chem. Phys. (**2013**), submitted.
6. V. Zazubovich and R. Jankowiak, *"Biophotonics of Photosynthesis"*, an invited book chapter for Photonics, volume 4, *Biophotonics*; Ed.: David Andrews, Publisher Wiley, **(2013),** submitted.
7. S. Savikhin and R. Jankowiak, *"Mechanism of Primary Charge Separation in Photosynthetic Reaction Centers"*, in The Biophysics of Photosynthesis, Springer, Eds. John Golbeck and Art van der Est (**2013**), submitted.
8. N. Herascu et al., *"Modeling of various optical spectra in the presence of uncorrelated excitation energy transfer in dimers and trimers with weak inter-pigment coupling"*, J. Phys. Chem. B, (**2013**) to be submitted in November.
9. A. Kell et al., *"On the Controversial Nature of the 825 nm Exciton Band in FMO Complexes"*, J. Phys. Chem. Letters, (**2013**), to be submitted in November.

Controlling Electron Transfer Pathways in Photosynthetic Proteins

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Overall research goals: Photosynthetic reaction centers (RCs) are protein-cofactor complexes that convert light energy into chemical energy in a series of extremely efficient transmembrane electron transfer reactions. The X-ray structures of RCs reveal two pseudo-symmetrical branches of cofactors (Fig. 1) that are functionally asymmetric; bacterial RCs use the A pathway exclusively. The goal of the project is to identify a mutant RC that utilizes the B pathway for quinone (Q_B) reduction with the same high yield as that of the A pathway in the native complex, thereby gaining fundamental insight into the factors necessary for *de novo* design of efficient multistep electron transfer. Previous attempts at rational design have provided neither the means nor understanding necessary to engineer efficient B-branch electron transfer.

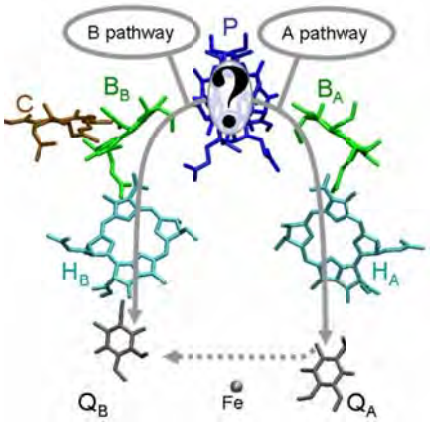

Figure 1. *Arrangement of cofactors in the bacterial RC.*

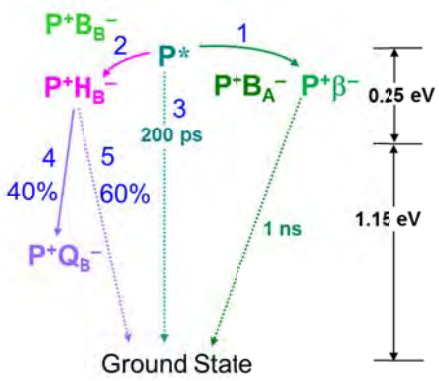

Figure 2. *States, target processes, and the native $P^+H_B^-$ branching.*

Towards this end, we developed (2009–2011) a semi-directed molecular evolution approach that streamlined both mutagenesis and RC isolation and feeds into a high-throughput time-resolved spectroscopic screen. By design, a large number of RC variants can be examined for the yield of $P^+Q_B^-$ arising from exclusive use of the B-side cofactors. Our experimental vehicle is the RC from *Rhodobacter capsulatus*. In short, our goal (Fig. 2) is to find mutant(s) in which the rates of reactions 2 and 4 are increased and those of reactions 1, 3 and 5 are decreased, thereby unlocking efficient B-side charge separation. Screening 150 initial mutants revealed polarizable residues near B_B that enhance B-side electron transfer. A selection of these mutants was the kernel of our first publication.[1]

Significant achievements 9/2011 – 9/2013: Continued improvements to our mutagenesis and screening methods have enabled the analysis of a total of over 450 mutants to date. Fig. 3 shows the results of the millisecond assay for the yield of $P^+Q_B^-$ relative to wild type (~100%) for a subset of nearly 300 mutants. The colors of the bars reflect different RC templates used and different residues targeted for saturation mutagenesis. Most mutants utilize a scaffold (YFHV; Fig. 3, left inset) that displays 22% B-pathway activity in the assay (Fig. 3; main, yellow bar). Clearly we have realized many mutants with significant improvement of utilization of the B-side electron transfer cofactors compared to the initial YFHV template.

In general, RCs that display increased B-pathway activity carry substitutions in residue near one or more of the three B-side cofactors, but sites near B_A have been targeted also in order to

handicap or disable reaction 1. Several mutants affect the initial charge-separation event, altering the balance between reactions 1 and 2 to favor B-side electron transfer. As mutagenesis targets expanded into other regions of the RC, we recently discovered that changing a single residue near H_B modifies efficiency of secondary electron transfer from H_B to Q_B. Mutation of M131 near H_B from a Val to a Glu shifts the competitive balance between reactions 4 and 5 from 40/60 (Fig. 2) to 80/20 (Fig. 3, right inset). This result obtains largely from slowing down reaction 5, the charge recombination process or "back reaction," from ~2 ns to ~8–9 ns. On the A side in wild-type RCs, the time constant for the corresponding $P^+H_A^-$ charge recombination process is ~10 ns and, most interestingly, the residue near H_A that is related by C_2 symmetry to M131 near H_B is a Glu (at L104).

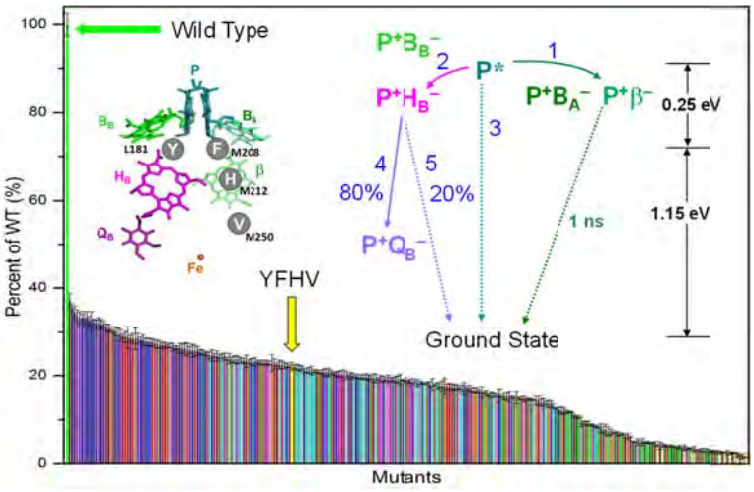

Figure 3. *Yield of $P^+Q_B^-$ relative to wild-type in 280 mutants (main panel); the YFHV template RC (top left); and improved $P^+H_B^-$ branching in RCs with M131Glu (top right).*

To date ~20 mutants from the millisecond high-throughput screen have been studied in detail by ultrafast time-resolved measurements in order to determine the rates and yields of reactions 1, 2, 3, 4, and 5. We are currently drafting a paper on a set of six mutants, including two bearing the M131Glu mutation, which our results suggest forms a hydrogen bond to H_B (as does the native Glu L104 to H_A). Our overall approach is clearly bearing fruit, providing new results and unanticipated insights into how to engineer and control the directionality of charge separation and the efficiencies of the electron-transfer processes.

Science objectives for 2013-2014 and beyond:

- Iterate combinations of mutations, pairing favorable substitutions and introducing substitutions within newly targeted regions.
- Mine datasets to correlate properties of substituted residues with observed photochemistry to discover parameters (through, e.g., principal component analysis) that direct outcomes, good or bad.
- Implement new ultrafast laser instrumentation to detail the initial photochemistry of a larger sampling of mutants.
- Select photocompetent, phenotypic revertants that grow using the B-side cofactors exclusively.

References to work supported by this project 2011-2013:

1. *High-throughput Engineering to Revitalize a Vestigial Electron Transfer Pathway in Bacterial Photosynthetic Reaction Centers,* K. M. Faries, L. L. Kressel, M. J. Wander, D. Holten, P. D. Laible, C. Kirmaier, and D. K. Hanson *J. Biol. Chem.* **2012**, *287*, 8507-8514.

Photoreceptor Regulation and Optimization of Energy Harvesting in *Nostoc punctiforme*

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Overall research goals: We seek to characterize and exploit the biliprotein photosensors of the phytochrome superfamily found in the model cyanobacterium *Nostoc punctiforme.* Our ongoing studies examine how representative members of this family function and elucidate the biological processes that they regulate. This project combines approaches ranging from ultrafast characterization of photochemical processes through biochemical analysis of signal transduction and genetic examination of this cyanobacterium. A longer-term goal of these studies is to use these light-sensing proteins as synthetic tools for regulating the expression of arbitrary target genes in response to the color or intensity of ambient light. Such tools can be used to tailor cyanobacteria for more efficient, sustainable, and carbon-neutral biological capture of sunlight and conversion and storage of that light as chemical energy.

Significant achievements 2012-2013: Work on phytochrome-related cyanobacteriochromes (CBCRs) from *N. punctiforme* has yielded exciting results. Phytochromes and CBCRs combine to provide complete coverage of the visible spectrum and near-UV for this organism. In published work, we have elucidated the photocycles for three of the five known CBCR subfamilies (two of which have been discovered in the course of this project), leading to a 'mix-and-match' model for generating CBCR diversity. Co-PI Larsen has shown that many CBCRs exhibit much higher photochemical quantum yields than do conventional phytochromes and has demonstrated novel CBCR reactivity on the ground state surface after de-excitation (second-chance initiation dynamics or SCID), work he is presenting in more detail. Co-PI Ames has now published backbone assignments for the photoproduct state of the CBCR NpF2164g3, a member of the insert-Cys subfamily discovered under this project in 2011. This first step toward structure determination already shows interesting differences relative to other CBCR subfamilies. Finally, we have also published successful expression and *in vivo* chromophore incorporation of a model CBCR in the *Chlamydomonas* chloroplast, an important step in developing CBCR-based sensors and regulatory circuits for eukaryotic photosynthetic systems.

