Title:

Light-driven quinone reduction in heliobacterial membranes

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ABSTRACT

Photosynthetic reaction centers (RCs) evolved >3 billion years ago and have diverged into Type II RCs reducing quinones and Type I RCs reducing soluble acceptors via iron-sulfur clusters. Photosystem I (PSI), the exemplar type I RC, uses modified menaquinones as electron transfer cofactors, but it has been controversial if the type I RC of heliobacteria (HbRC) uses its two bound menaquinones in the same way. The sequence of the quinone-binding site in PSI is not conserved in the HbRC, and the recently solved crystal structure of the HbRC does not reveal a quinone in the analogous site. We found that illumination of heliobacterial membranes resulted in reduction of menaquinone to menaquinol, suggesting that the HbRC can perform a function thought restricted to type II RCs. Experiments on membranes and live cells are consistent with the hypothesis that the HbRC preferentially reduces soluble electron acceptors (*e.g.* ferredoxins) in low light, but switches to reducing lipophilic quinones in high light, when the soluble acceptor pool becomes full. Thus, the HbRC may represent a functional evolutionary intermediate between PSI and the Type II RCs.

KEYWORDS

Heliobacteria, reaction centers, quinone, type I reaction center, type II reaction center

INTRODUCTION

All known RCs have a common architecture in which 5 transmembrane helices from each of two similar (or identical) membrane-embedded polypeptides bind the cofactors required for light-driven electron transfer (ET) through the complex. The type II RCs are heterodimeric and the two cofactor branches are functionally asymmetric, with one specialized for charge separation and electron transfer to the quinone on the other branch (Q_B), which becomes fully reduced to a quinol after two light-driven ET events and can then diffuse into the membrane as the terminal product. The Type I RCs reduce soluble electron carriers, such as ferredoxin and flavodoxin.

In Photosystem I (PSI), the best understood Type I RC, there is a [4Fe-4S] cluster called F_X shared between the polypeptides, and the phylloquinone on either branch can be used as an ET intermediate to it from the chlorins (where charge separation takes place). The other known Type I RCs are homodimeric and are found in phototrophic bacteria that use them to drive cyclic photophosphorylation. Among these are the Heliobacteriaceae, the only phototrophic family in the Firmicutes. The heliobacterial RC (HbRC) is the simplest photosynthetic reaction center: it is composed of a homodimer of the PshA polypeptide in perfect C_2 symmetry, along with one copy of the single transmembrane-helix PshX polypeptide on each side (Gisriel et al. 2017). The primary electron donor, P_{800}, a dimer of bacteriochlorophyll (BChl) *g′* molecules, is at the C_2 symmetry axis on the external side of the membrane, while the F_X [4Fe-4S] cluster is on the other side (see **Fig. 1**). Between them are a pair of chlorins on each side of the symmetry axis: a BChl *g* and a 8^1-hydroxychlorophyll a_F (8^1-HO-Chl *a*); the latter is often called A_0, in analogy to the corresponding cofactor in PSI. The F_X cluster has recently been shown to be the terminal acceptor in the HbRC and is capable of reducing a variety of soluble acceptor proteins (Romberger et al. 2010; Romberger and Golbeck 2010, 2012).

Heliobacteria use menaquinone (MQ), which has the same headgroup as phylloquinone, as their sole membrane quinone (Hiraishi 1989; Sarrou et al. 2012). Although the HbRC contains up to 2

bound MQs, they seem to be bound loosely and dissociate from the HbRC rather easily (Trost and Blankenship 1989; Sarrou et al. 2012; Chauvet et al. 2013). The role of MQ in the HbRC has been controversial. Some electron paramagnetic resonance experiments have been interpreted as evidence for the involvement of a semiquinone species during forward ET within the HbRC (Muhiuddin et al. 1999; Miyamoto et al. 2008; Kondo et al. 2015). However, optical studies have found no signs of such a species (Brettel et al. 1998), and HbRCs lacking MQ or membranes depleted of MQ by ether extraction are not blocked in ET to the F_X cluster (Kleinherenbrink et al. 1993; Chauvet et al. 2013). The recently published 2.2-Å resolution structure of the HbRC reveals that the A_0 and F_X cofactors are closer together than they are in PSI by ~4 Å, potentially allowing ET between them to occur with the experimentally observed ~700-ps life time without requiring an intermediate cofactor (Gisriel et al. 2017). Although a quinone was not observed in the structure, consistent with its absence as measured by HPLC, there was an unassigned electron density to one side of A_0 that appeared to have an isoprenyl tail; the headgroup, however, appeared tetrahedral and was inconsistent with the shape of a naphthoquinone. In **Figure 1** is a model of where a MQ would be if its isoprenyl tail were in the same position as that of the unassigned electron density.

This indicates that ET within the HbRC is very different from the case in PSI and raises the question: what is the role of the quinone within the HbRC? The fact that MQ is bound loosely by the HbRC raises the possibility that it serves as an alternate electron acceptor, similar to the mobile quinones of type II RCs. We have tested this hypothesis by illuminating heliobacterial membranes and examining the redox state of the quinone pool directly.

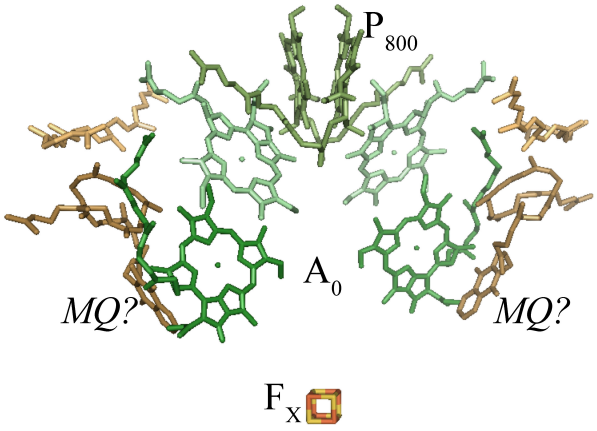

Figure 1. Schematic of ET cofactors in the HbRC. P_{800}, a pair of BChl *g*, is the primary electron donor and the F_X iron-sulfur cluster is the terminal electron acceptor. The A_0 cofactor (8^1-HO-Chl *a*) serves as an electron transfer intermediate during the process. Unlike PSI, there is no tightly-bound quinone in the HbRC structure. However, to one side of A_0 there is an unassigned electron density that appears to have an isoprenyl tail. A menaquinone molecule was fit to this density to visualize where it might bind.

