FINAL TECHNICAL REPORT
GRANT NAME
GRANT NUMBER DE-FG02-08ER20314
TITLE OF THE PROJECT The Type 1 Homodimeric Reaction Center in *Heliobacterium modesticaldum*
PI'S NAME AND TITLE John H. Golbeck, Professor
PERIOD OF PERFORMANCE Sept. 15, 2010 to Sept. 14, 2013

A total of **6** peer-reviewed papers, plus **1** review article, **1** book chapter, and **1** edited special issue of a journal devoted to homodimeric Type I RCs have resulted from the work. The title and authorship of the relevant publication or manuscript are listed in this final technical report.

I. Observation of Variable Fluorescence in Living Cells

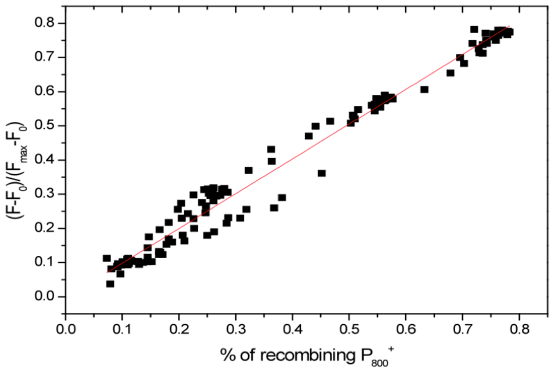

Figure 1. Correlation of rise of fluorescence and fast back-reacting state in illuminated *H. modesticaldum* cells. The fraction of back-reacting RCs vs. the variable fluorescence measured at various time points during the fluorescence induction at three different light intensities.

It had been reported that heliobacteria emit a variable fluorescence signal, a property commonly associated with oxygenic phototrophs (Collins, Redding et al. 2010). In this grant period, we uncovered the source of the signal. Part of this work was performed by one of the PIs (Redding) while on sabbatical.

I.A. Fluorescence Induction and Correlation with Charge Recombination in vivo

The critical finding was that fluorescence emission from heliobacterial cells was found to increase during exposure to actinic light, although its characteristics were very different from that in oxygenic phototrophs (Collins, Redding et al. 2010). Using pump-probe spectroscopy in the nanosecond timescale, we found that illumination of heliobacterial cells with bright light for a few seconds will put them into a state in which a significant fraction of the RCs undergo charge recombination from $P_{800}^+A_0^-$ with a time constant of ~20 ns. The fraction of RCs in the rapidly back-reacting state correlated very well with the variable fluorescence (**Figure 1**), indicating that nearly all of the increase in fluorescence can be explained by charge recombination from $P_{800}^+A_0^-$, some of which regenerates the singlet excited state.

I.B. Time-resolved fluorescence emission

This hypothesis was tested directly by time-resolved fluorescence studies in the ps and ns timescales. The major decay component in cells had a 25-ps decay time, representing trapping by the RC. Treatment with dithionite resulted in the ~200-fold increase of a 18-ns decay component. It represented only 0.5% of the decay, but this quantitatively accounts for the increase in steady-state fluorescence. We concluded that strong illumination of heliobacterial cells results in saturation of the electron acceptor pool, leading to reduction of the acceptor side of the RC and the creation of a back-reacting RC state that gives rise to delayed fluorescence. Based on this *in vivo* data, we cannot identify the cofactor that is reduced, but based on the results described below, we can ascribe it to F_x with some confidence. This would indicate that photoaccumulation of F_x^- can be readily formed in heliobacterial cells.