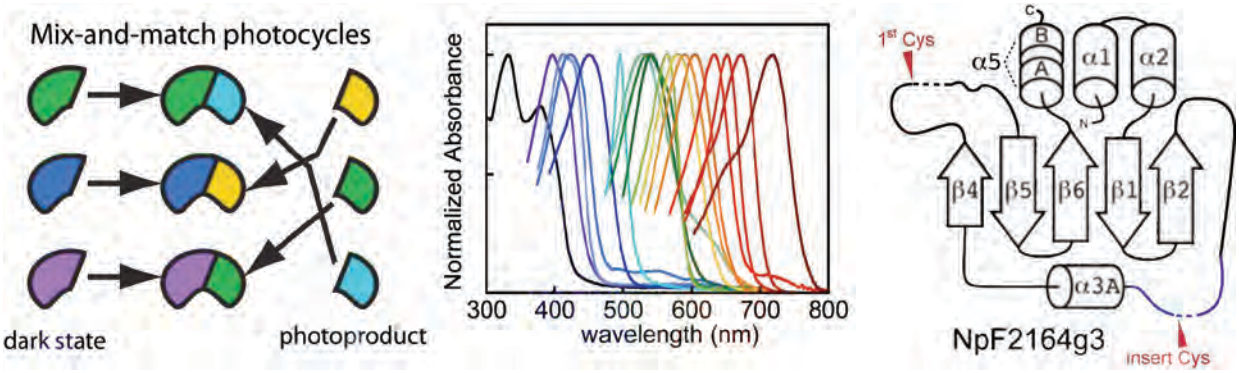

Figure 1. Left Panel: Schematic of the mix-and-match model for CBCR photocycles. In this model, each CBCR subfamily has only a few allowed dark-state and photoproduct spectral responses; combining the two generates a larger group of allowed photocycles. Center Panel: Allowed spectral responses for phytochromes and CBCRs from *N. punctiforme* are shown. This photoreceptor superfamily provides complete coverage of the 'photosynthetically useful' spectrum for *N. punctiforme.* Right Panel: Experimentally determined secondary structure of the CBCR NpF2164g3 in the photoproduct state (publication #13, page 2).

Science objectives for 2014-2015:

- Completion of structure determination for the NpF2164g3 photoproduct, providing the first structural information for a member of this subfamily and for a C5-unsaturated bilin chromophore in the photoproduct state. Backbone assignments are complete. Our next steps are side-chain assignments and chromophore spectroscopy to identify key protein-chromophore constraints for successful structure determination.
- Completion of unpublished work elucidating the photocycles for the remaining two subfamilies. Extensive site-directed mutagenesis has identified key hydrophobic residues in representative members of both groups that are shared with those in a well-understood CBCR subfamily, implicating a conserved mechanism for tuning the photoproduct state in all three subfamilies.
- Completion of genetic and photobiological analysis of *N. punctiforme* phototaxis. We have now identified phenotypes for both of the photosensory MCP proteins in *N. punctiforme* taxis operons, NpR6012 and NpF2164. This result implicates complex regulation of movement by multiple photosensors in this organism.

References to work supported by this project 2012-2013:

1. P. W. Kim, L. H. Freer, N. C. Rockwell, S. S. Martin, J. C. Lagarias, and D. S. Larsen, "Femtosecond photodynamics of the red/green cyanobacteriochrome NpR6012g4 from *Nostoc punctiforme*. 1. Forward dynamics." Biochemistry, **51**: 608-618 (2012).
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3. P. W. Kim, L. H. Freer, N. C. Rockwell, S. S. Martin, J. C. Lagarias, and D. S. Larsen, "Second-chance forward isomerization dynamics of the red/green cyanobacteriochrome NpR6012g4 from *Nostoc punctiforme*." J. Am. Chem. Soc. **134:** 130-133 (2012).
4. N. C. Rockwell, S. S. Martin, A. G. Gulevich, and J. C. Lagarias, "Phycoviolobilin formation and spectral tuning in the DXCF cyanobacteriochrome subfamily." Biochemistry **51**: 1449-1463 (2012).
5. N. C. Rockwell, S. S. Martin, and J. C. Lagarias, "Mechanistic insight into the photosensory versatility of DXCF cyanobacteriochromes." Biochemistry **51**: 3576-3585 (2012).
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Novel Photodynamics in Phytochrome & Cyanobacteriochrome Photosensory Proteins

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Overall research goals: The goal of this project is to use transient spectroscopies to uncover the mechanisms necessary for engineering cyanobacteria for light-regulated energy production. These experiments examine photochemical and conformational changes in the phytochromes and cyanobacteriochrome (CBCR) biliprotein photosensors on a timescale of femtoseconds to milliseconds, using (1) ultrafast broadband transient absorption spectroscopy and (2) a novel time-resolved FRET technique capable of characterizing long-range conformational dynamics in response to light activated photochemistry.

Significant achievements 2009-2012: We characterized the primary and secondary photodynamics of over 15 phytochrome and cyanobacteriochrome bilin-binding domains with dispersed femtosecond transient absorption spectroscopy. Signals were interpreted within a multi-population global analysis approach to construct underlying sample-dependent target models describing the evolution and photoactivity of transient populations. The dynamics of the Cph1, RcaE, NpR6012g4 and NpF2164g6 were subsequently dissected with dispersed pump-dump-probe spectroscopy, which resolved reactive ground-state species, excited-state equilibria, static and dynamic inhomogeneity in the photodynamics of these bilin-proteins.

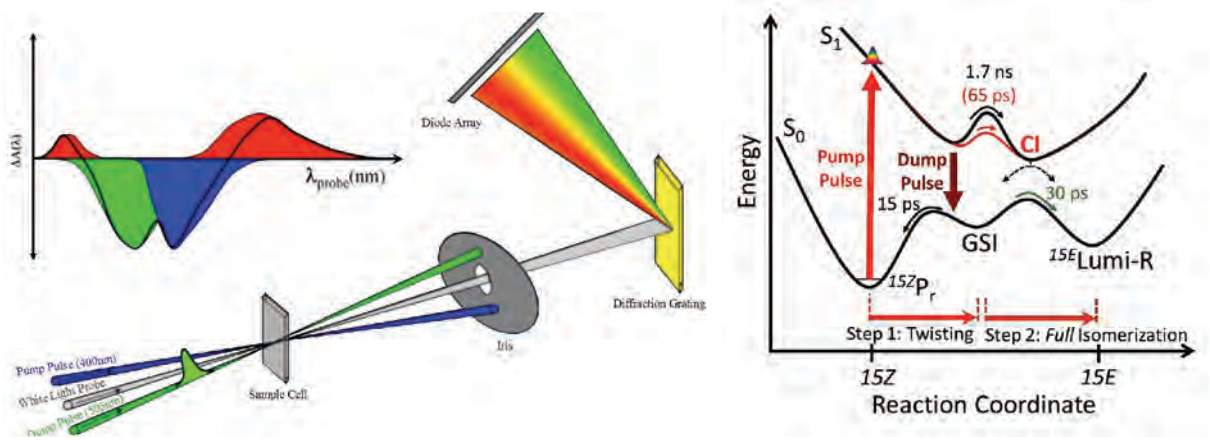

Figure 1. Left Panel: Schematic of the dispersed pump-dump-probe setup (with 400-nm pump, 500-nm dump and broadband whitelight probe). Inset: model transient absorption spectra. Right Panel: Potential Energy Surface of the forward reaction of NpR6012g4 based on target model of the dispersed pump-dump-probe. The dump pulse generates the reactive ground-state intermediate responsible for the second-chance initiation dynamics (SCID) and high quantum yield of this CBCR domain.

Science objectives for 2013-2014:

- Research in the next year will focus on two objectives: characterizing the primary (<10 ns) ultrafast excited-state dynamics of a subset of CBCRs, and characterizing the secondary (> 10 ns) ground-state dynamics of the same CBCRs. Key to the success of these goals is the application of complementary spectroscopic techniques for exploring transient electronic and vibrational properties. Each of the proposed techniques utilize the same detection system and software infrastructure, but probe different features of the photo-induced dynamics by applying different laser pulse sequences.

- Although primary dynamics (<10 ns) often dictate the overall efficiency of the photosensory response, significant secondary dynamics occur afterward. Ground-state evolution of the chromophore and surrounding protein matrix is coupled to formation of a stable product state and modulation of the biological signaling state of the output domain that interfaces with cellular signal transduction networks. We will to track this evolution from the 10 ns to the 100 ms timescale for selected photocycles.
- There is a correlation between behavior of the chromophore as assessed by circular dichroism (CD) and other properties, but almost nothing is known about the dynamics of the CD signals during the photocycle. We intend to measure CD signals, permitting us to extract CD spectra for the intermediates and hence learn how the bilin chromophore is evolving. In combination with structural information, this can identify key residues interacting with the chromophore D-ring during photoconversion.
- The visible signals will be complemented with transient vibrational (mid-IR and Raman) signals. We have been constructing the proposed ultrafast mid-IR transient absorption spectrometer, using sum-frequency-mixing of the IR probe light with a nonlinear mixing crystal to generate visible light that our existing detector systems can resolve. We have recently increased the spectral resolution of this instrument 10-fold (from 5 cm^{-1} to 0.45 cm^{-1}).

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Spatial Regulation of Chloroplast Division and Physiological Significance

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Overall research goals: The proliferation of chloroplasts by division increases chloroplast numbers and compartment size during leaf growth and development, and is therefore essential for achieving maximum photosynthetic productivity. A striking feature of the division process is that it is restricted to the middle of the chloroplast, producing a large population of small organelles of consistent size and shape. The aims of the funded research are to probe the mechanisms by which spatial regulation of chloroplast division-site positioning is achieved, investigate the relationship between chloroplast morphology, chloroplast movement and photosynthesis, and identify genetic loci potentially involved in the control of chloroplast size, number and cell coverage. Together, these studies will improve our mechanistic understanding of how chloroplast division-site placement and overall morphology are regulated in plants and suggest hypotheses regarding how chloroplast form and function interact to influence photosynthetic performance.

Significant achievements 2011-2013:

Spatial regulation of division-site placement: The first step in chloroplast division is formation of a stromal contractile ring composed of two tubulin-like cytoskeletal proteins, FtsZ1 and FtsZ2, that coassemble in heteropolymers. Both proteins are homologs of bacterial FtsZ, which forms a cytokinetic ring during cell division. Assembly of the chloroplast FtsZ ring ("Z ring") is restricted to the mid-plastid in part by MinD1 and MinE1, which evolved from closely related cyanobacterial cell division proteins, and by ARC3 (Figure 1A), a protein unique to plants. We carried out a series of genetic analyses in *Arabidopsis* to investigate the role of ARC3 in Z-ring positioning and ascertain its functional relationship to MinD1 and MinE1. The results support a model wherein ARC3 plays a central role in division-site placement by directly inhibiting Z-ring assembly at non-division sites *in vivo*, primarily through interaction with FtsZ2 in heteropolymers, and suggest that ARC3 activity is spatially regulated by MinD1 and MinE1 to permit Z-ring assembly only at the middle of the chloroplast (Figure 1B). The findings also indicate that ARC3 supplanted cyanobacterial MinC, which has been lost from plants, as the direct inhibitor of Z-ring assembly in the complex system of proteins that regulates Z-ring positioning in chloroplasts (Zhang et al, 2013).