EXPERIMENTAL PROCEDURES

Cells and materials: The Ice1 strain of *Heliobacterium modesticaldum* (Kimble et al. 1995), generously provided by Prof. Michael Madigan (Southern Illinois Univ.), was used for all studies. Liquid cultures of *H. modesticaldum* were grown anaerobically to late exponential phase in PYE medium (Kimble et al. 1995). Benzyl viologen (1,10-dibenzyl-4,40-bipyridinium dichloride), NADH, and azoxystrobin were purchased from Sigma-Aldrich. Terbutryn was a generous gift from Dr. James Allen (Arizona State University).

Isolation of membranes: Late-log cells were harvested by centrifugation at 5000 x *g* and resuspended in 50 mM MOPS (pH 7.0) under an atmosphere of 95% N_2/5% H_2. Lysozyme was

added to 100 µg/mL and after 30 min incubation at room temperature cells were lysed by sonication on ice in the dark on the bench (*i.e.* under air). The lysate was monitored by light microscopy every 5 cycles (1 minute at 12 W interspersed with 4 minutes on ice) until cells were fully lysed. Unbroken cells and cell wall material were removed by centrifugation at 12500 x *g* for 5 minutes. The supernatant was transferred to ultracentrifuge tubes in a glovebox and all subsequent steps were performed anoxically under very low green light. Membranes were pelleted by centrifugation at 200,000 x *g* for 30 minutes. Membranes were resuspended in the anaerobic chamber in 50 mM MES (pH 6.0) with 20% glycerol to a final concentration of 38 µM BChl *g* and flash-frozen in liquid N_2. The BChl *g* concentration in acetone extracts of membranes was determined via visible spectrophotometry using a gas-tight cuvette and $\varepsilon_{788} = 76$ mM^{-1} cm^{-1} at 788 nm (Sarrou et al. 2012). Membrane aliquots were stored under liquid nitrogen.

MQ reduction assay: All manipulations were performed in an anaerobic chamber in the dark. Unless otherwise noted, membrane samples were diluted to 15 µM BChl *g* in a degassed buffer containing 50 mM MES (pH 6.0), 20 mM ascorbate, and 20 mM $MgSO_4$. Each sample was exposed to light for 5 seconds and immediately extracted by addition of 20 volumes of degassed acetone. After vigorous mixing, they were promptly centrifuged at 14,200 x *g* for 1 minute, loaded into a gas-tight Hamilton syringe, removed from the glovebox and immediately loaded onto the HPLC (see below). Sample illumination was provided by a frequency-doubled continuous wave Nd-YAG laser (532 nm) with a maximal output of 860 mW; with a 7-mm aperture, this translates to 100 mmol photons m^{-2} s^{-1}. It was typically run in pulsed mode at 1 kHz with a 500-µs pulse length (*i.e.* 50% power); the pulse lengths were shortened to lower power further (*e.g.* 10% power, or 10 mmol m^{-2} s^{-1}, corresponded to using 100-µs pulses at 1 kHz). After addition of inhibitors (BV, azoxystrobin, terbutryn), membranes were allowed to incubate at room temperature in the dark for 20 minutes before illumination to allow equilibration with the added reagent. To test the effect of pH, membranes were washed and resuspended in various buffers at 50 mM concentration: acetate (pH 5), MES (pH 6), MOPS (pH 7), Tricine (pH 8). To explore the effect of changing temperature, tubes with membranes in the standard buffer were incubated in a water bath for 10 minutes immediately before laser exposure.

Pigment analysis by HPLC: Samples were injected into a Quaternary Gradient LC-2000plus-LPG HPLC (Jasco) using a C-18 Column (Phenomenex Ultrasphere 250 mm L x 4.6 mm ID packed with 5-µm particles) for separation. Pigments were eluted isocratically using 17:1 methanol/hexane at a flow rate of 1.5 mL min^{-1}. Integration of the peaks in the chromatograms allowed calculation of the number of pigments per HbRC. The abundance of each pigment was calculated from the area of the peak (750 nm for BChl g, 246 nm for MQH_2, 260 nm for MQ), using the extinction coefficients of these pigments at those wavelengths (76 cm^{-1} mM^{-1}, 15 cm^{-1} mM^{-1} and 45 cm^{-1} mM^{-1}, respectively (Kroger and Dadak 1969)). The integrated MQ and MQH_2 peak areas were used to calculate the ratio of MQH_2 to MQ in the extract. Peaks associated with MQ and MQH_2 were assigned based on their UV-visible absorbance spectrum, as per (Sarrou et al. 2012).

In vivo BChl* g *fluorescence: Log-phase cultures were used without prior concentration. Terbutryn was added from a 100 mM stock in DMSO to final concentrations of 10 – 100 µM. The "no addition" control contained an amount of DMSO equivalent to the highest concentration of terbutryn tested (*i.e.* 0.1% DMSO final concentration). Fluorescence traces were collected as described (Redding et al., 2014).

RESULTS

Light-dependent MQ reduction in heliobacterial membranes

Heliobacteria use MQ exclusively as their quinone (Hiraishi 1989). In *Heliobacterium modesticaldum*, the major quinone is MQ-9 with a smaller amount of MQ-8 (Sarrou et al. 2012). Although the MQ pool is ~50-60% reduced in membranes prepared anoxically, one can control its reduction state by simple treatments. If the membranes are briefly exposed to air in the dark during purification (see Experimental Procedures for details), the quinone pool is found to be almost completely oxidized (~3% MQH_2; see **Table 1**). This is likely due to the action of the cytochrome (cyt) *bd*-type quinol oxidase complex, predicted to be encoded by the genome (Sattley and Blankenship 2010; Sattley et al. 2008), which can scavenge O_2 by reducing it to water using MQH_2 as reductant. Such air-treated membranes were used for all subsequent experiments.

The genome also encodes a NAD(P)H:MQ oxidoreductase that should be able reduce MQ using NAD(P)H as reductant (Sattley and Blankenship 2010; Sattley et al. 2008). In accordance with that prediction, incubation of air-treated membranes with NADH in the dark resulted in reduction of roughly half the MQ in the pool to MQH_2 in 10 minutes (**Table 1**). Without NADH, very slow MQ reduction occurred in the dark, such that ~8% MQH_2 was attained after 24 hour. This is likely due to reduction by H_2 present in the anoxic glovebox (~5% of the atmosphere), perhaps via the membrane-bound [NiFe] hydrogenase (Sattley and Blankenship 2010; Sattley et al. 2008). All experiments described below employed membranes within 2 hours of thawing.

