Resolution and Reconstitution of a Bound Fe−S Protein from the Photosynthetic Reaction Center of *Heliobacterium modesticaldum*†

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ABSTRACT: The photosynthetic reaction center of *Heliobacterium modesticaldum* (HbRC) was isolated from membranes using *n*-dodecyl β-D-maltopyranoside followed by sucrose density ultracentrifugation. The low-temperature EPR spectra of whole cells, isolated membranes, and HbRC complexes are similar, showing a single Fe−S cluster with *g* values of 2.067, 1.933, and 1.890 after illumination at 20 K, and a complex spectrum attributed to exchange interaction from two Fe−S clusters after illumination during freezing. The protein containing the Fe−S clusters was removed from the HbRC by washing it with 1.0 M NaCl and purified by ultrafiltration over a 30 kDa cutoff membrane. Analysis of the filtrate by SDS−PAGE showed a major band at ∼8 kDa that was weakly stained with Coomassie Brilliant Blue and strongly stained with silver. The optical spectrum of the oxidized Fe−S protein shows a maximum at 410 nm, and the EPR spectrum of the reduced Fe−S protein shows a complex set of resonances similar to those found in 2[4Fe-4S] ferredoxins. The HbRC core was purified by DEAE ion-exchange chromatography and resolved by SDS−PAGE. The purified HbRC was composed of a band at ca. 40 kDa, which is identified as PshA, and several additional proteins. The isolated Fe−S protein rebinds spontaneously to purified HbRC cores, and the light-induced EPR signals of the Fe−S clusters are recovered. The flash-induced kinetics of the HbRC complex show two kinetic phases at room temperature, one with a lifetime of 75 ms and the other with a lifetime of 15 ms. The 75 ms component is lost when the Fe−S protein is removed from the HbRC complex, and it is regained when the Fe−S protein is rebound to HbRC cores. Thus, the 75 ms kinetic phase is derived from recombination of a terminal Fe−S cluster with $P798^+$, and the 15 ms kinetic phase is derived from recombination with an earlier acceptor, probably F_X. We suggest that the bound Fe−S protein present in the HbRC be designated PshB.

The major focus of research in photosynthesis is to understand the mechanisms that plants and bacteria use to convert sunlight into chemical bond energy. All known photosynthetic reaction centers (RCs)[1] can be divided into two main groups: type I, in which an Fe−S cluster is the terminal electron acceptor, and type II, in which a quinone is the terminal electron acceptor. Each of these two types of RCs can be subdivided into two additional subgroups: photosystem II (PS II) and the purple bacterial RC are members of the type II group, and photosystem I (PS I) and the homodimeric RCs of Heliobacteriaceae and Chlorobiaecae are members of the type I group. The latter are the only homodimeric RCs known in nature (*1*, *2*). Atomic-resolution structures are available for the purple bacterial RC (*3*) and for PS I (*4*), and models for PS II based on X-ray data taken at a resolution of 3.5−3.8 Å are becoming available (*5*, *6*). However, no detailed structural information is yet available for the type I homodimeric RCs.

Heliobacteria are strictly anaerobic, spore-forming members of the Firmicutes. They are photoheterotrophic organisms that grow on a very limited range of carbon sources (*7*) and contain a unique photosynthetic pigment called bacteriochlorophyll *g*, which contains a vinyl group on ring I and an ethylidine group on ring II. These features give the microorganism its characteristic absorbance spectrum that confers a selective advantage in dealing with its specific growth environment (*7*, *8*). Even though heliobacteria stain Gram-negative, an analysis of the G-C content of its 16S ribosomal RNA indicates that these microorganisms are Gram-positive. This makes them the first identified Gram-positive microorganisms to perform photosynthesis (*9*). Unlike cyanobacteria, which have two types of photosynthetic RCs, heliobacteria have only one type, with Fe−S clusters as the terminal electron acceptors. This places them in the type I class of RCs (*10*).

In the 22 years that has followed their discovery, research on heliobacteria has proceeded at a steady pace. The heliobacterial RC (HbRC) was shown to contain 35−40 antenna bacteriochlorophyll *g* molecules (*11*, *12*) and a primary donor consisting of a bacteriochlorophyll *g*′ dimer

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[1] Abbreviations: RCs, reaction centers; PS I, photosystem I; PS II, photosystem II; Fe−S, iron−sulfur; EPR, electron paramagnetic resonance; HbRC, heliobacterial reaction center; PshA, photosynthetic reaction center protein from heliobacteria; PshB, Fe−S protein in the HbRC.