II. A Homogeneous Preparation of HbRC Cores

The preparation of HbRC cores we previously published (Heinnickel, Agalarov et al. 2006) was sufficiently pure for spectroscopic studies, but it contained a few protein contaminants and was therefore too impure for crystal growth. In this grant period, we improved on the purification by adding a cation exchange chromatography step. We found that this removed the contaminating proteins, which were identified by mass spectrometry to be membrane-bound cytochrome c_{553} and four different ABC-type transporters (**Figure 2**). The purified PshA$_2$ homodimer was found to bind 20 bacteriochlorophyll (BChl) *g*, two BChl *g′*, two 8^1-OH-Chl a_F, and one 4,4′-diaponeurosporene. The ratio of 22 (BChl *g*/*g′*)/P$_{800}$ agrees nicely with the 21.6 ± 1.1 (BChl *g*/*g′*) /P$_{800}$ determined earlier by a method that used as its basis the 4 irons and 4 sulfurs associated with F$_X$ in each HbRC core (Heinnickel, Agalarov et al. 2006). There are ~1.6 menaquinones per HbRC in the high-purity preparation. The HbRC core was active in charge separation and exhibited a trapping time of 23 ps as judged by time-resolved fluorescence studies. The decay of the charge separated states was ~15 ms, as seen earlier (Heinnickel, Agalarov et al. 2006). We also discovered that high-purity HbRCs were very stable and could be purified without conversion of BChl *g* to Chl *a* in the presence of O$_2$ under very low light intensities. The following paper resulted from this study.

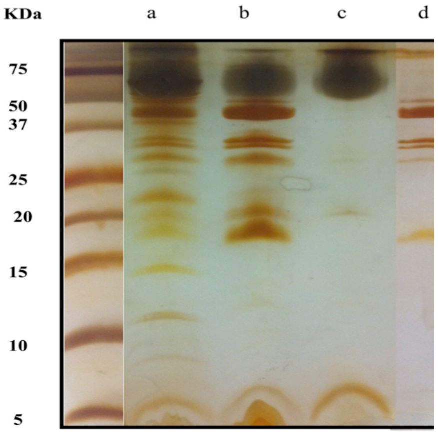

Figure 2. Purification of the HbRC core. Silver-stained SDS-PAGE gel of complexes: sucrose gradient fraction (**a**). Previous preparation (**b**) was loaded onto CM-Sepharose, washed (**d**) and eluted with MgSO$_4$ (**c**). PshA indicated with arrow.

Sarrou, I.; Khan, Z.; Cowgill, J.; Lin, S.; Brune, D.; Romberger, S.; Golbeck, J. H.; Redding, K. E. (2012) Purification of the photosynthetic reaction center from *Heliobacterium modesticaldum*. *Photosynth. Res. 111*, 291-302.

III. Expression and Purification of Electron Donor and Acceptor Proteins

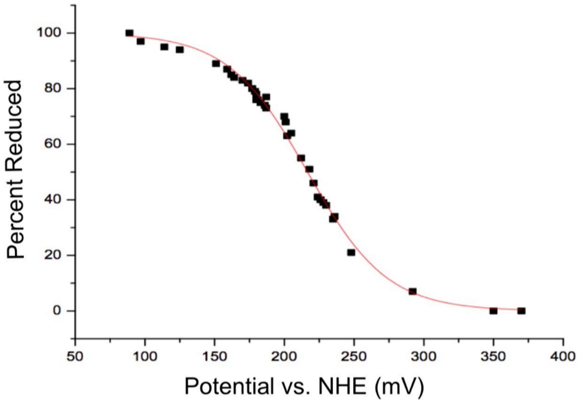

Figure 3. Redox titration of recombinant cyt c_{553} with unconstrained fitting to the Nernst equation (n = 0.91±0.02, E$_m$ = 217±1 mV vs. NHE).

Two of the peculiarities of the HbRC are the presence of a membrane bound cytochrome (cyt) c_{553} as the electron donor to P$_{800^+}$ (Oh-oka, Iwaki et al. 2002) and the presence of F$_A$ and F$_B$, on two loosely-bound bacterial dicluster ferredoxins, PshBI and PshBII (Romberger and Golbeck 2010). In the current grant period, we studied the thermodynamic and kinetic properties of these donor and acceptor proteins.