To test the hypothesis that the HbRC can reduce MQ to MQH_2, we illuminated membranes in buffer containing ascorbate as electron donor and then extracted them with acetone immediately after. We found that even a few seconds of light was sufficient to observe significant reduction of the quinone pool (**Fig. 2**). The time resolution of our experiments was limited, but we found that within about 1 s of illumination, the MQH_2 level was relatively constant (not shown); we were unable to test shorter illumination times. Increasing laser power resulted in higher MQH_2 levels (**Fig. 3A**), consistent with a role for the HbRC.

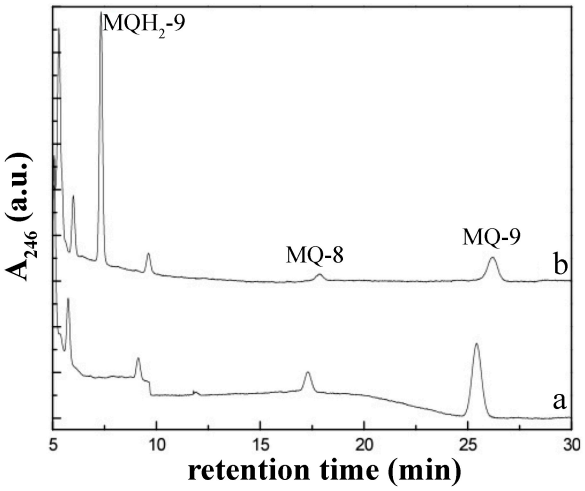

Figure 2. HPLC chromatogram of acetone-extracted heliobacterial membranes measured at 246 nm. Membranes left in the dark (a) or exposed to light (50 mmol m^{-2} s^{-1} at 532 nm) for 5 s (b) were immediately extracted and ran under the same conditions. MQH_2-9 elutes at ~7 min. MQ-8 at ~17 min. MQ-9 elutes at ~26-min. (MQH_2-8 is not visible by this procedure.)

For the experiments reported here, we quantified MQ-9 and MQH_2-9 and report the reduction

level as the percent of the MQ-9 pool that is present as menaquinol. Although MQ-8 was visible in the chromatogram (**Fig. 2**), MQH_2-8 co-eluted with the bacteriochlorophyll *g* peak, precluding its quantitation. MQ-9 is by far the major component of the MQ pool in this species (Sarrou et al. 2012), as it was previously seen in *Heliobacterium chlorum* (Hiraishi 1989). Since there is no reason to assume that MQ-8 will behave differently than MQ-9, the ratio (MQH_2-9)/(MQ-9 + MQH_2-9) should be a very good proxy for the overall menaquinone pool reduction level.

Preliminary characterization of MQ photoreduction in heliobacterial membranes

Since reduction of MQ to MQH_2 is a two-electron process, it would require a first charge separation to generate the $P_{800}^{+}F_X^{-}$ state, followed by re-reduction of P_{800}^{+} to allow a second charge separation. Charge recombination of the $P_{800}^{+}F_X^{-}$ state occurs with a decay time of ~15 ms (Heinnickel et al. 2006), but the membrane-attached cyt c_{553} can reduce P_{800}^{+} in less than ~1 ms (Oh-oka et al. 2002; Redding et al. 2014). It is therefore expected that photo-accumulation of the reduced F_X cluster (F_X^{-}) could occur efficiently in illuminated membranes containing reduced cyt c_{553} and lacking soluble electron acceptors from the F_X cluster. As the cyt *c* was largely oxidized after exposure to air, ascorbate was used as a sacrificial electron donor to cyt c_{553} (Kashey et al. 2014); importantly, ascorbate cannot reduce MQ directly (see **Table 1**). If ascorbate was omitted from the reaction, MQ reduction in the light was blocked (**Fig. 3A**), demonstrating that an electron donor is important either as a donor to the RC or to prime the system for subsequent electron transfer reactions resulting in quinone reduction.

The membranes should be largely devoid of soluble electron carriers that could direct electrons away from the HbRC after a single turnover. Consistent with this, washing of the membranes with 0.1 M Na_2CO_3 (pH 11) buffer, a fairly harsh procedure that removes extrinsic proteins (Cowgill 2012), only slightly lowered MQ photoreduction activity of the membranes (**Table 1**). Not only are soluble electron acceptors unnecessary for MQ reduction, one would expect that an acceptor from F_X would compete with MQ for electrons. Addition of benzyl viologen (BV), a known acceptor from F_X (Collins et al. 2010; Kashey et al. 2014), inhibited reduction of MQ to MQH_2 in a concentration-dependent manner (**Fig.3B**). This is attributed to a decrease in the steady-state level of F_X^{-}, consistent with the hypothesis that excitation of a center in the $P_{800}F_X^{-}$ state would be required for production of MQH_2.

Modulating the pH of the samples before light exposure should bias MQ reduction and MQH_2 oxidation in a predictable manner, as protons are consumed by the former and produced by the latter. As predicted, lower MQH_2 levels were obtained as the pH was raised from 5 to 8 (**Fig. 3C**). Varying temperature could affect the speed at which MQH_2 and MQ can dissociate from and associate with their binding pockets, affecting the rate of reduction by the HbRC and the rate of its reoxidation by other membrane complexes. Raising the temperature resulted in a higher level of MQH_2 at steady state (**Fig. 3D**). Thus, while one would expect that both MQ reduction and oxidation could be accelerated by an increase in temperature, the former process seems to be more strongly accelerated by the increase of temperature in this range.