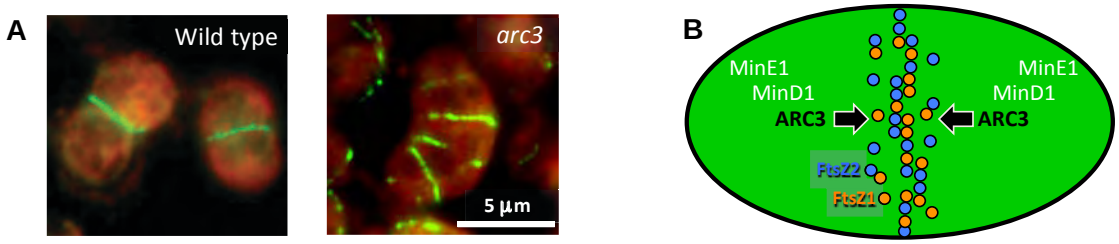

Figure 1. Central role of ARC3 in chloroplast Z-ring placement. (A) Single Z rings (green) assemble in the middle of chloroplasts (red) in wild-type *Arabidopsis* plants; multiple rings form in *arc3* mutants, indicating ARC3 functions as a Z-ring assembly inhibitor. (B) Z-rings form only at the mid-plastid through the combined activities of ARC3, MinD1 and MinE1. ARC3 functions as the direct assembly inhibitor downstream MinD1 and MinE1.

Relationship between chloroplast morphology, chloroplast movement and photosynthesis: Symmetric division produces chloroplasts of similar size and shape, which may enhance photosynthetic performance by optimizing chloroplast movement in response to changing light conditions. We are exploiting our extensive collection of *Arabidopsis* chloroplast division mutants, representing an array of distinct chloroplast-morphology phenotypes, to explore the relationship between chloroplast morphology, chloroplast movement, and various photosynthetic parameters. Previous studies of light-induced chloroplast movements have relied on spectrophotometric measurements of red-light transmittance and microscopic analysis in detached leaves exposed to blue light. In collaboration with David Kramer and Jeffrey Cruz, we have developed a new imaging platform capable of making continuous, simultaneous measurements of chloroplast movements and chlorophyll fluorescence across all leaves in multiple intact plants. Plants are exposed to differing white-light exposures and the time-resolved reflectance of red light is measured. Recording chloroplast movements in whole plants in white light enhances the physiological relevance of such measurements. Initial experiments indicate that red-light reflectance measurements accurately reproduce chloroplast movement responses previously measured by red-light transmittance, and suggest that measurements of photosynthetic efficiency and nonphotochemical quenching are affected by chloroplast movements. Several different mutants with drastically enlarged chloroplasts exhibit impaired movement and reduced photosynthetic efficiency, particularly in fluctuating light (Dutta et al, in preparation).

Science objectives for 2013-2014:

- ARC3 has several distinct domains and regions that have been implicated in its interactions with other chloroplast division proteins. We will use a combination of genetic and biochemical analysis to define the functions of selected regions towards understanding the mechanisms by which ARC3 regulates Z-ring and division-site positioning in chloroplasts.
- To address how chloroplast morphology affects chloroplast movement and photosynthetic performance, we will test the extent to which the magnitude and kinetics of chloroplast movement in mutants with different chloroplast morphologies are correlated with photosynthetic parameters and photoinhibition under continuous and various frequencies of fluctuating illumination.

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Interdisciplinary Research and Training Program in the Plant Sciences

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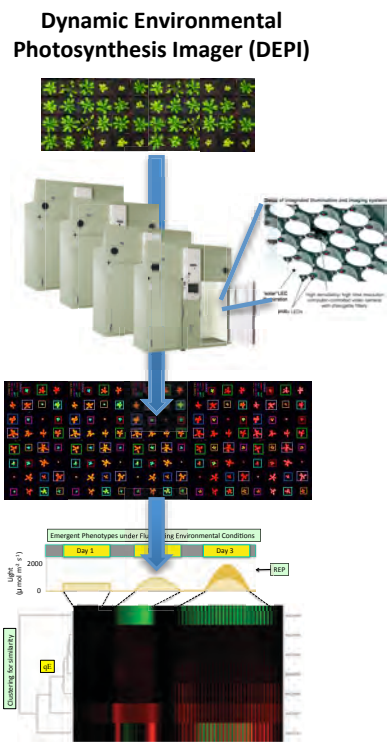
Overall research goals: During the current funding period, research has focused on the biogenesis, biochemistry, and biophysics of cellular energy systems (Brandizzi, Hu, Keegstra, Kramer, Montgomery, and Wolk); the interactions of photosynthetic organisms with the environment (He, Howe, Kramer, Montgomery, and Thomashow); and the biosynthesis of plant cell walls (Brandizzi, Keegstra and Walton). In addition, we are engaged in the development of novel technologies (Kramer and colleagues) to monitor photosynthesis in plants and microorganisms. Three of these enabling technologies are: 1) the Dynamic Environmental Phenotyping Image (DEPI) for high-throughput analysis of photosynthetic parameters (e.g., photosynthetic efficiency, photochemical quenching, photoinhibition), and growth under highly controlled, but dynamic environmental conditions (e.g., fluctuating light intensity and temperature); 2) the environmental Photobioteactor (ePBR) for probing photosynthetic, growth and biomass quality phenotypes in photosynthetic microorganisms, which are increasingly important for bioenergy applications; and 3) the Phenometrix Analysis System (PAS) for collecting, storing, and analyzing the streams of phenotyping data generated from PPP and APP.



Emergent photosynthetic phenotypes

Recent achievements 2012-2013: During the past two-year funding period, the PRL faculty published 62 articles reporting research supported by DOE-BES grant DE-FG02-91ER20021. A complete listing of publications can be found at the PRL website (http://www.prl.msu.edu/). Recent highlights include: establishing that light-dependent regulation of c-di-GMP and reactive oxygen species levels in cyanobacteria are central to fine-tuning adaptive responses that optimize photosynthetic efficiency (Agostoni et al., 2013; Singh et al., 2013); determining a role for phytochromes in regulating plastid gene expression through light-dependent anterograde signaling (Oh and Montgomery, 2013); showing that an [FeFe]-hydrogenase from *Shewanella oneidensis* MR-1 is active in *Anabaena* sp. strain PCC 7120 grown in air, but only when heterocysts are formed (Gärtner et al., 2012); demonstrating that cyclic electron flow is rapidly regulated by a new mechanism to balance the ATP/NADPH demands of algal carbon concentrating mechanisms (Lucker et al., 2013); defining a new mechanism for regulating the storage of thylakoid proton motive force (Ioannidis et al., 2012); defining the roles and mechanisms for redox and metabolic regulation of the chloroplast ATP synthase (Kohzuma et al., 2012, 2013); developing improved algorithms for measuring leaf growth (Tessmer et al., 2013) and analyzing time-series expression

data (Rosa et al., 2012); establishing roles for Arabidopsis dynamin-related proteins in the division of three energy organelles—chloroplasts, mitochondria and peroxisomes (Aung and Hu (2012); demonstrating that three Arabidopsis peroxisome membrane proteins—RING peroxins—are E3 ubiquitin ligases that function in conjunction with two ubiquitin receptor proteins (Kaur et al., 2013); providing functional evidence for the existence of contacts sites between the ER and the chloroplasts (Mehrshahi et al., 2013); identifying the N-methyl myristoylase responsible for post-translational modification of the ARF-GTPases that control traffic at the Golgi apparatus, the cellular site where most of the carbohydrates used in cell wall biosynthesis are synthesized (Renna et al., 2013); determining the mechanism of nuclear localization of JAZ transcriptional repressors involved in jasmonate signaling that regulates growth and defense (Withers et al., 2012); identifying JAZ repressors as the host targets of a bacterial effector that activates jasmonate signaling (Jiang et al., 2013); elucidating a mechanism by which alternative transcript splicing regulates the jasmonate response (Moreno et al., 2013); and establishing a role for three Calmodulin Binding Transcription Activator (CAMTA) transcription factors in regulating the biosynthesis of salicylic acid and growth at low temperature (Kim et al., 2013)

The past year has also included establishment of the MSU Center for Advanced Algal and Plant Phenotyping (CAAPP). CAAPP, which is co-funded by DOE-BES and a $5M commitment from MSU, aims to enhance the impact of the PRL efforts to develop "transformative phenotyping technologies," by bringing together investigators with a wide breadth of expertise to address the complex problems of next-generation phenotyping. Our goal is to develop CAAPP into an international point of destination for scientists to come and perform sophisticated phenotyping analyses relating to photosynthesis, growth and other aspects of energy-related research.

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Thylakoid membrane biogenesis in cyanobacteria

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Overall research goals:

The main aim of the project is to understand thylakoid membrane biogenesis. In previous work we have observed that very few ribosomes are associated with both cytoplasmic and thylakoid membranes, thus raising the question how membrane proteins are replaced or integrated and how thylakoid membranes get made in the first place. Also, chlorophyll is required for thylakoid formation in both plants and cyanobacteria, for reasons as yet unknown. If chlorophyll is depleted, for example by growing a *chlL*$^-$ mutant that is impaired in the light-independent pathway of protochlorophyllide reduction in darkness, large inclusions are seen in the cell, from which membranes emanate upon chlorophyll synthesis in the light. In normal growth conditions such large inclusions are not observed. Our working hypothesis is that under normal growth conditions membranes are formed from smaller inclusions (vesicles), and we are investigating the players involved in making these vesicles and synthesizing thylakoid membranes as indicated in the next sections.

Significant achievements 2012-2013:

- Overexpression of the *slr1090* gene led to overproduction of vesicles near the thylakoid and cytoplasmic membranes (Figure 1), in line with our hypothesis that the Slr1090 protein is involved in vesicle formation related to membrane biogenesis.
- Slr1090 does not appear to be necessary for thylakoid formation as the *slr1090*$^-$ strain is normal in its phenotype. This suggests that there may be multiple pathways to generate thylakoid membranes, which would explain why thylakoid membrane biogenesis has remained elusive for many decades.
- ClpB1 overexpression led to a major increase in tolerance of *Synechocystis* to rapid heating of the culture. Thylakoid morphology and characteristics of the photosynthetic apparatus were comparable to wild type.
- Overexpression of other chaperones led to overall improved thermotolerance regardless of the heating rate of the culture, but the ultrastructure of the cells remained largely normal.
- Following previous project goals, small open reading frames were identified in *Synechocystis* that coded for SCPs (Small Cab-like Proteins). These ORFs were expressed and in antisense orientation and overlapping with important genes (coding for ferrochelatase and pyruvate dehydrogenase). This may provide yet another level of regulatory interplay between chlorophyll and cellular metabolism.