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(*13*). The primary acceptor (A_0) is an 8^1-hydroxychlorophyll *a* molecule (*14*). Although it was suggested that the PshA homodimer contains all of the HbRC antenna chlorophylls (*15*), it is still uncertain whether additional chlorophyll proteins are associated with the RC complex. The participation of a quinone is controversial, with some studies arguing for (*16*) and some against (*17*−*19*) its participation as the intermediate electron transfer cofactor. An Fe−S cluster similar to F_X has been detected in urea-treated HbRCs using time-resolved optical spectroscopy (*20*), and Fe−S clusters similar to F_A and F_B have been detected in membranes by EPR spectroscopy (*21*, *22*). However, the absence of a gene that encodes a low-molecular mass Fe−S protein in the major gene cluster that encodes the photosynthetic proteins has led to difficulties in identifying a protein that plays a role similar to that of PsaC in harboring the terminal Fe−S clusters in HbRCs (*23*).

The primary aim of this work is to begin characterization of the proteins that contain bound Fe−S clusters in the HbRC. One of the problems in studying this organism has been the difficulty of isolating intact HbRCs. Heliobacteria are strict anaerobes, and the Fe−S clusters are particularly unstable in the presence of oxygen. We approached this issue using a strategy similar to our studies on type I RCs from *Chlorobium tepidum* (*24*). We first determined the light-induced EPR spectra of the terminal Fe−S clusters in freshly frozen whole cells of *Heliobacterium modesticaldum*, a mild thermophile that grows optimally at 50−52 °C (*25*). We then used these spectra as a benchmark to optimize the isolation conditions for membranes and HbRCs, with the retention of the light-induced EPR spectrum of the Fe−S clusters as the primary criterion of intactness. This guaranteed that the properties of the bound Fe−S clusters were from an undamaged protein.

We report here that in addition to the PshA homodimer, the isolated HbRC complex contains at least several additional proteins. One is an 8 kDa, bound Fe−S protein, which can be removed from the HbRC complex at moderate ionic strength. The resolved Fe−S protein can be rebound to the purified HbRC cores, thus regaining light-induced charge separation between P798 and the Fe−S clusters. The spectroscopic properties of the HbRC complex, the HbRC core, and the isolated Fe−S protein are described in this paper.

MATERIALS AND METHODS

Growth of Heliobacterial Cultures. A culture of *H. modesticaldum* was generously provided by M. Madigan (Southern Illinois University, Carbondale, IL). Liquid cultures of *H. modesticaldum* were grown anaerobically in PYE medium as described in ref *25*, except that resazurin, an oxygen reporter dye, was added to a final concentration of 0.001%. Banks of eight fluorescent bulbs provided white light illumination. The medium was degassed and allowed to incubate in an anaerobic chamber with an atmosphere of 10% hydrogen and 90% nitrogen (Coy Labs, Grass Lake, MI) until it was devoid of oxygen, as indicated by the color of the resazurin. The medium was sealed in Hungate bottles and autoclaved. The bottles were inoculated using the Hungate technique, and the culture was allowed to grow at 43 °C. Growth at a temperature lower than the optimum resulted in fewer broken cells (*26*).

Isolation of HbRC Complexes. All manipulations were performed anaerobically. Plasticware and glassware were placed in the anaerobic chamber 3 h prior to use and tested with a resazurin solution to verify that any residual oxygen had been removed. Cells grown to late-exponential phase were harvested anaerobically at 10000*g* and resuspended in 50 mM MOPS buffer (pH 7). Whole cells were lysed by sonication, and membranes were pelleted by centrifugation at 200000*g*. A variety of detergents were used to solubilize the membranes, including Triton X-100, digitonin, Deriphat 160c, *n*-dodecyl β-D-maltopyranoside, and *n*-octyl β-D-glucopyranoside. On the basis of the efficiency of solubilization, the stabilization of the chlorophyll-containing RC complexes, and retention of the light-induced EPR resonances of the Fe−S clusters, *n*-dodecyl β-D-maltopyranoside was chosen as the optimum detergent. Membranes were solubilized with 1% *n*-dodecyl β-D-maltopyranoside for 1 h; intact membranes were removed by centrifugation at 200000*g*. The supernatant was loaded onto a 5 to 20% sucrose gradient and ultracentrifuged at 28 000 rpm in an SW-28 rotor for 16 h. A brownish-green fraction was collected from the gradient.