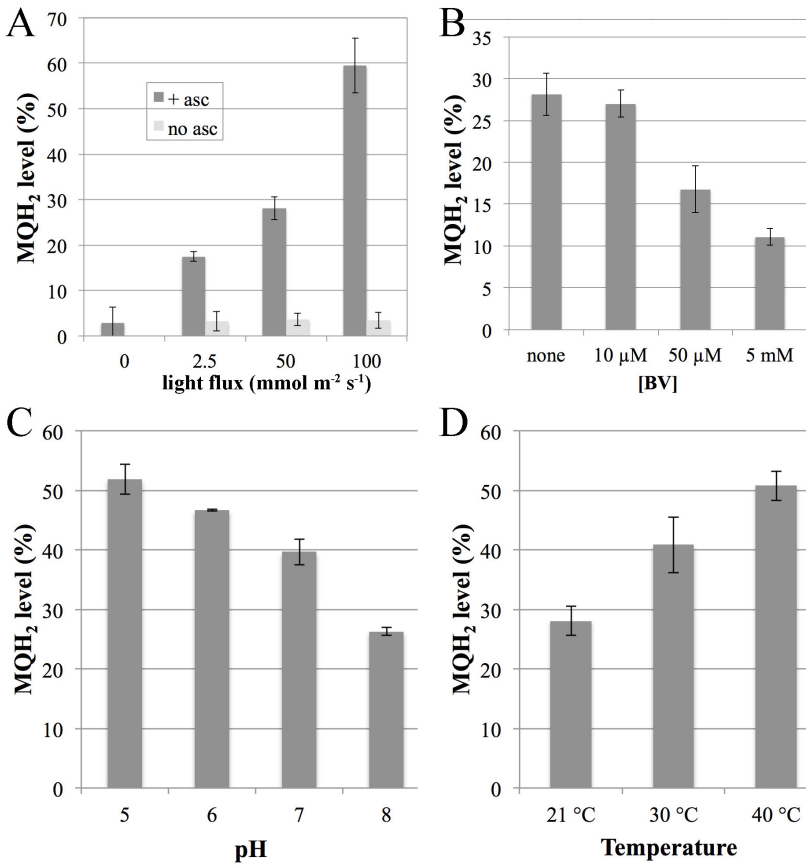

Figure 3. Characterization of MQ photoreduction in heliobacterial membranes. Unless otherwise indicated, MQ reduction levels were quantified by HPLC after 5-s exposure to 532-nm light at 50 mmol m^{-2} s^{-1} in MES buffer (pH 6) containing 20 mM ascorbate. **A:** Laser power was modulated as indicated and the buffer either contained (dark grey) or omitted (light gray) ascorbate as electron donor. **B:** Membranes were pre-incubated with various amounts of benzyl viologen. **C:** Membranes were washed and resuspended in different buffers before illumination. **D:** Membranes were pre-incubated at different temperatures before illumination.

Competition between MQ reduction and MQH_2 oxidation in heliobacterial membranes

The fact that the quinone reduction levels obtained in our experiments were both reproducible and time-independent after ~1 s suggested that the quasi-steady-state level of MQ pool reduction was the result of a competition between reduction of MQ and oxidation of MQH_2. The cyt b_6c complex is expected to be the major contributor to MQH_2 oxidation in the light, as the HbRC would constantly oxidize cyt c_{553}, the electron acceptor of the cyt b_6c complex. We tested this hypothesis by addition of an inhibitor of the cyt b_6c complex. After testing several Q_o-site inhibitors, we found that azoxystrobin is a relatively potent inhibitor of heliobacterial cyt b_6c activity ($K_{1/2} \approx 5$ µM; see Supplementary Information for details). The addition of 100 µM azoxystrobin to the light-driven MQ reduction assay resulted in a doubling of the MQH_2 reduction level (55% vs 26% MQH_2; **Table 1, Fig. 4**), consistent with the hypothesis that the cyt b_6c complex is reoxidizing MQH_2 in the heliobacterial membranes during the assay.

By the same logic, an inhibitor of MQ reduction by the HbRC would be expected to have the opposite effect. Competitive inhibitors that act at the Q_B site have been useful in the analysis of

Type II RCs. There are no known inhibitors that bind at the phylloquinone-binding site of PSI, which is unsurprising given that the PhQ is tightly bound in the pocket and serves as an intermediate in ET to F_X, not as an exchangeable acceptor. Exchange of the quinone from this site with exogenous quinones has been seen to occur only if it has become doubly reduced, removed by solvent extraction, or replaced with a foreign quinone (Sieckman et al. 1991; Rustandi et al. 1990; Ostafin and Weber 1997; Johnson et al. 2000; Johnson et al. 2001; Lefebvre-Legendre et al. 2007; McConnell et al. 2011). If MQ is fully reduced to quinol by the HbRC and then exchanges with the MQ pool in the membrane, then the quinone-binding site of the HbRC would be somewhat analogous to the Q_B site in Type II RCs. Terbutryn is a triazine type inhibitor of the Q_B site in both the purple bacterial RC and PSII (Michel et al. 1986; Arntz and Trebst 1986) and blocks light-driven quinone reduction *in vivo* and *in vitro*. We tested its effects on MQ photoreduction in heliobacterial membranes. High laser power was used to maximize the MQH_2 steady-state level. Addition of terbutryn inhibited MQ reduction in a dose-dependent manner (**Fig. 4, Table 1**). We observed no effect of terbutryn upon P_{800} photo-oxidation yields or upon the rate of decay of P_{800}^+ by charge recombination of $P_{800}^+F_X^-$ with a single laser flash (data not shown), consistent with the lack of effect of quinone removal upon charge separation in heliobacterial membranes (Kleinherenbrink et al. 1993) and isolated HbRCs (Chauvet et al. 2013).

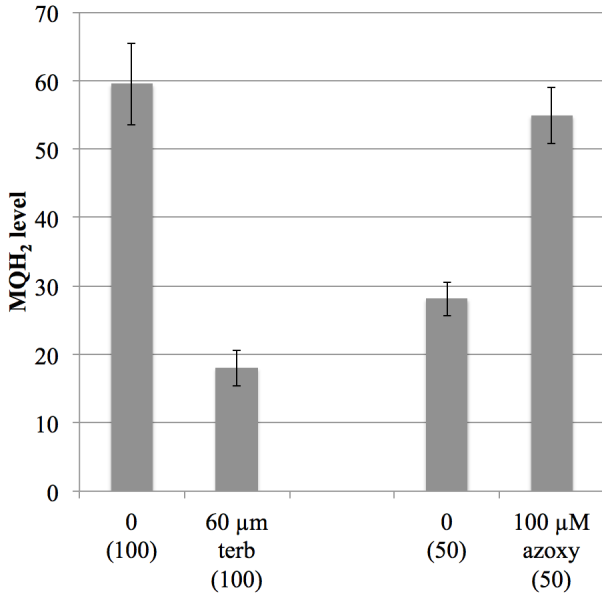

Figure 4. Effect of inhibitors upon MQ reduction in heliobacterial membranes. Terbutryn (at 60 µM) or azoxystrobin (at 100 µM) or were added to membranes and allowed to incubate in the dark for 20 minutes before illumination and solvent extraction. In order to see more easily the inhibitory effect of terbutryn, membranes were illuminated at 100 mmol m^{-2} s^{-1}, while the membranes used to test the effect of azoxystrobin were illuminated at the standard 50 mmol m^{-2} s^{-1}. Control samples (0) contained an equal amount of solvent as the inhibitor sample.