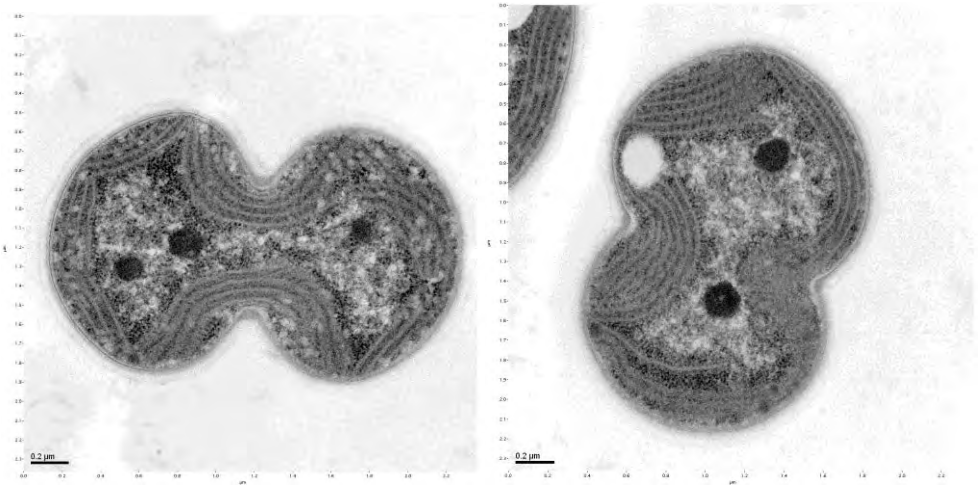

Figure 1. Representative electron micrographs of a cell of the *slr1090* overexpression (left) and deletion (right) strains. Note the large number of apparent small vesicles near the thylakoid membranes in the overexpression strain that are absent in the deletion mutant. The wild type (not shown here) has intermediate amounts of small vesicles. Large electron-transparent inclusions like in the cell of the deletion strain in the right panel are present in the overexpression strain as well but were not captured in the particular section shown to the left. The thickness of sections is 70 nm, which is about 5% of the diameter of a cell.

Science objectives for 2013-2014:

- Further investigate the function of Slr1090, including determination of membrane biosynthesis kinetics and appearance in mutants lacking both ChlL and Slr1090.
- Investigate the role of membrane protein integration factors (including Slr1531 and Slr2102) to determine their involvement in the thylakoid biogenesis process.
- Try to develop and test a working hypothesis regarding the parallel pathways that appear to be available in *Synechocystis* for thylakoid membrane formation.

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Geometric and Electronic Structure of the Mn_4Ca Cluster in Photosystem II and the Changes During the Catalytic Cycle

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Overall research goals: The specific questions that are the focus of our studies are: 1) What is the geometric and electronic structure and the changes of the Mn_4Ca cluster as it traverses the enzymatic cycle driven by the absorption of four photons? 2) What is the mechanism of the water-oxidation reaction that is catalyzed by the Mn_4Ca cluster? We are using steady state and time-resolved X-ray spectroscopy and crystallography methodologies at synchrotron and X-ray free electron laser sources in pursuit of these goals.

Significant achievements 2011-2013:

1) X-ray spectroscopy was used to study the Mn_4Ca cluster through the entire S-state cycle. The data shows distinct structural changes in the S_2 to S_3 transition, which are important for the mechanism of water-oxidation and oxygen evolution. The structure of the Mn_4Ca cluster from X-ray spectroscopy is similar to the 1.9 Å structure based on X-ray crystallography, but there are some important differences. We have combined data from both methodologies to propose the structural changes in the S-states, that has important implications to O-O bond formation and the mechanism of water oxidation (Fig. 1).
2) We have collected RIXS of PS II from all the S-states and the data shows that the charge is delocalized as the complex steps through the S-states. Detailed theory/simulations of the electronic structure are underway.

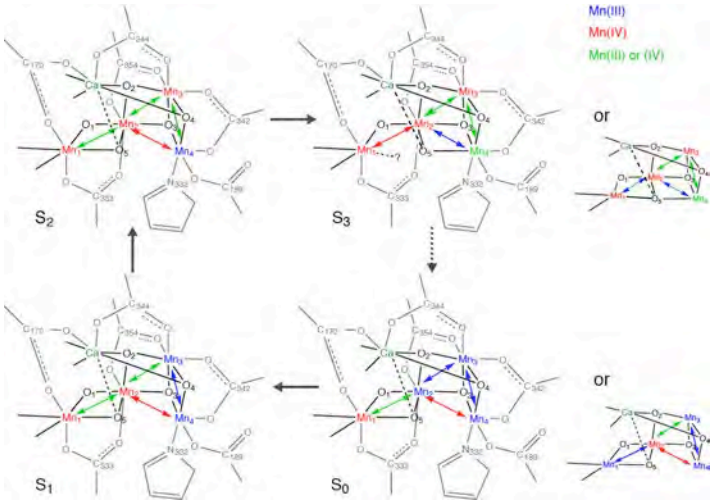

Fig.1. Possible structural changes during the S state transitions are illustrated. Note that the focus here is to accommodate the EXAFS distance changes, and possible protonation states (at oxo-bridging and terminal water molecules) or changes in the ligand environment (type of ligands and ligation modes) are not included in the figure. The Mn-Mn distances at ~ 2.7 Å are indicated by green arrows, ~ 2.8 Å by blue arrows and ~ 3.2 Å by red arrows. The dashed line indicates that it may not be a bond. For the S_3 and the S_0 state two possible models are presented. Mn atoms are shown in blue (Mn^{III}), red (Mn^{IV}) or magenta (Mn^{III} or Mn^{IV} possible), Ca in green.

3) We collected polarized Mn EXAFS from Ca-depleted samples of PS II in the S_1, S_2 and S_3-Ca-depleted states. The results show that removal of Ca does not significantly perturb the Mn cluster geometry. Although, Ca is required for the S_2 to S_3 transition and for O_2 evolution, it is surprising that removal of Ca does not significantly alter the structure of the Mn cluster.
4) X-ray spectroscopy studies of inorganic complexes have been very productive. We studied the heteronuclear Mn-Ca/Sr cubane-like complexes that are very good mimics of the structure present in PS II. Mn^{V} low and high spin states were investigated using X-ray emission spectroscopy. *In situ* X-ray spectroscopy/electrochemistry of water oxidation by a Ni oxide and both oxidation/reduction by a Mn oxide catalyst were used to determine the structural motifs of the catalysts.

Science objectives for 2013-2014:

- We will continue our solution and single-crystal EXAFS of the S_2, S_3 and S_0 states. The distance resolution that can be obtained from these results will be combined with the the XFEL data to understand structural changes and the mechanism and the O-O bond formation.
- The question of the presence/absence of isomeric structures in the S_2 (g=4.1 and g=2 S_2 states) and S_3 states will be investigated with X-ray spectroscopy.
- We will use Ca X-ray spectroscopy to determine the role of Ca as the Mn_4Ca complex traverses the catalytic cycle.
- We will continue to pursue studying inorganic systems of relevance to PS II with X-ray methods, especially, *in situ* X-ray emission and absorption spectroscopy of electrochemical water-oxidation catalysts.

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Session VII

Molecular Regulation of Photosynthetic Carbon Dioxide Fixation in Nonsulfur Purple Bacteria

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Overall research goals: The research objectives are to elucidate the molecular and biochemical mechanisms by which CO_2 fixation is controlled in nonsulfur purple photosynthetic bacteria. These studies involve specific studies to: (1) determine how a master transcriptional activator protein (CbbR) affects CO_2 fixation (*cbb*) gene expression in *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris*; (2) probe biochemical mechanisms as to how CbbR interacts with other regulatory proteins to maximize CO_2 fixation; and (3) determine the consequences of CO_2 fixation control and its reciprocal influence on other redox balancing processes in the cell.

Significant achievements (2011-2013): In *Rb. sphaeroides*, CbbR and RegA (PrrA) are transcriptional regulators of the cbb_I and cbb_{II} (Calvin-Benson-Bassham) CO_2 fixation pathway operons. Both proteins interact specifically with promoter sequences of the *cbb* operons. RegA has four DNA binding sites within the cbb_I promoter region, with the CbbR binding site and RegA binding site 1 overlapping each other. Previous progress reports and publications showed that these transcriptional regulators interact to form a specific RegA-CbbR protein complex on the DNA; we established that CbbR must first bind to the DNA at its specific binding site before RegA could interact with the CbbR-DNA complex. We have recently completed studies to define the residues in RegA that are required for specific interactions with CbbR, which is bound to the DNA. These residues are illustrated within the structure of RegA (Fig. 1). RegA greatly enhances the ability of CbbR to bind the cbb_I promoter. The N-terminal receiver domain and the DNA binding domain of RegA were shown to interact with CbbR. Residues in α-helix 7 and α-helix 8 of the DNA binding domain (helix-turn-helix) of RegA directly interacted with CbbR, with α-helix 7 positioned immediately above the DNA and α-helix 8 located in the major groove of the DNA.

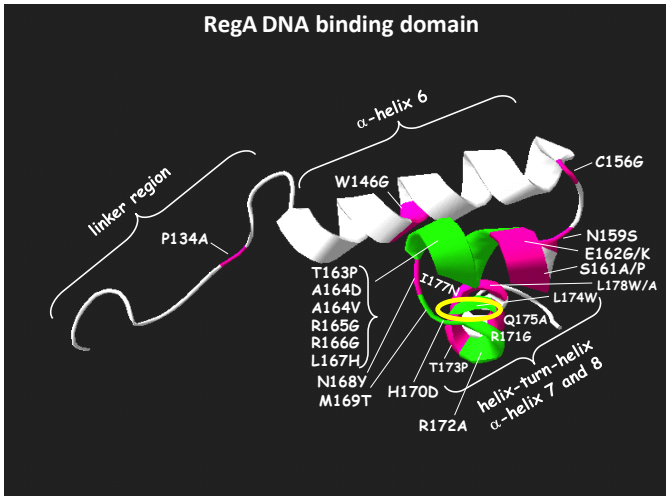

Fig. 1. Structural model of the RegA DNA binding domain (DBD) from *Rb. sphaeroides* (Luguri *et al*. 2003). RegA mutant residues highlighted in green abolish interaction with CbbR. Mutated residues highlighted in magenta do not abolish interaction with CbbR. Yellow circle identifies region where residues of the DBD of CbbR likely interact with residues of the DBD of RegA.