III.A. Cytochrome c_{553} as Electron Donor

We made a recombinant version of cyt c_{553} to have a donor to P$_{800^+}$ with faster kinetics than either ascorbate or dithionite. A soluble version was made by replacing the lipid-attachment site (Albert, Rutherford et al. 1998) with a hexahistidine (His$_6$) tag located immediately after the introduced signal peptide and signal peptidase cleavage site. The protein was induced by addition of IPTG in an *Escherichia coli* strain over-expressing the cytochrome *c* maturation system (Feissner, Richard-Fogal et al. 2006). A large amount of cyt c_{553} is obtained from the periplasm of such cells, and a single pass through an immobilized metal affinity chromatography (IMAC) column is sufficient to obtain pure protein. Mass spectrometry confirmed that the purified protein was of the expected molecular weight. The optical spectra of the oxidized and reduced protein appear identical to those of native cyt c_{553}, which we have purified in small amounts. The EPR spectra of the native and recombinant proteins in the oxidized state also appear very similar, and are of low-spin hemes. The measured reduction potential of the recombinant protein is +217 mV vs. NHE (**Figure 3**), which is very similar to the reported value for cyt c_{553} purified from another heliobacterium (Albert, Rutherford et al. 1998). As expected, the recombinant cyt c_{553} is an efficient electron donor to P_{800}^{+}.

III.B. HM1_1461 (PshBII) and HM1_1462 (PshBI) as Loosely-Bound Electron Carriers

We showed previously that the protein containing the F_A and F_B clusters was encoded by the downstream member of a dicistronic operon composed of HM1_1461 and HM1_1462. These genes encode two polypeptides similar to bacterial dicluster ferredoxins, which we have named PshBII and PshBI, respectively. PshBI (HM1_1462) is a 54-residue polypeptide with an estimated pI of 3.53, and PshBII (HM1_1461) is a 54-residue polypeptide with an estimated pI of 4.86 (Heinnickel, Shen et al. 2007). The two polypeptides are 61% homologous (33 identical and 5 similar residues) and each contain two CxxCxxCxxxC motifs. In the current grant period, we cloned the HM1_1461 gene, overexpressed a His_6-tagged version of the apoprotein in *E. coli*, and purified it by IMAC. We showed that the holoprotein harbors 8 iron and 8 sulfides, consistent with the presence of two [4Fe–4S] clusters. Incubation of the reconstituted holoprotein with HbRC cores resulted in a lifetime of the charge-separated state characteristic of electron transfer to the F_A and F_B clusters. These results suggest that similar to the previously characterized PshBI protein (Heinnickel, Shen et al. 2007), PshBII is capable of functioning as an electron acceptor. Unlike PS I, which contains the tightly bound PsaC subunit, two ferredoxins, PshBI and PshBII, interact with the HbRC core as loosely bound electron carriers. The following paper resulted from this work.

Romberger, S. P., Castro, C., Sun, Y., and Golbeck, J. H. (2010) Identification and characterization of PshBII, a second F_A/F_B-containing polypeptide in the photosynthetic reaction center of *Heliobacterium modesticaldum*, *Photosynth. Res. 104*, 293-303.

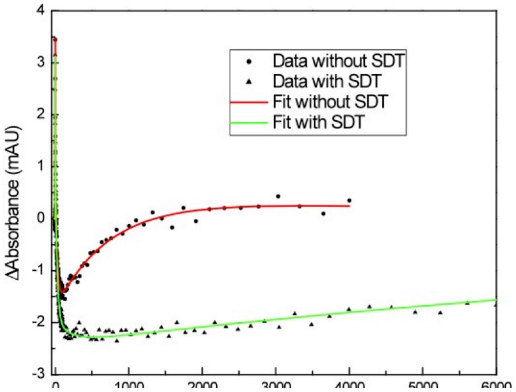

Figure 4: Transient absorption kinetics at 675 nm for HbRCs reduced with either 10 mM dithionite at pH 10 (red) or 10 mM ascorbate and 20 μM PMS at pH 10 (green).