Evidence of MQ reduction *in vivo*

To test the idea that MQ reduction has physiological significance, we examined the effect of terbutryn upon the phenomenon of fluorescence induction in living heliobacteria. We have previously identified the $P_{800}F_X^-$ state as the one emitting fluorescence (Redding et al. 2014). Upon strong illumination, the electron acceptor pool for the HbRC becomes exhausted in a matter of seconds, leading to a rise of both reduced F_X (F_X^-) and fluorescence emission from BChl *g*. Addition of a soluble electron acceptor from F_X^- (BV) inhibited fluorescence induction (Collins et al. 2010), consistent with this idea. Therefore, if MQ serves as an alternate electron acceptor from the HbRC, then MQ reduction would quench fluorescence emission, and one would predict that blocking MQ reduction would result in higher fluorescence. Addition of

terbutryn at 10-30 µM to live *H. modesticaldum* cells led to both a faster rise of fluorescence and a higher steady-state fluorescence level upon strong illumination (**Fig. 5A**), consistent with this hypothesis.

We found that higher terbutryn concentrations (>100 µM) led to a smaller rise in fluorescence (**Fig. S4A**), and that at these higher concentrations terbutryn can inhibit the cyt b_6c complex (**Fig. S4B**). We had previously found that inhibition of cyt b_6c with stigmatellin lowered *in vivo* BChl *g* fluorescence, and this was explained as a lowering of charge separation due to accumulation closed RCs (i.e. P_{800}^{+}). This phenomenon also explains the effect of terbutryn at high concentrations. It is likely that this secondary effect also explains why inhibition of light-dependent quinone reduction becomes less effective at higher terbutryn concentrations (**Table 1**).

Given that the electron acceptor pool is in a more reduced state in high-light conditions, one would expect that MQ reduction by the HbRC would be more important in high light than in low light. Consistent with this hypothesis, the effect of terbutryn (100 µM) on the growth of heliobacteria was very apparent when grown under high light, but almost negligible under low light (**Fig. 5B**). These results give support to the idea that direct MQ reduction by the HbRC is an important physiological process under conditions when the soluble electron acceptor pool (*e.g.* ferredoxins) becomes limiting, which seems to be the case in high light (Redding et al. 2014).

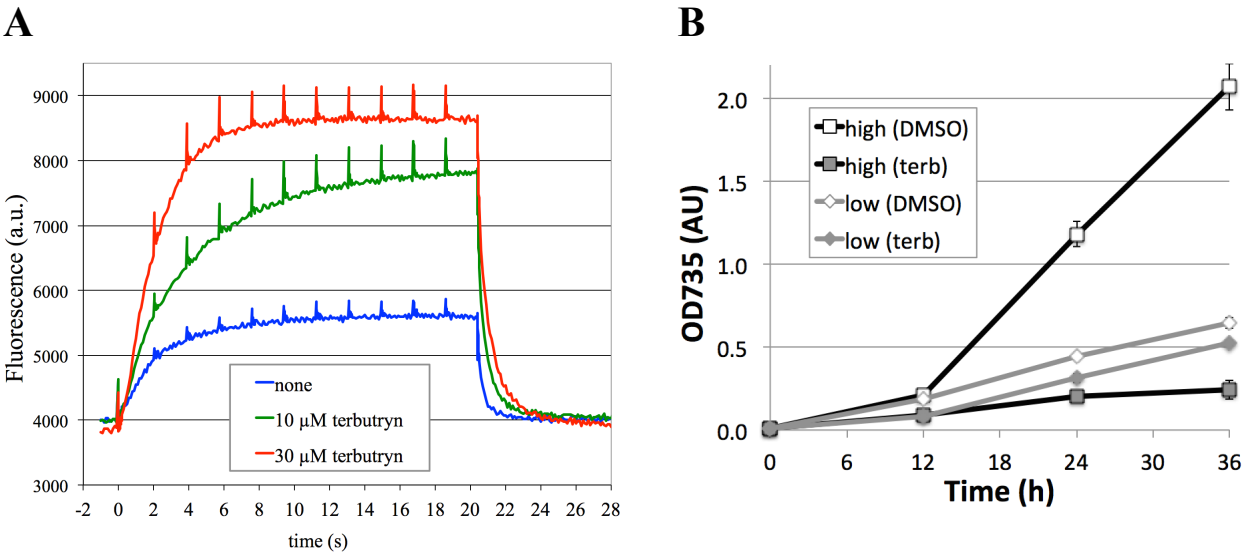

Figure 5. Effect of terbutryn *in vivo*. Panel **A:** In vivo BChl *g* fluorescence from heliobacterial cultures containing no addition (blue) or terbutryn at 10 µM (green) or 30 µM (red). At time 0, cells were illuminated with orange LEDs (1.7 mmol photons m^{-2} s^{-1}); every ~2 s, the actinic light was increased to 4.3 mmol photons m^{-2} s^{-1} for 40 ms. **Panel B:** Growth of heliobacteria under 780-nm light at low (gray diamonds; 0.5 W m^{-2}) or high (black squares; 5 W m^{-2}) flux in media containing either 100 µM terbutryn (filled symbols) or solvent (empty).

DISCUSSION

The results presented here demonstrate that reduction of menaquinones in the heliobacterial membrane pool occur rapidly upon the onset of illumination. It is important to stress that these results do not prove that the HbRC is the site of MQ reduction. It is possible that reducing equivalents are transferred elsewhere and used to reduce MQ. However, carbonate treatment of

the membranes at pH 11, which opens vesicles and removes extrinsic proteins, had a small effect on MQ photoreduction activity (**Table 1**). Thus, if reducing equivalents are transferred from the HbRC to a MQ reduction site, the process would have to make use of a membrane-bound protein that could accept electrons from the F_X cluster. No such protein is known. Furthermore, the presence of such an acceptor in membranes would result in a decrease of charge recombination of $P_{800}^+F_X^-$ after a laser flash, which we have not observed. Thus, if such a protein exists, it would have to be present at low stoichiometry relative to the HbRC. For the remaining discussion, we will explore the hypothesis that the HbRC is the site of MQ reduction. Rigorous testing of this hypothesis will be the focus of future experiments.