In *Rps*. *palustris,* CbbR interacts with two response regulator proteins, CbbRR1 and CbbRR2, that are part of a unique three protein "two-component" regulatory system. In addition, it was found that various metabolic intermediates influence these interactions. The response regulator proteins cannot by themselves bind to DNA. Rather, they exert their influence by interacting specifically with CbbR, which binds to a specific promoter sequence and positively affects *cbb* transcription. Depending on which response regulator protein, CbbRR1 or CbbRR2, as well as the presence or absence of key effector molecules, determines whether CO_2 fixation (*cbb*) gene expression is positively or negatively controlled. Protein-protein interactions were

illustrated via a number of techniques, including cross-linking studies, gel mobility shift assays, quantitative bacterial two-hybrid analyses, and Surface Plasmon Resonance (SPR) (Fig. 2). SPR was particularly useful as it enabled accurate determination of the rate constants for these interactions, with each protein, in the presence or absence of the effector molecules.

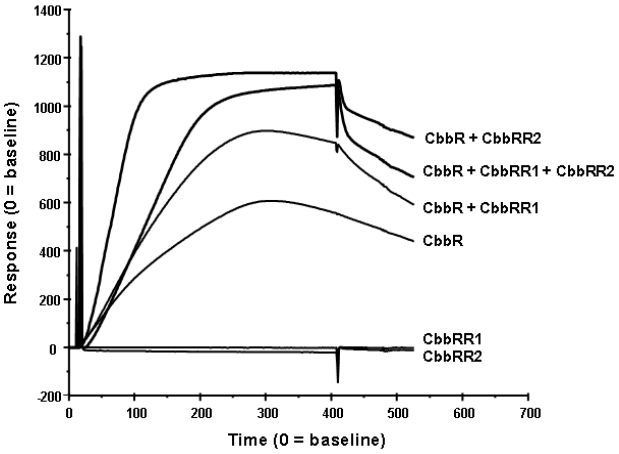

Fig. 3. SPR analyses indicate that CbbR binding to specific promoter sequences on a BiaCore sensor chip is influence by CbbRR1 and CbbRR2 and various effectors (refs. 3,5,6 below)

Clearly, from results with two different systems, *Rb. sphaeroides* and *Rps. palustris*, it is apparent that CO_2 fixation gene expression involves a complex interplay between a major transcription factor (CbbR) as well as a response regulator protein from a two-component regulatory system that is organism-specific. Effector metabolites were also shown to influence transcription factor interactions and subsequent *cbb* gene transcription

A third major advance during this grant period was the elucidation of the consequences and reciprocal control of the *cbb* system with other systems; e.g., the nitrogenase (*nif*) complex.

Science objectives for 2013-2014: There will be several approaches taken under the rubric of the major goal to discern the mechanism and consequences of transcription factor complexes in controlling CO_2 fixation (*cbb*) gene expression, including:
(1) further mutational analysis to determine amino acid residues required for specific interactions between the transcriptional apparatus and the major and ancillary regulator proteins
(2) determining how the regulatory protein complex interacts with RNA polymerase.
(3) continuing studies on the biochemical basis for reciprocal control of the *nif* and *cbb* systems.

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***** Gauri Joshi and Ryan Farmer obtained their Ph.D. degrees supported by this project.

Structure/Function of the Novel Proteins LCIB and LCIC in the Chlamydomonas CCM

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Overall research goals: Our goal is to understand the function of a novel protein, LCIB, as an essential component of the microalgal CO_2-concentrating mechanism (CCM). Because LCIB is a novel, soluble chloroplast stromal protein lacking any recognizable domains or any apparent homologs outside of the "green lineage" algae (e.g., green algae and diatoms), and a small number of cyanobacteria and bacteria, the specific nature of its critical role in the CCM still is somewhat unclear. We hypothesize that LCIB and the very similar protein LCIC form a multimeric complex that traps stromal CO_2 and converts it vectorially into stromal HCO_3^-. This vectorial conversion of CO_2 into HCO_3^- could prevent loss of stromal CO_2 released by HCO_3^- dehydration in the thylakoid lumen and also may capture external CO_2 diffusing into the stroma by trapping it as HCO_3^-. The experimental plan of this proposal includes the following specific aims:

1. Investigate the function of LCIB, LCIC and the LCIB/LCIC complex in the microalgal CCM using a combination of reverse and forward genetics.
2. Investigate the structure/function of LCIB/LCIC complexes both *in vivo* and *in vitro*.
3. Determine the structure of LCIB, LCIC and the LCIB/LCIC complex.

Significant achievements for 2012-13:

1) **Investigate the function of LCIB, LCIC and the LCIB/LCIC complex in the microalgal CCM using a combination of reverse and forward genetics.** We have developed a gene activation system for over-expression of genes in Chlamydomonas, including LCIA, LCIB and Ci transporters. This system is based on the **T**ranscription **A**ctivator-**L**ike **E**ffector (TALE) of secreted by *Xanthomonas* bacteria into the cells of their host plant. The TALEs bind to specific DNA promoter sequences and activate host plant genes to the benefit of the bacteria. So far, we have demonstrated this TALE system functions in Chlamydomonas by targeting and activating the ARS2 and ARS1 genes as proof-of-concept reporters.

   We previously identified several second-site suppressor mutations that bypass the need for LCIB (suppressors of the air-dier phenotype of *lcib* mutants) in low-CO_2 grown Chlamydomonas. We used deep sequencing (using Illumina NextGen sequencing) of bulk segregant progeny to map one of these, *su1*, to a narrow region of chromosome 16 and identified the likely mutation as a unique SNP in a low-CO_2-inducible gene. We are trying to confirm disruption of this gene as being responsible for the suppression phenotype of *su1* by using artificial micro RNA (amiRNA) knockdown of the putative *SU1* to recapitulate the suppression of an *lcib* mutant phenotype. Preliminary results from the amiRNA knockdown of *SU1* indicate that we have identified the responsible mutation.

2) **Investigate the structure/function of LCIB/LCIC complexes formed *in vivo* and *in vitro*.** In order to unravel the LCIB-LCIC interaction in their *in vivo* context, we have used strep-tagged LCIB transgenic lines to investigate the LCIB-LCIC complex formation using affinity pull-down assays. Even though distinct LCIB localization was observed in high CO_2- and very low CO_2-grown cells, no differences in the stoichiometry or composition of LCIB-LCIC complex formation in response to CO_2 were observed, which suggests that association and dissociation of LCIB-LCIC may not be involved in localization changes of the complex in response to different

CO_2 concentrations. In agreement with this interpretation, we did not observe any size differences of the LCIB-LCIC complex purified from high or low CO_2 grown cells by native gel electrophoresis.

Even though the sequence alignment shows that LCIB and LCIC are highly conserved, chimeric LCIB-LCIC fusion proteins we have tested so far cannot restore LCIB function in *lcib* mutants, indicating that some variant regions present in LCIB are important for its functions associated with Ci accumulation. We also have constructed 13 mutant species of LCIB comprising substitutions and deletions of highly conserved sites in native LCIB sequence. Site directed mutagenesis of LCIB has revealed that most these highly conserved amino acid residues, including two specific cysteines, are essential for LCIB function. However, deletion of the C-terminal variant amino acid sequences of LCIB has no apparent impact on LCIB function or on its re-localization upon CO_2 concentration changes, suggesting the region containing these variant amino acids is not an important functional domain of LCIB.

In vitro treatment of the affinity-purified LCIB-LCIC complex with reducing reagents did not impact the complex stability, so intermolecular disulfide bonds appear not to be required for complex formation. The essential cysteines may be part of the LCIB active site or be essential for intramolecular disulfide formation to maintain its structure or its interaction with LCIC.

We also have generated transgenic LCIB and LCIC over-expression Arabidopsis plants. The LCIB and LCIC have been introduced sequentially into the single plants. We have confirmed the over-expression of LCIB, although we haven't been able to confirm the LCIC expression in these plants yet. Once over-expression of both LCIB and LCIC confirmed, we will investigate whether a complex can be formed between these two proteins in Arabidopsis.

3) **Determine the structure of LCIB, LCIC and the LCIB/LCIC complex (Smith).**
We continued to try to obtain crystals of LCIB, but have thus far only been able to get very small micro crystals. As has been done with other difficult proteins, we have been trying to complex with Fab fragments from antibodies to LCIB. This, with some of our other projects, allows the protein to be coated with Fabs and can block those interactions that can interfere with crystallization. We also have determined it is likely that smaller fragments created in the thrombin cleavage of LCIB-GST fusions are poisoning the crystallization. We have created a GST expression vector with an rTEV protease site in between the GST and the LCIB. We have performed digestion of the highly-expressed, soluble LCIB, and the product looks very clean, and far better than with the thrombin cleavage. Our goal now is to start producing large amounts of soluble LCIB for crystallization alone and with Fabs attached. Ee feel that this new expression vector will finally lead to a break through.

Objectives for 2013-2014:

- Apply TALE activation to LCIB, LCIC and other CCM components to probe the impact of their over-expression.
- Further probe the structure/function of LCIB/LCIC by investigating functions of conserved amino acid sites and function domains of LCIB, as well as by expression of the LCIB-LCIC complex *in vitro* or in other system, such as in bacteria or high plants.
- Investigate the nature of LCIB/LCIC aggregation upon expression in transgenic Arabidopsis, as well as the impact of LCIB-LCIC expression in Arabidopsis on Ci assimilation.
- Succeed in crystallization of LCIB. This would yield immediate structural information, which should inform all other aspects of the project and provide insight into LCIB function.

Integration of the light and dark reactions of oxygenic photosynthesis

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Overall Research Goals:

The inducible high affinity carbon concentrating mechanism (CCM) is studied to understand how it is integrated with the light and dark reactions of photosynthesis. The basic hypothesis was that a tight integration is achieved through specific regulatory interactions between photosynthetic metabolites and the transcriptional regulatory proteins that control the expression of the structural genes for the inducible CCM. While such interactions had been hypothesized, the actual mechanisms had remained unresolved. *The DOE-supported analysis revealed that metabolic intermediates of the light and dark reactions, notably NADP$^+$ and α-ketoglutarate (α-KG), of cyanobacterial photosynthetic metabolism act as allosteric effectors of the DNA-binding proteins which modulate the expression of the CCM genes* (Daley et al, 2012). The research objectives involve:

1. Study the interactions between effector metabolites, transcriptional regulator, and the regulatory DNA sequences of the genes in the inducible CCM. Define the basic mechanics of the regulatory circuits of the CCM, which will benefit by a new collaboration with an expert in the structural and functional analysis, of the LysR-type transcriptional regulators involved (Prof. Cory Momany, U Georgia).
2. Connect transcriptional regulation findings with physiological analysis that probes how the deduced regulatory circuitry explains certain cellular responses to changing light and inorganic carbon availability conditions. Here we have made very good progress tracking the redox state of the cell, notably, the NAD(P)H/NAD(P)$^+$ ratio during the onset of inorganic carbon limitation.
3. Construction and analysis of experimental systems that will test the function of the CCM and test the feasibility of assembling genetically chimeric forms of the CCM. This will allow teasing apart some of the unique regulatory and enzymatic features of the CCM and will provide insight into the feasibility of one day transplanting the cyanobacterial CCM into eukaryotes.