III.C. Reduction of Flavodoxin from Synechocystis sp. *PCC 6803*

The finding that PshBI and PshBII are loosely bound proteins implies that F_X is the terminal Fe/S cluster in the HbRC. If true, then a variety of mobile electron transfer proteins should be able to accept electrons from F_X^-. Using HbRC cores, we found that the F_X cluster directly reduces cyanobacterial flavodoxin (Fld) without the involvement of F_A or F_B. (Fld was used as a proxy for soluble redox proteins, even though a gene encoding Fld has not been identified in the annotated *H. modesticaldum* genome.) Fld reduction by the HbRC was inhibited by addition of PshBI and PshBII, a finding consistent with competition for access to F_X^-. In contrast, P_{700}-F_X cores from PS I require the presence of the PsaC (i.e. the F_A/F_B clusters), for reduction of Fld or ferredoxin. This finding indicates that that F_X^- should be capable of donating electrons to a wide variety of yet-to-be characterized soluble redox partners. The following paper resulted from this work.

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.**

IV. Ultrafast Spectroscopy of Primary and Secondary Charge Separation

The involvement of phylloquinone in forward electron transfer is well established for PS I but evidence for the participation of a quinone in the HbRC is still equivocal. Because we had perfected a protocol to chemically reduce F_x, we were poised to carry out transient absorption studies to determine the identity of the cofactor that immediately precedes F_x. We used a pump-probe setup to study light-induced charge separation and electron transfer in purified HbRCs and obtained transient spectra for the HbRC in the range of 630-830 nm. Global fitting resolved two kinetic components: a 25-ps component assigned to primary charge separation ($RC^* \rightarrow P_{800}^{+}A_0^{-}$), similar to what has been reported previously for membranes or particles from other heliobacterial species (Neerken and Amesz 2001), and a ~600-ps component assigned to secondary electron transfer ($P_{800}^{+}A_0^{-} \rightarrow P_{800}^{+}F_X^{-}$). The features in the decay-associated spectra are consistent with the assignment of A_0 to the 8^1-OH-Chl a_F found in the HbRC, as previously suggested (Neerken and Amesz 2001). If the HbRCs are treated with dithionite at pH 10, the ~600-ps decay is replaced with a ~18-ns decay (**Figure 4**), which we attribute to charge recombination of $P_{800}^{+}A_0^{-}$; this was the lifetime of the event we measured *in vivo* when cells were in the high-fluorescence state (see Progress Report section I). The quinone content and reduction state were assessed before and after the experiment, and MQ was found to be present in the HbRC. This is an important result, as it demonstrates that when F_x is reduced, no electron transfer occurs beyond A_0, even though a quinone is present. Thus, the function of the quinone remains unknown.

V. Studies on the Conversion of BChl *g* to Chl *a*

The macrocycle of BChl *g* is a tautomer of Chl *a*, in which the double bond in ring II is moved to the ethyl substituent at carbon 8 (Brockmann and Lipinski 1983, Michalski, Hunt et al. 1987); see **Figure 5**). BChl *g* converts to Chl a_F (where F is farnesyl) in the presence of light and O_2 (Brockmann and Lipinski 1983, Gest and Favinger 1983). This changes the color of the pigment from brownish-green to emerald-green. We were interested in determining how the conversion of BChl *g* to Chl a_F affects activity.

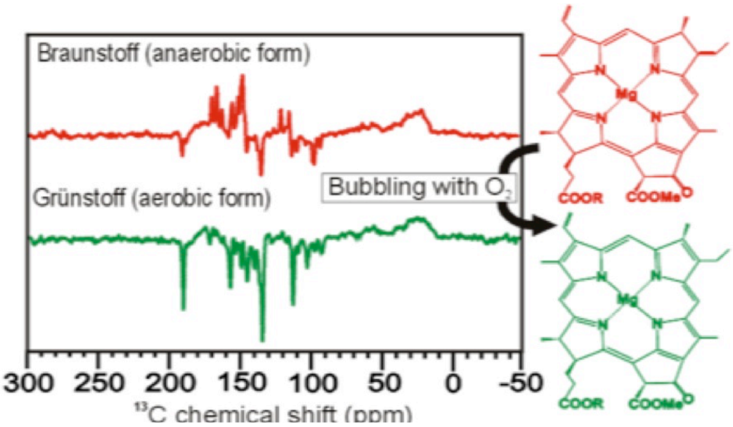

Figure 5. CIDNP spectra of whole cells of *Heliobacterium mobilis* before (red) and after (green) conversion of 90% of BChl *g* to Chl *a*F. The macrocycles of BChl *g* and BChl a_F are shown on the right.