We have previously estimated that there are 5-6 MQ per RC in the membranes of *H. modesticaldum* (Sarrou et al. 2012). Since a RC would bind only 2 MQ, and a MQ pool reduction level of up to 80% can be obtained (**Table 1**), this implies that there must be exchange of MQ and MQH_2 at the site of reduction. This site is currently unknown. If the MQ binding site is indeed at the location of the unassigned electron density in the structure, as suggested in **Figure 1**, then it would be in a position to be reduced by A_0 but not pass the electron to F_X in the sub-nanosecond timescale. When the soluble acceptors are limiting (*e.g.* in the cell when the ferredoxin pool is reduced or in isolated membranes), the reduced F_X cluster accumulates, as demonstrated in living cells (Redding et al. 2014); charge separation in this state would yield $P_{800}^+A_0^-F_X^-$. One possibility is that MQ is reduced by A_0^-, and the resulting semiquinone oxidizes F_X^-, yielding a quinol. Of course, the second electron transfer step, at least, would have to be accompanied by protonation. How protons reach the MQ reduction site is unknown at this point.

The striking structural similarity between the core of PSI, PSII, and the purple bacterial RC has led to the conclusion that the Type I and Type II RCs share a common evolutionary origin (Rutherford and Nitschke 1996; Schubert et al. 1998). That is, the RC has evolved only once and the original RC was almost surely homodimeric. All known Type II RCs (PSII, and the RCs of purple proteobacteria and Chloroflexi) are heterodimeric, as their function dictates a specialization of the two cofactor branches: one for charge separation, and one for stabilization of the semiquinone anion (*i.e.* preventing charge recombination before the second electron arrives to fully reduce it to a quinol). Although PSI is also a heterodimer, it has been shown that it is functionally symmetric at the core, in that both cofactor branches are used for ET to the F_X cluster (Guergova-Kuras et al. 2001; Rappaport et al. 2006; Redding and van der Est 2006). Although the phylloquinone cofactor of PSI has a headgroup (2-methylnaphthoquinone) identical to that of MQ, differing only in the isoprenoid tail substituent, it is not normally reduced to a quinol. However, PSI can reduce the embedded quinone when the latter is replaced with a quinone of higher potential (plastoquinone; (McConnell et al. 2011)), albeit very inefficiently. It is thought that PSI has evolved to use the embedded 2-methylnaphthoquinone exclusively as an ET intermediate to the F_X cluster and its structure and function prohibits double reduction of the quinone to quinol (Srinivasan and Golbeck 2009; Srinivasan et al. 2009; Srinivasan et al. 2011).

All of the other Type I RCs are homodimeric and exist in anoxygenic phototrophic bacteria (Heliobacteria, Chlorobi, Chloroacidobacteria) that presumably employ cyclic photophosphorylation using ferredoxin as RC electron acceptors and either ferredoxins or NAD(P)H as electron donors to a complex I-like enzyme that pumps protons across the membrane as the electrons are transferred to the MQ pool. Our results suggest that the HbRC represents a functional intermediate between PSI and the Type II RCs, preferentially reducing soluble (one-electron) acceptors when they are available, and reducing their embedded quinones

when the soluble acceptors become limiting. Thus, there might be two possible cyclic electron transport pathways in heliobacteria – a larger cycle using complex I under conditions when the soluble acceptor pool is oxidized and a smaller cycle resembling the cycle in purple bacteria when the soluble acceptor pool becomes reduced (**Fig. 6**). The smaller cycle may be the ancestral one. The larger cycle would represent a new pathway allowed by the ability of Type I RCs to reduce ferredoxins via their FeS cluster(s), thus opening up a new cycle involving NADH dehydrogenase and additional proton pumping with a concomitant increase in ATP production. Thus, one would expect heliobacteria to preferentially use the larger cycle. However, the ability to reduce MQ as an alternate electron acceptor would allow them to continue to make ATP (albeit at a lower ATP/photon ratio) in conditions when the ferredoxin acceptor pool is temporarily full.

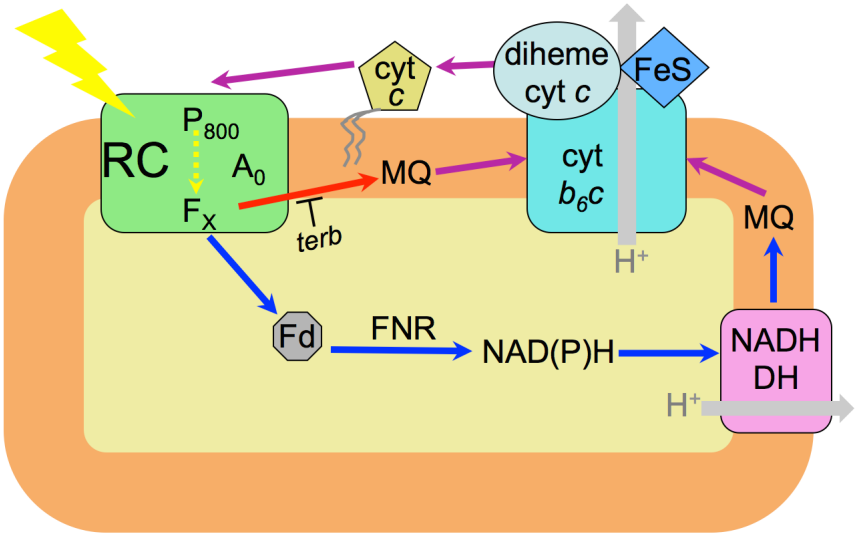

Figure 6. Proposed cyclic electron transfer pathways in heliobacteria. The yellow lightning bolt represents excitation by light, and the dotted yellow arrow is light-driven electron transfer within the RC. The blue arrows depict the likely major pathway from the F_X cluster of the HbRC to the MQ pool via cellular ferredoxins (Fd), ferredoxin:$NADP^+$ oxidoreductase (FNR), the cellular NAD(P)H pool, and NAD(P)H dehydrogenase (NADH DH). The red arrow indicates the MQ photoreduction process proposed here, in which the HbRC reduces internal MQ in the absence of soluble electron acceptors. Purple arrows indicate reactions in common to the two cycles: cyt b_6c oxidizes MQH_2 and reduces the membrane-attached cyt c_{553}, which serves as electron donor to P_{800} of the HbRC. Proton pumping (thick gray arrows) will occur at cyt b_6c in both cycles, but at NADH DH only in the larger cycle.

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AUTHOR CONTRIBUTIONS

Trevor Kashey did the majority of the work described on quinone reduction, a project whose feasibility was first demonstrated by preliminary experiments performed by John Cowgill. Dustin Luu created the assay for cyt b_6c function and used it to analyze the effects of inhibitors. This work was performed in the laboratory of Kevin E. Redding under his supervision.