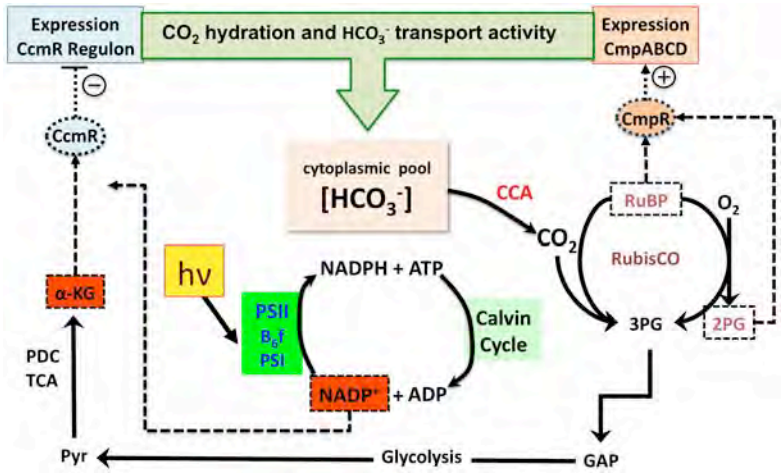

Figure 1. Model for the CCM regulatory network within *Synechocystis* showing CcmR and CmpR. Regulatory molecules and interactions are indicated with dashed lines, metabolic fluxes indicated with solid arrows. Ligand molecules for CcmR and CmpR are enclosed in dashed boxes. Using Surface Plasmon Resonance (SPR) Shawn Daley showed that NADP$^+$ and α-KG (red dashed boxes) function as co-repressors for CcmR. The depletion of these metabolites results in the de-repression CCM transcriptional expression to alleviate the scarcity of inorganic carbon (Ci).

Major Accomplishments 2012-2013

1. Real-time and biochemical tracking of physiological cues for gene expression:

The NADPH/NADP$^+$ ratio now appears key in the coordination of the expression of the CCM, carbon status, and the light reactions (Daley et al, 2012). Continuous measurements of chlorophyll *a* and NAD(P)H fluorescence during limitation of inorganic carbon now provide a window into the dynamics of the redox state of two key functional components of the photosynthetic mechanism: 1.) the plastoquinone (PQ) pool of the photosynthetic membranes and 2.) NADPH/NADP$^+$pool of the cytoplasm. Our work has defined NADP$^+$ and α-KG as 'regulating the regulator' insofar as the concentration of these metabolites allosterically control the transcription factor repressing the CCM genes. So far, real-time tracking of NAD(P)H is proving feasible and very interesting as exemplified in **Figure 2.**

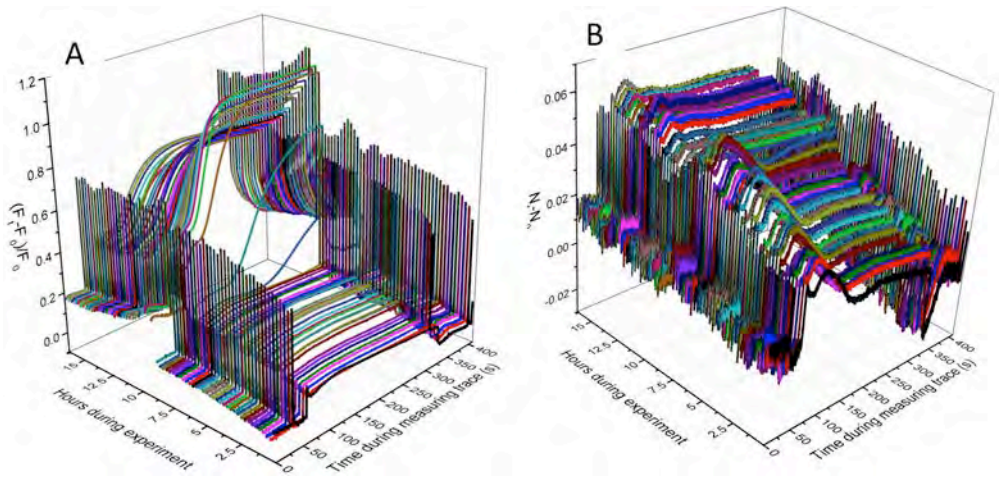

Figure 2. Carbon limitation affects the redox state of the PQ (Panel A) and NAD(P)H (Panel B) pools reflecting the progressive filling of metabolite capacity with electrons generated by the light reactions. Wild-type cells show a dramatic decline in photochemical quenching upon Ci limitation reflecting an over-reduction of the PQ pool due to loss of electron sink capacity. This constitutes a 'congestion' of the normal routes of photosynthetic electrons that occurs when inorganic carbon, the destination of photosynthetic reduction, becomes limiting. We think of this as diminished *'absorptive flux capacity'.* Of particular interest is the post-actinic overshoot (overshoot oxidation, left panel) of the steady-state NADPH/$NADP^+$ ratio in C_i replete cells (dark blue transient): Noting that the Calvin cycle is turned off in the dark and activated in the light, the overshoot is hypothesized to reflect high rates of NADPH consumption in the seconds after the light is off before the Calvin cycle is down-regulated yet NADPH production has abated due to darkness.

Significance: Tracking metabolism during C_i limitation is important because it helps resolve how photosynthetic cells cope with potentially catastrophic accumulation of excess photosynthetic reductant on the short-term and mobilize a long-term response by adjusting gene expression. Furthermore, having real-time data of key metabolites will become important as researchers attempt to validate computational metabolic models (flux-balance, metabolomics) and attempt to re-route metabolism for the production of desired products.

2. Biochemical analysis of metabolism: Determination of changing NADPH/$NADP^+$ Ratios and [α-KG]

During this last funding period, we have developed rapid-quench metabolite extraction techniques and utilized quantitative assays to show that NADPH/$NADP^+$ ratios become very high as predicted by the

Significance: Real-time spectroscopic tools are very powerful, yet we did not have direct evidence that the changing signals were indeed NADPH or perhaps some other fluorescent molecules accumulating under Ci-limitation. The biochemical experiments are now allowing us to assign the spectroscopic transients to NADPH. Now we are working out the assays for determining α-KG. Why are there two metabolic signals for the induction of the CCM via the transcriptional regulator CcmR? Based upon some earlier metabolomic data, it appears that α-KG levels may have rather different kinetic responses to Ci-limitation. We will test the hypothesis that the two metabolites ensure correct regulation under different conditions or during different phases of the adaptation process.

3. Other results to be reported: Structural analysis of the putative reverse carbonic anhydrase, CupA has been initiated and progress will be reported.

Publications:

Daley SME, Kappell AD, Carrick MJ, Burnap, RL (2012) Regulation of the cyanobacterial CO_2-concentrating mechanism involves internal sensing of $NADP^+$ and α-ketogutarate levels by transcription factor CcmR. PLoS ONE 7(7): e41286. doi:10.1371/journal.pone.0041286

Burnap RL, Nambudiri R, Holland S. (2013) Regulation of the carbon-concentrating mechanism in the cyanobacterium Synechocystis sp. PCC6803 in response to changing light intensity and inorganic carbon availability. *Photosynth Res.* 2013 Aug 29. [Epub ahead of print]

Manuscripts:

Holland S., Kappell, A., and Burnap, RL Redox changes accompanying inorganic carbon limitation in *Synechocystis* sp. PCC 6803 (in preparation).

Session VIII

Photosynthetic Hydrogen: Is a Tyrosine Radical Involved in Make the [FeFe] Hydrogenase Catalytic H-Cluster?

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The radical *S*-adenosylmethionine (SAM) enzyme HydG lyses free L-tyrosine to produce CO and CN^- for the assembly of the catalytic H-cluster of [FeFe] hydrogenase. We use electron paramagnetic resonance (EPR) spectroscopy to detect and characterize HydG reaction intermediates generated with a set of ^2H, ^{13}C, and ^{15}N nuclear spin labeled tyrosine substrates. We propose a detailed reaction mechanism (Fig 1) in which the radical SAM reaction, initiated at an N-terminal [4Fe-4S] cluster,generates a tyrosine radical bound to a C-terminal [4Fe-4S] cluster. Heterolytic cleavage of this tyrosine radical at the C_{alpha}-C_{beta} bond forms a transient 4-oxidobenzyl (4OB·) radical and a dehydroglycine bound to the C-terminal [4Fe-4S] cluster. Electron and proton transfer to this 4OB· radical forms *p*-cresol with the conversion of this dehydroglycine ligand to Fe-bound CO and CN^-, a key intermediate in the assembly of the [2Fe] subunit of the H-cluster (1).

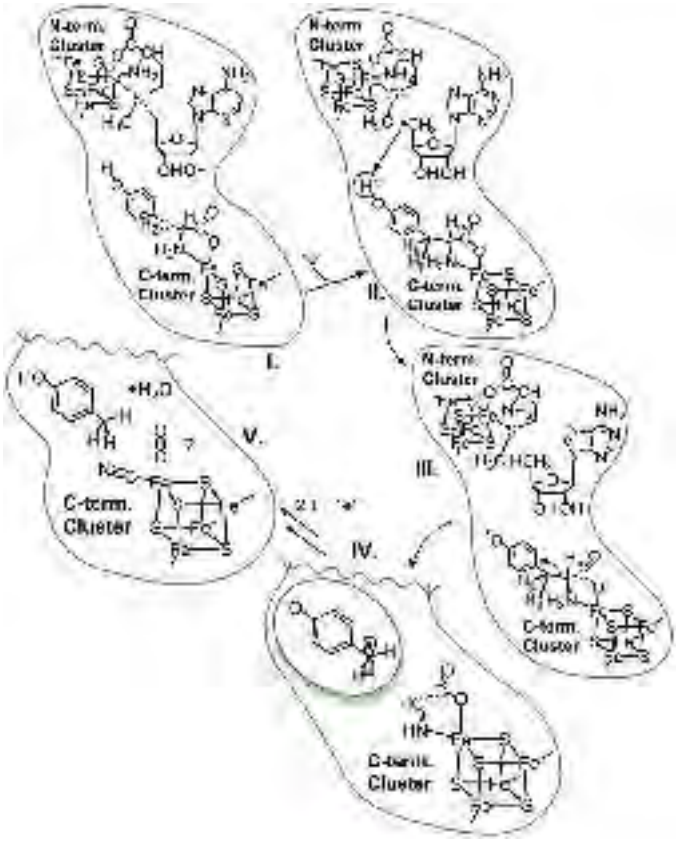

Figure 1: Tyrosine radical scission in forming Fe-bound CO and CN^- from tyrosine

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Redox Protein Hybrid Architectures for Solar Chemical Energy Conversion

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Overall research goals: This program is targeted at the synthesis of biomimetic hybrid assemblies for solar fuels applications by combining biological and chemical synthetic approaches. Key goals include: testing of concepts for exploiting *E. coli* expressed, multiheme c-cytochromes recruited from *Geobacter* to serve as molecular wire frameworks for bio-hybrid synthesis; characterization of the mechanisms for abiotic cofactor insertion into the multi-heme architectures; characterization of the photochemistry for abiotic cofactors within protein host sites; resolution of fundamental mechanisms for multi-step solar energy to fuels conversion in bio-hybrid architectures. This research has impact for metalloprotein design, fundamental mechanisms for multi-step solar energy conversion, and the development of new approaches for solar fuels catalysis in artificial photosynthesis. The results give insight both into physical parameters guiding the design of biohybrids and natural photosynthetic protein assemblies.