In collaboration with Dr. Jörg Matysik (U. of Leiden), we carried out photochemically induced dynamic nuclear polarization (photo-CIDNP) studies of whole cells of *Heliobacterium mobilis*. The purpose of the study was to determine whether the conversion of BChl *g* to Chl a_F affected the ability of the HbRC to carry out primary charge separation. The photo-CIDNP effect is an intrinsic property of light-induced charge separation in photosynthesis. The non-Boltzmann nuclear spin polarization is detected as a strongly enhanced ^{13}C or ^{15}N magic angle spinning NMR signal. The non-Boltzmann spin distribution is achieved by the transfer of the electron spin polarization from the primary radical pair to the nucleus by hyperfine interaction. Our studies showed that the chemical shifts of the absorptive and emissive signals remain almost identical between the native samples and those in which ~90% of the BChl *g* had been converted to Chl a_F (**Figure 6**). The decay of the donor triplet was unchanged, suggesting that its localization does not change. These observations are best explained if the primary electron donor (P_{800}) remains unchanged, while the intermediate accessory pigment is converted from BChl *g* to Chl a_F. Thus, it appears that primary charge separation is retained after conversion of the majority of BChl g to Chl a_F. The following paper appeared from this work.

Thamarath, S. S., Alia, A., Daviso, E., Mance, D., Golbeck, J. H., and Matysik, J. (2012) Whole cell nuclear magnetic resonance characterization of two photochemically active states of the photosynthetic reaction center in heliobacteria. *Biochemistry 51*, 5763-5773.

VI. Creating a Transformation System for *Heliobacterium modesticaldum*

In this funding period, we had proposed to use genetic transformation to make targeted deletion mutants. We established methods to clone heliobacterial cells in medium solidified with GelRite, which is a prerequisite for a transformation system. While we could obtain colonies in medium containing kanamycin after electroporation with a plasmid containing a thermostable kanamycin-resistance gene that works in a variety of Gram-positive bacteria (Mai and Wiegel 2000), the putative transformants were unstable. Although the appearance of such colonies was dependent upon the presence of the plasmid, their resistance was low and most of them were lost in the next round of growth; the few survivors grew poorly and were lost in the subsequent round. While we do not fully understand this phenomenon, we have observed it several times. We also made constructs that should be integrated into the genome, but never obtained any transformants.

VII. Redox Titration of the [2Fe-2S] Proteins in the Chlorosome Envelope of *C. tepidum*

Chlorobaculum tepidum, a green sulfur bacterium, contains a homodimeric Type I RC. However, unlike heliobacteria, it also contains a large antenna structure called the chlorosome. In this study, the EPR spectral properties and the midpoint potentials of the membrane-localized CsmI, CsmJ, and CsmX proteins were measured in single, double, and triple mutants that produce chlorosomes containing two, one, or none of these Fe/S proteins. This study completes work on *C. tepidum*. The following paper appeared from this work.

Johnson, W., Li, H., Frigaard, N.U., Golbeck, J.H. and Bryant, D. '[2Fe-2S] proteins in chlorosomes: redox properties of CsmI, CsmJ, and CsmX of the chlorosome envelope of *Chlorobaculum tepidum' (in review)*.

VIII. Double Reduction of Quinones by Photosystem I (PS I)

We initiated a study of the effect of point mutations and quinone substitution in the quinone-binding site of PS I. We made the surprising discovery that PS I could doubly reduce plastoquinone-9 inserted at this site to plastoquinol. This study completes work on PS I that had remained unfinished from the preceding grant period. The following paper appeared from this work.

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.

IX. Review articles, Book Chapters, and Edited Books

Romberger, S.P., and Golbeck, J.H. (2010) The Bound Iron-Sulfur Clusters of Type-I Homodimeric Reaction Centers, *Photosynth. Res. 104*, 333-346.