COMPETING FINANCIAL INTERESTS STATEMENT

None of the authors have any financial interests related to this work.

FIGURE LEGENDS

(Figures are embedded along with their legends in the Main Text.)

TABLES

Table 1. Menaquinol levels under different conditions

Condition[1]	Light flux (mmol m^{-2} s^{-1})	MQ reduction level (%)[2]
Dark Assay[3]		
Oxically prepared membranes	0	2.8 ± 2.5
+ 0.5 mM NADH (10 min)	0	44 ± 2.5
Light Assays[1]		
20 mM ascorbate	2.5 50 100	17.5 ± 1.1 28.1 ± 2.5 59.5 ± 6
No ascorbate	2.5 50 100	3.2 ± 2.1 3.6 ± 1.3 3.4 ± 1.7
Carbonate-washed membranes	50	22.3 ± 1.2
No additions (standard conditions)	50	27.8 ± 1.9
Addition of DMSO (control)	50	26.4 ± 2.8
+ 10 μM BV	50	27 ± 1.6
+ 50 μM BV	50	16.8 ± 2.8
+ 5 mM BV	50	11.1 ± 1.0
+ 100 μM azoxystrobin	50	54.9 ± 4.1
+ 6 μM terbutryn	100	42 ± 4.8
+ 60 μM terbutryn	100	18 ± 2.6
+ 600 μM terbutryn	100	12 ± 6.2
30 °C	50	40.9 ± 4.7
40 °C	50	50.8 ± 2.5
40 °C, 300 μM azoxystrobin, pH 5	100	80.5 ± 4.9

[1]Unless otherwise noted, all samples contained air-treated membranes corresponding to 15 μM BChl *g* suspended in a buffer containing 50 mM MES (pH 6.0), 20 mM $MgSO_4$, and 20 mM sodium ascorbate. They were illuminated for 5 s at 50 mmol photons m^{-2} s^{-1} before immediate extraction with acetone, and then analyzed by HPLC (see Materials and Methods for details). Temperature of the sample was 21 °C before illumination, unless otherwise indicated.

[2]MQ-9 and MQH_2-9 were quantified separately from the chromatogram and the reduction levels was calculated as (MQH_2-9)/(MQH_2-9 + MQ-9). Values reported are averages (± standard deviation) of at least 3 independent assays.

[3]Samples were not illuminated. Membranes treated with NADH were incubated in the dark for 10 min.

ELECTRONIC SUPPLEMENTARY MATERIAL

This contains information on the assay for cyt b_6c activity and its use to assess the effectiveness of inhibitors. It includes four figures (S1, S2, S3, S4).

REFERENCES

Arntz B, Trebst A (1986) On the role of the Q_B protein of PS II in photoinhibition. FEBS Lett 194:43-51

Brettel K, Leibl W, Liebl U (1998) Electron transfer in the heliobacterial reaction center: evidence against a quinone-type electron acceptor functioning analogous to A1 in photosystem I. Biochim Biophys Acta 1363 (3):175-181

Chauvet A, Sarrou J, Lin S, Romberger SP, Golbeck JH, Savikhin S, Redding KE (2013) Temporal and spectral characterization of the photosynthetic reaction center from *Heliobacterium modesticaldum*. Photosynth Res 116 (1):1-9. doi:10.1007/s11120-013-9871-9

Collins AM, Redding KE, Blankenship RE (2010) Modulation of fluorescence in *Heliobacterium modesticaldum* cells. Photosynth Res 104 (2-3):283-292. doi:10.1007/s11120-010-9554-8

Cowgill J (2012) Characterization of the electron acceptors of the Type-I photosynthetic reaction center of Heliobacterium modesticaldum. M.S., Arizona State University, Ann Arbor

Gisriel C, Sarrou I, Ferlez B, Golbeck JH, Redding KE, Fromme R (2017) Structure of a symmetric photosynthetic reaction center-photosystem. Science 357 (6355):1021-1025. doi:10.1126/science.aan5611

Guergova-Kuras M, Boudreaux B, Joliot A, Joliot P, Redding K (2001) Evidence for two active branches for electron transfer in photosystem I. Proc Natl Acad Sci U S A 98 (8):4437-4442.

Heinnickel M, Agalarov R, Svensen N, Krebs C, Golbeck JH (2006) Identification of F_X in the Heliobacterial Reaction Center as a [4Fe-4S] Cluster with an S = 3/2 Ground Spin State. Biochem 45 (21):6756-6764

Hiraishi A (1989) Occurrence of menaquinone is the sole isoprenoid quinone in the photosynthetic bacterium *Heliobacterium chlorum*. Arch Microbiol 151:378-379

Johnson TW, Shen GZ, Zybailov B, Kolling D, Reategui R, Beauparlant S, Vassiliev IR, Bryant DA, Jones AD, Golbeck JH, Chitnis PR (2000) Recruitment of a foreign quinone into the A(1) site of photosystem I - I. Genetic and physiological characterization of phylloquinone biosynthetic pathway mutants in Synechocystis sp PCC 6803. J Biol Chem 275 (12):8523-8530

Johnson TW, Zybailov B, Jones AD, Bittl R, Zech S, Stehlik D, Golbeck JH, Chitnis PR (2001) Recruitment of a foreign quinone into the A(1) site of photosystem I - Invivo replacement of plastoquinone-9 by media- supplemented naphthoquinones in phylloquinone biosynthetic pathway mutants of synechocystis sp PCC 6803. J Biol Chem 276 (43):39512-39521

Kashey TS, Cowgill JB, McConnell MD, Flores M, Redding KE (2014) Expression and characterization of cytochrome c_{553} from *Heliobacterium modesticaldum*. Photosynth Res 120 (3):291-299. doi:10.1007/s11120-014-9982-y

Kimble LK, Mandelco L, Woese CR, Madigan MT (1995) Heliobacterium modesticaldum, sp. nov., a thermophilic heliobacterium of hot springs and volcanic soils. Arch Microbiol 163 (4):259-267

Kleinherenbrink FA, Ikegami I, Hiraishi A, Otte SCM, Amesz J (1993) Electron transfer in menaquinone-depleted membranes of *Heliobacterium chlorum*. Biochim Biophys Acta 1142:69-73

Kondo T, Itoh S, Matsuoka M, Azai C, Oh-Oka H (2015) Menaquinone as the Secondary Electron Acceptor in the Type I Homodimeric Photosynthetic Reaction Center of Heliobacterium modesticaldum. J Phys Chem B 119 (27):8480-8489. doi:10.1021/acs.jpcb.5b03723