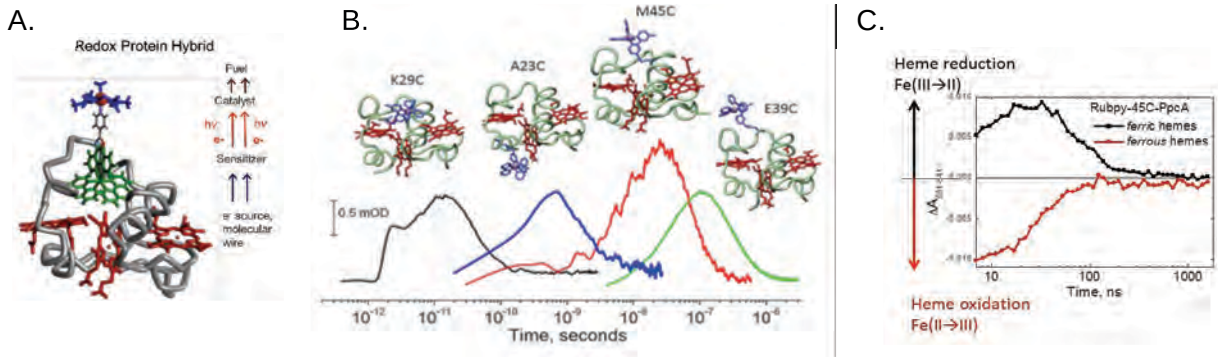

Figure 1. **A**. Design target for a biohybrid comprised of a synthetic photosensitizer-catalyst linked to cytochrome c7. The multiheme c-cytochrome serves as a source of multiple electrons for hydrogen evolution. **B.** Control on the rate of transient, light-induced heme reduction in cysteine-linked, Ru(bipyridy)$_3$-cytochrome c7 derivatives, using single site cysteine mutations at selected locations The positive absorption traces show 551 nm – 540 nm heme absorption transients for selected Ru(bpy)$_3$-cyt c7 conjugates. The structure above each kinetic trace shows a molecular dynamics equilibrium structure. **C.** Demonstration of differences in photo-oxidative and photo-reductive electron transfer light-induced electron transfer in the Ru(bpy)$_3$- K45C-cyt c$_7$ construct. The kinetic traces show heme 551 nm - 540 nm heme absorption changes measured with all hemes either poised reduced (red trace) or oxidized (black) before the laser flash.

Significant achievements 2011-2013: We have mapped out site-dependent photo-sensitized electron transfer to cofactor hemes in the tri-heme Geobacter PpcA using Ru(bpy)$_3$ derivatives that are covalently linked to cysteine residues placed at a variety of positions on the cytochrome c7 surface through site-directed mutagenesis. Rates of electron transfer were found to vary from 10^{11} s^{-1} to 10^{6} s^{-1} depending upon the site and pathway for electron transfer. In all cases, forward electron transfer was found to compete with heme-dependent, non-electron transfer excited-state quenching. Photochemical quenching processes are found to track in parallel the site-dependent electron transfer, indicating that both processes follow similar pathway

dependences. These results establish a criterion for constructing photocatalytic pathways in multi-heme proteins, one that requires multi-step electron transfer to prevent heme-based sensitizer quenching and rapid charge recombination pathways. Comparable studies of electrostatically-directed photosensitizer-cytochrome c7 assembly showed that the three surface exposed hemes provide similar but more efficient pathways for quenching and transient electron transfer that function on the 10^{-12} s timescale, and further induced multimerization of the cytochrome assemblies.

Science objectives for 2013-2014:

- Synthesis of hybrids that covalently connect catalyst-sensitizer dyads using both hydrogen and oxygen evolving catalysts. Fast electron transfer within catalyst-sensitizer dyads will be tested as a mechanism to rapidly convert initial excited-states to charge separated states and offer the opportunity to use multi-heme proteins as sources and sinks for electrons in solar fuels catalysis.
- Test peptide designs that use amino acid specific metal-binding and covalent linking chemistries to organize photosensitizer-catalyst assemblies on the cytochrome c7 proteins. Initial designs will utilize His-Cys pairs for porphyrin and cobaloxime site-specific attachments.
- Construct single site, heme-deleted mutants in multi-heme cytochromes c7, and reconstitution with Zn-porphyrin and chlorophyll derivatives and corresponding catalyst dyads for light-initiated catalysis.

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Photobiohybrid Solar Fuels

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Overall research objectives: The long-term goal of this project is to understand the process of solar energy conversion into H_2 in model photochemical systems that combine light harvesting nanoparticles with hydrogenases. Light-driven production of H_2 occurs naturally in photosynthetic microbes, where hydrogenases couple to low potential reductant pools to help maintain electron flow under anaerobic-aerobic transitions. The ubiquitous role of H_2 as an energy carrier in microbial systems is underscored by the significant structural diversity among the different hydrogenase enzyme classes. Corresponding functional properties, including active site coordination, substrate transfer pathways and domain compositions of selected enzymes models are being investigated towards developing a broader fundamental understanding of hydrogenase diversity.

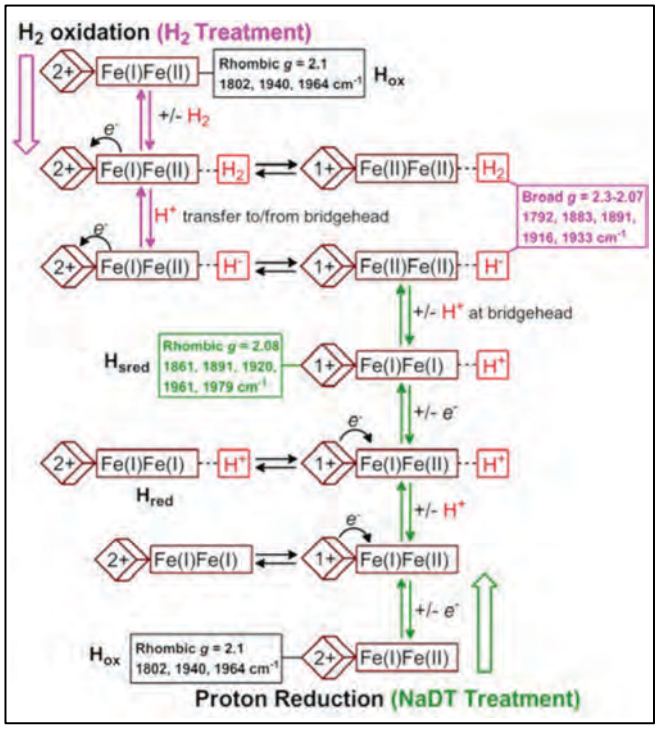

Figure 1. Summary of H-cluster intermediates proposed for CrHydA1 under H_2 or NaDT (proton transfer to the bridgehead amine is not pictured). Prominent EPR and FTIR spectroscopic signals (CO peaks) are indicated in the boxes, along with previously assigned states (i.e., H_{ox}, H_{red}, and H_{sred}). The cube represents the $[4Fe\text{-}4S]_H$ subcluster, while FeFe represents the $2Fe_H$ subsite. Electron-transfer between the two subsites is indicated by the curved arrows (proximal/distal Fe positions of the subsite are not specified).

Significant achievements 2011-2013: (i) In collaboration with the Moore and Gust group at ASU, clostridial [FeFe]-hydrogenase was studied on Au electrodes bearing self-assembled thiol monolayers (SAMs). Binding was mediated between positively charged patches on the hydrogenase and carboxylate groups on the SAM. Single-molecule images were obtained in an electrochemical STM and showed the tunneling currents increased under an applied bias, which led to an estimated lower limit k_{cat} value of 20,000 s^{-1}, in combination by macroscopic voltammetry. (ii) Clostridial [FeFe]-hydrogenase and mercaptopropionic acid (MPA) capped CdS/CdTe nanoparticles form complexes where the nanoparticle adsorbs to the hydrogenase ferredoxin-binding surface, adjacent to the distal [4Fe-4S]-cluster. Ultrafast measurements showed photoexcited, interfacial ET involves exchange from nanoparticles into the distal [4Fe-4S] cluster and occurs at ~10^7 s^{-1}, rates that are much faster than photochemical H_2 production turnover. (iii). The free energies along proton-transfer (PT) pathways in [FeFe]-hydrogenase were investigated using QM/MM and umbrella sampling techniques. Key residues

were identified along with pK_a estimations from a thermodynamics integration method and used to model PT profiles to the H-cluster. (iv) The [FeFe]-hydrogenase from the green alga *Chlamydomonas reinhardtii*, consisting of only the catalytic H-cluster, was studied using EPR and FTIR spectroscopy. The spectra revealed several unreported paramagnetic and IR signals under reducing conditions, which have been modeled into a revised catalytic scheme (Figure 1) incorporating electron exchange between the two H-cluster ($[4Fe\text{-}4S]_H$ and $2Fe_H$) sub-sites during turnover.

Science objectives for 2013-2014:

- Theoretical exploration of H-cluster diiron center conformations and potential intermediate structures are being developed towards modeling and identifying discrete candidate states, including basic vibrational spectra and EPR *g*-tensor calculations.

- The characterization of proton-transfer mutants of algal [FeFe]-hydrogenase by FTIR and EPR is underway. So far the spectral results assigned to the H-cluster show similar features to the wild-type enzyme, but with differential enrichment of sub-populations under reduction. Future work will aim to understand these differences in the context of the model being developed for H_2 activation by the catalytic site H-cluster.