In this invited book chapter, the PI (Golbeck) argues that at the advent of O_2 evolution, a mobile bacterial dicluster ferredoxin similar to PshBI and PshBII in heliobacteria initially served to transfer electrons directly from the F_X cluster to target proteins. At that point, the O_2-sensitive F_A and F_B clusters became vulnerable to denaturation at the onset of oxygenic photosynthesis. In response to this threat, a tight binding interface developed between what ultimately became PsaC and the Type I RC core, thereby making it possible for the F_A and F_B clusters to survive in the presence of O_2. The need for an alternative, mobile electron carrier led to the evolution of an O_2-insensitive [2Fe-2S] plant-type ferredoxin. The development of a PsaD-like protein to generate a binding site for ferredoxin broke the perfect C_2-symmetry of the homodimeric reaction center and, due to the need for asymmetric binding, provided the selective pressure that led to the differentiation of the RC core into separate PsaA and PsaB polypeptides. Much of the new information on the HbRC was published in the following comprehensive review article.

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.

One of the PIs (Golbeck) edited a Special Issue of *Photosynthesis Research* devoted to Type I RCs. The issue contained chapters on Taxonomy, Phylogeny, and Genomics; Proteomics and Biochemistry; Cytochromes; Light-Harvesting Systems; Reaction Centers; and Evolution.

Phototrophs that Contain Homodimeric Type I Reaction Centers (2010) J. H. Golbeck, ed., *Photosynth. Res. (Special Issue), 104*, 101-372, Springer, The Netherlands.

References

Albert, I., et al. (1998). "The 18 kDa cytochrome c553 from *Heliobacterium gestii*: gene sequence and characterization of the mature protein." *Biochemistry* **37**(25): 9001-9008.

Brockmann, H. and A. Lipinski (1983). "Bacteriochlorophyll g. A new bacteriochlorophyll pigment from Heliobacterium chlorum." *Archives of Microbiology* **136**(1): 17-19.

Brockmann, H. J. and A. Lipinski (1983). "Bacteriochlorophyll g. A new bacteriochlorophyll from *Heliobacterium chlorum*." *Arch Microbiol* **`36**: 17-19.

Collins, A. M., et al. (2010). "Modulation of fluorescence in *Heliobacterium modesticaldum* cells." *Photosynth Res* **104**(2-3): 283-292.

Feissner, R. E., et al. (2006). "ABC transporter-mediated release of a haem chaperone allows cytochrome *c* biogenesis." *Mol Microbiol* **61**(1): 219-231.

Gest, H. and J. L. Favinger (1983). "*Heliobacterium chlorum*, an anoxygenic brownish-green bacterium containing a `new' form of bacteriochlorophyll." *Arch Microbiol* **136**: 11-16.

Heinnickel, M., et al. (2006). "Identification of F(X) in the Heliobacterial reaction center as a [4Fe-4S] cluster with an S = (3)/(2) ground spin state." *Biochemistry* **45**(21): 6756-6764.

Heinnickel, M., et al. (2007). "Identification and characterization of PshB, the dicluster ferredoxin that harbors the terminal electron acceptors F_A and F_B in *Heliobacterium modesticaldum*." *Biochemistry* **46**(9): 2530-2536.

Mai, V. and J. Wiegel (2000). "Advances in development of a genetic system for *Thermoanaerobacterium spp.*: expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into the chromosome." *Appl Environ Microbiol* **66**(11): 4817-4821.

Michalski, T. J., et al. (1987). "Bacteriopheophytin *g*: Properties and some speculations on a possible primary role for bacteriochlorophylls *b* and *g* in the biosynthesis of chlorophylls." *Proc Natl Acad Sci, USA* **84**(9): 2570-2574.

Neerken, S. and J. Amesz (2001). "The antenna reaction center complex of heliobacteria: composition, energy conversion and electron transfer." *Biochim Biophys Acta* **1507**(1-3): 278-290.

Oh-oka, H., et al. (2002). "Electron donation from membrane-bound cytochrome c to the photosynthetic reaction center in whole cells and isolated membranes of *Heliobacterium gestii*." *Photosynthesis Research* **71**(1-2): 137-147.

Romberger, S. P. and J. H. Golbeck (2010). "The bound iron-sulfur clusters of Type-I homodimeric reaction centers." *Photosynth Res* **104**(2-3): 333-346.