Kroger A, Dadak V (1969) On the Role of Quinones in Bacterial Electron Transport. European Journal of Biochemisty 11:328-340

Lefebvre-Legendre L, Rappaport F, Finazzi G, Ceol M, Grivet C, Hopfgartner G, Rochaix JD (2007) Loss of phylloquinone in Chlamydomonas affects plastoquinone pool size and photosystem II synthesis. J Biol Chem 282 (18):13250-13263. doi:M610249200 [pii]
10.1074/jbc.M610249200

McConnell MD, Cowgill JB, Baker PL, Rappaport F, Redding KE (2011) Double reduction of plastoquinone to plastoquinol in photosystem 1. Biochem 50 (51):11034-11046. doi:10.1021/bi201131r

Michel H, Epp O, Deisenhofer J (1986) Pigment-protein interactions in the photosynthetic reaction centre from Rhodopseudomonas viridis. Embo J 5 (10):2445-2451

Miyamoto R, Mino H, Kondo T, Itoh S, Oh-Oka H (2008) An electron spin-polarized signal of the $P_{800}^{+}A_1(Q)^{-}$ state in the homodimeric reaction center core complex of *Heliobacterium modesticaldum*. Biochem 47 (15):4386-4393

Muhiuddin IP, Rigby SE, Evans MC, Amesz J, Heathcote P (1999) ENDOR and special TRIPLE resonance spectroscopy of photoaccumulated semiquinone electron acceptors in the reaction centers of green sulfur bacteria and heliobacteria. Biochem 38 (22):7159-7167

Oh-oka H, Iwaki M, Itoh S (2002) Electron donation from membrane-bound cytochrome *c* to the photosynthetic reaction center in whole cells and isolated membranes of *Heliobacterium gestii*. Photosyn Res 71 (1-2):137-147

Ostafin AE, Weber S (1997) Quinone exchange at the A_1 site in Photosystem I in spinach and cyanobacteria. Biochim Biophys Acta 1320:195-207

Rappaport F, Diner BA, Redding K (2006) Optical measurements of secondary electron transfer in Photosystem I. In: Golbeck J (ed) Photosystem I: The Plastocyanin:Ferredoxin Oxidoreductase in Photosynthesis. Kluwer Academic Publishers, Dordrecht, pp 223-244

Redding K, van der Est A (2006) The Directionality of Electron Transfer in Photosystem I. In: Golbeck J (ed) Photosystem I: The Plastocyanin:Ferredoxin Oxidoreductase in Photosynthesis. Kluwer Academic Publishers, Dordrecht, pp 413-437

Redding KE, Sarrou I, Rappaport F, Santabarbara S, Lin S, Reifschneider KT (2014) Modulation of the fluorescence yield in heliobacterial cells by induction of charge recombination in the photosynthetic reaction center. Photosynth Res 120 (1-2):221-235. doi:10.1007/s11120-013-9957-4

Romberger SP, Castro C, Sun Y, Golbeck JH (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 (2-3):293-303. doi:10.1007/s11120-010-9558-4

Romberger SP, Golbeck JH (2010) The bound iron-sulfur clusters of type-I homodimeric reaction centers. Photosynth Res 104 (2-3):333-346. doi:10.1007/s11120-010-9543-y

Romberger SP, Golbeck JH (2012) The F_X iron-sulfur cluster serves as the terminal bound electron acceptor in heliobacterial reaction centers. Photosynth Res 111:285-290. doi:10.1007/s11120-012-9723-z

Rustandi RR, Snyder SW, Feezel LL, Michalski TJ, Norris JR, Thurnauer MC, Biggins J (1990) Contribution of vitamin K1 to the electron spin polarization in spinach photosystem I. Biochem 29 (35):8030-8032

Rutherford AW, Nitschke W (1996) Photosystem II and the quinone-iron-containing reaction centers: comparisons and evolutionary perspectives. In: Baltscheffsky H (ed) Origin and evolution of biological energy conversion. VCH Publishers, New York, pp 143-175

Sarrou I, Khan Z, Cowgill J, Lin S, Brune D, Romberger S, Golbeck JH, Redding KE (2012) Purification of the photosynthetic reaction center from *Heliobacterium modesticaldum*. Photosynth Res 111 (3):291-302. doi:10.1007/s11120-012-9726-9

Sattley WM, Blankenship RE (2010) Insights into heliobacterial photosynthesis and physiology from the genome of Heliobacterium modesticaldum. Photosynth Res 104 (2-3):113-122. doi:10.1007/s11120-010-9529-9

Sattley WM, Madigan MT, Swingley WD, Cheung PC, Clocksin KM, Conrad AL, Dejesa LC, Honchak BM, Jung DO, Karbach LE, Kurdoglu A, Lahiri S, Mastrian SD, Page LE, Taylor HL, Wang ZT, Raymond J, Chen M, Blankenship RE, Touchman JW (2008) The genome of Heliobacterium modesticaldum, a phototrophic representative of the Firmicutes containing the simplest photosynthetic apparatus. J Bacteriol 190 (13):4687-4696

Schubert W-D, Klukas O, Saenger W, Witt HT, Fromme P, Krauss N (1998) A common ancestor for oxygenic and anoxygenic photosynthetic systems: a comparison based on the structural model of photosystem I. J Mol Biol 280 (2):297-314

Sieckman I, van der Est A, Bottin H, Setif P, Stehlik D (1991) Nanosecond electron transfer kinetics in photosystem I following substitution of quinones for vitamin K1 as studied by time resolved EPR. FEBS Lett 284 (1):98-102

Srinivasan N, Golbeck JH (2009) Protein-cofactor interactions in bioenergetic complexes: the role of the A1A and A1B phylloquinones in Photosystem I. Biochim Biophys Acta 1787 (9):1057-1088

Srinivasan N, Karyagina I, Bittl R, van der Est A, Golbeck JH (2009) Role of the hydrogen bond from Leu722 to the A1A phylloquinone in photosystem I. Biochem 48 (15):3315-3324

Srinivasan N, Santabarbara S, Rappaport F, Carbonera D, Redding K, van der Est A, Golbeck JH (2011) Alteration of the H-bond to the A(1A) phylloquinone in Photosystem I: influence on the kinetics and energetics of electron transfer. J Phys Chem B 115 (8):1751-1759. doi:10.1021/jp109531b

Trost JT, Blankenship RE (1989) Isolation of a photoactive photosynthetic reaction center-core antenna complex from *Heliobacillus mobilis*. Biochem 28 (26):9898-9904