- A novel [FeFe]-hydrogenase identified in the genome of *Clostridium perfringens* with a ruberythrin domain has been expressed and purified, and the EPR and FTIR properties are being characterized. Based on the structural composition we hypothesize the enzyme couples H_2 oxidation to H_2O_2 reduction, which would be a unique biochemical function to have evolved among the more O_2 sensitive [FeFe]-hydrogenases. The functional properties will be tested using H_2 uptake and H_2O_2 reduction assays.

- We have been characterizing the electron-transfer and photocatalytic properties of complexes between clostridial [FeFe]-hydrogenase and CdS or CdTe nanoparticles. Future efforts will begin to examine how nanoparticle size scales control ET rates and H_2 production quantum yields towards understanding the thermodynamic and kinetic control of solar conversion in these systems.

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> University Grants: "This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, under Award # <*insert grant award number*>."
>
> National Laboratory FWP projects: "This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division."

2. Research solely supported by a single grant or FWP from DOE BES, that includes coauthors supported by graduate student/postdoctoral merit-based award fellowships.

When applicable, the acknowledgement should also indicate the support of graduate students and/or postdoctoral researchers provided by personal fellowship awards. Some examples are shown below:

> University Grants: "This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences under Award # <*insert grant award number*>. A.B.C. acknowledges a graduate fellowship through the DOE Office of Science Graduate Fellowship program. D.E.F. acknowledges a postdoctoral fellowship from the Human Frontier Science program."
>
> National Laboratory FWP Projects: "This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences, Chemical Sciences, Geosciences, and Biosciences Division. G.H.I. acknowledges support through the Glenn Seaborg postdoctoral fellowship. J.K.L. acknowledges support from the DOE Science Undergraduate Laboratory Internship (SULI) program."

3. Research solely supported by a single grant or FWP from DOE BES that included work at a multi-user facility.

When a multi-user facility (e.g., national user facilities funded by DOE-SC or shared facilities or research instrumentation funded by NSF or another agency) was also used for the research, the publication should include appropriate acknowledgement of the facility or instrumentation and its supporting agency. The following illustrative example is given for a scenario involving a BES-supported university grant and two multi-user facilities:

> "This work was supported by the U.S. Department of Energy, Office of Science, Basic Energy Sciences under Award # <*insert grant award number*>. Neutron scattering was carried out at the Spallation Neutron Source and High Flux Isotope Reactor which are supported by DOE, Office of Science, BES. Small-angle x-ray scattering work was carried out at UCSB-MRL which was supported by the NSF-MRSEC program."

4. Research supported by multiple funding sources.

When the work was only partially supported by a single DOE BES grant or FWP, it is important to clearly state which aspect of the reported research was funded by DOE BES and which other part of the work was supported by each of the other acknowledged sponsors. Although DOE does not require (or expect) that a dominant funding sponsor be indicated in the acknowledgement, a word such as "primarily" can be included to indicate such a distinction.

As indicated by the following examples, different approaches may be used to distinguish funding sources and research supported for scenarios involving a BES-funded university grant. DOE BES expects that at least one of these approaches will allow the authors to communicate what portion of the research was supported by their BES grant/FWP while simultaneously adhering to the guidelines for acknowledgement for each of the other funding agencies involved in supporting the published research.

4a. Multiple funding sources with distinction by scope/specific aspects of the research

"Research <*insert "primarily" if appropriate and desired*> supported by the U.S. Department of Energy (DOE), Office of Science, Basic Energy Sciences (BES), under Award # <*insert grant award number*> (neutron scattering studies), by the National Science Foundation (NSF) under Award # <*insert grant award number*> (computational studies), and by the National Institutes of Health (NIH) under Award # <*insert grant award number*> (synthesis of samples). "

[Note that BES expects that distinction by scope/specific aspects of the research will be possible in many cases and that PIs will typically use this type of delineation.]

4b. Multiple funding sources with distinction by institution

"Work at <*insert Institution #1*> was supported by the U.S. Department of Energy (DOE), Office of Science, Basic Energy Sciences (BES) under Award # <*insert grant award number*>. Work at <*insert Institution #2*> was supported by DOE, Office of Science, BES under Award # <*insert grant award number*>."

[Note that BES expects that PIs will use this form of acknowledgement only when it is clear from the publication what scope of work was done at which institution.]

4c. Multiple funding sources with distinction by individual

"Work by <*Investigator #1*> was supported by the U.S. Department of Energy (DOE), Office of Science, Basic Energy Sciences (BES) under Award # <*insert grant award number*>. Work by <*Investigator #2*> was supported by the National Science Foundation (NSF), Division of Materials Research (DMR) under Award # <*insert grant award number*>."

[Note that if support is listed by individual author, an individual should not be shown as being funded by more than one sponsor. Although further delineation by scope of research may be possible in a particular case, simply listing multiple funding sources for a single individual generally suggests that the sponsors are supporting the same research. Similarly, listing all of the sponsors that have provided salary support for any individual but were not directly supporting the research in the publication is NOT an acceptable practice.

See questions 2 and 3 in the Q&A section at the end of this document.]

5. Multiple funding sources with distinction by institution (or investigator) combined with use of a multi-user facility

   As an illustration of the many combination scenarios that are possible, the following is an example of a proper acknowledgement for a BES-funded university grant in this scenario:

   "This work at <*insert name of Institution #1*> is supported by the Office of Naval Research grant # <*insert grant award number*> and the work at the <*insert name of Institution #2*> is supported by the U. S. Department of Energy, Office of Science, Basic Energy Sciences under Award # <*insert grant award number*>. Transmission electron microscopy was performed at the Northwestern University's NUANCE Center which is supported by NSF-NSEC <*insert grant award number*>, NSF-MRSEC <*insert grant award number*>, the Keck Foundation, the State of Illinois, and Northwestern University. The National Institute of Standards and Technology, U.S. Department of Commerce, provided the neutron research facilities; the SPINS spectrometer is supported in part by NSF under Agreement No. <*insert agreement number*>."

To reiterate, the aforementioned are only examples and recommendations for acknowledging DOE BES funding. If you have any questions or have a situation that is not covered, please ask your Program Manager for guidance prior to publication submission.

Examples of Improper Acknowledgements:

Please note that simply presenting a list of supporting agencies in a shared acknowledgement should not be done because it is impossible to discern the level and scope of BES support relative to that of other agencies as in the following examples. Such acknowledgements may also lead one to conclude that all the listed agencies supported the same research, i.e., duplicate funding. Similarly, listing all of the salary support for a particular individual without further delineation suggests that all the listed agencies supported the same research and/or could include funding sources that were not directly supporting the published research.

1. "This work was supported by DOE grant XXXXXXX, NSF grant DMR-YYYYYYY, and NIH grant GM-ZZZZZZZ."

2. "This work has been financially supported by the Office of Naval Research, primarily through the TEC-MURI program, and by the DARPA NMO program as well as the <name of the EFRC>, an Energy Frontier Research Center (EFRC) funded by the U.S. DOE Office of Basic Energy Sciences."

3. "This work was supported by DOE grant XXXXXXX and NSF (CHE-YYYYYYY) as well as the NSF MRSEC (DMR-ZZZZZZZ) at the Advanced Materials Research Center of <*insert name of Institution #1*>. A.B.C. acknowledges support through the NSF Graduate Research Fellowship program. D.E.F. is grateful for support by DOE Early Career Award XXXXXXX; Office of Naval Research Award WWWWWWW; the <name of the EFRC>, an Energy Frontier Research Center (DOE Award DE-SC0000000); and NSF (CHE-YYYYYYY)."

Please remember:

- **A lack of clear delineation of support (in the acknowledgements of publications) is a potential basis for non-renewal and/or termination of funding support.**
- **In some recent cases, it has also led to investigations by the Inspector General's Office and prosecution.**

FAQs – to be updated as new questions are submitted

1. We submitted a manuscript for publication with a detailed acknowledgement as requested by BES but the journal wouldn't allow the in-depth description of research funding. How can I ensure that an acknowledgement provides sufficient information regarding multiple funding sources and associated research if the journal where the paper will be published does not permit detailed descriptions of the research funding for the acknowledgement?

   If the publication has space limitations (*i.e.,* limits the amount of text) and/or restricts the language to be used in the acknowledgement, you may use the supplemental/supporting material section of the manuscript to provide specific details on the funding for the research to be published.

   If the journal policy limits or otherwise restricts the acknowledgement section such that the required BES information cannot be included at all (*e.g.,* in the supporting material), then you can annotate the acknowledgement in the annual progress report submitted to BES. The goal is to ensure that DOE (as well as other funding agencies) has a record that clearly delineates the research supported by BES funding from that supported by other sources.

2. Must all authors acknowledge a funding source or may unfunded authors be omitted in the acknowledgment section? Our author list includes undergraduates who were not paid but contributed to the project as part of their laboratory research.

   Individuals who are authors on the publication but are unfunded (*e.g.* undergraduates who are working in the lab to get research experience, etc.) should not be included in the funding details of the publication's acknowledgement section since there is no funding agency or source to recognize.

3. One of the authors on our publication is a faculty member whose students are paid through BES funds. However, the faculty member does not receive any salary support from BES or from any other agency for the research reported in the project. Should the acknowledgement list the grant(s) that provides salary support for this faculty member?

   The acknowledgement should only include funding that contributed directly to the research reported in the publication. In the above example, the funding source for the students and postdoctoral researchers who conducted the study should be acknowledged, but the funding source for their faculty mentor would normally not be included (assuming this was covered by the faculty's university salary). Also, if the faculty member's salary funds come from a grant supporting studies unrelated to the research in the publication, that grant should not be listed in the acknowledgement section. If the faculty member needs salary support in order to contribute to the BES project/s, this should have been considered in allocation of BES funds.

4. I am at a DOE National Laboratory and I'm preparing a publication describing our latest results from research supported by BES through an EFRC, an FWP, and the Fuels from Sunlight Energy

Innovation Hub (the Joint Center for Artificial Photosynthesis). Since an award number is not applicable to national laboratory funding, how do I distinguish the different sources of BES funding in the publication acknowledgement?

The multiple funding sources should be distinguished by the scope/specific aspects of the research. An example would be:

Research is supported by U.S. Department of Energy (DOE), Office of Science, Basic Energy Sciences (BES), Chemical Sciences, Geosciences, and Biosciences Division (photochemical measurements); by <insert name of EFRC here> (material synthesis and screening), a DOE BES Energy Frontier Research Center; and by the Joint Center for Artificial Photosynthesis (theoretical studies of membrane assemblies), the DOE BES Fuels from Sunlight Energy Innovation Hub.