Purification of the Bound Fe−S Protein. HbRCs isolated by sucrose density ultracentrifugation were incubated with 1 M NaCl for 1 h and ultrafiltered over a 30 kDa molecular mass cutoff membrane (PM30, Millipore). The proteins that passed through the membrane were collected, and the process was repeated a second time. The filtrate, which contained low-molecular mass proteins, was concentrated over a 3 kDa molecular mass cutoff membrane (YM-3, Millipore) and desalted on a PD-10 column (Amersham Biosciences). The HbRC core that was retained on the ultrafiltration membrane was also desalted on a PD-10 column and concentrated over a 30 kDa molecular mass cutoff membrane (PM-30, Millipore). Fe−S clusters were reconstituted anaerobically according to the following protocol. The resolved Fe−S protein was incubated for 20 min with 100 mM β-mercaptoethanol in 50 mM Tris buffer (pH 8.3). Ferrous ammonium sulfate was added dropwise to a final concentration of 180 μM, and the solution was allowed to incubate for 20 min. After the mixture turned a slightly brown color, sodium sulfide was added dropwise to a final concentration of 180 μM and the solution was allowed to incubate overnight at 4 °C. The reconstituted Fe−S protein was desalted by ultrafiltration over a YM-3 membrane to remove all iron and sulfide and concentrated in the same step.

Low-Temperature X-Band EPR Spectroscopy. Low-temperature EPR spectroscopy was performed using a Bruker ECS-106 X-band spectrometer equipped with an Oxford liquid helium cryostat and temperature controller. The spectrometer conditions were as follows: temperature, 20 K; power, 126 mW; microwave frequency, 9.47 GHz; receiver gain, 2×10^4; modulation amplitude, 10 G at 100 kHz. Spectra were obtained by averaging 10 scans. HbRCs were concentrated to an absorbance of ~75 OD at 788 nm and loaded into the EPR sample tubes. To ensure that oxygen had not damaged the sample, the absorbance at 788 nm, which is a measure of bacteriochlorophyll *g*, needed to be at least 5 times greater than the absorbance at 670 nm, which is a measure of chlorophyll *a*; otherwise, the sample was discarded. Samples were chemically reduced with 10 mM sodium hydrosulfite in 100 mM glycine at pH 10.0. In all

cases, the oxidized spectrum (no additions) was subtracted from the reduced spectrum to yield a difference spectrum. A 250 W tungsten lamp focused to fill the grid of the resonator did not provide sufficient light to elicit the full amplitude of the Fe−S resonances. An argon ion laser was used instead, which was operated at 2.5 W in all-lines mode, and a 3-fold beam expander was used to fill the grid of the resonator. Light-induced electron transfer was assayed according to one of two protocols. One electron can be promoted from P798 to the Fe−S clusters by illuminating the sample frozen in darkness and illuminated at 20 K. Two or more electrons can be promoted from P798 to the Fe−S clusters by freezing the sample slowly during illumination. The latter was accomplished by fitting the EPR tube with a vacuum adaptor and illuminating the tube with an argon ion laser outside of the EPR resonator under conditions that allowed the sample to be frozen slowly to 77 K. The sample was then quickly transferred to the EPR resonator, which was maintained at 20 K.

Time-Resolved Optical Spectroscopy in the Visible and Near IR. The kinetics of $P798^+$ reduction were measured by monitoring the flash-induced absorbance after a laser flash. In brief, the sample was probed with a continuous measuring beam at 798 nm isolated from a 400 W tungsten−halogen lamp with a $^1/_4$ m monochromator. A shutter allowed the light to reach the sample 10 ms prior to the laser flash. The beam was monitored using a reverse-biased Si photodiode that was shielded from stray light with an 800 nm interference filter. The signal from the photodiode was amplified by a Tektronix AM502 differential amplifier and digitized using a DSA601 Tektronix digital oscilloscope. The data were sent to a Macintosh computer via an IEEE-488 bus (National Instruments). The electronic bandwidth of the detection system was fixed at 1 MHz. The sample was excited by a Nd:YAG laser operating in the second harmonic ($\lambda = 532$ nm) with a 7 ns pulse duration and an energy of $\sim$2 mJ/cm^2. Typically 8−10 transients were recorded and averaged. Samples were placed in a quartz anaerobic cuvette with an optical path length of 10 mm. The samples were in 50 mM MOPS buffer (pH 7.0) at an absorbance of 0.5 OD at 788 nm. The kinetic traces were analyzed by fitting a multiexponential decay using the Marquardt least-squares algorithm (Igor Pro, Lake Oswego, OR).

RESULTS

Isolation of an Intact HbRC Retaining the Light-Induced EPR Signals of Fe−S Clusters. Low-temperature X-band EPR spectra of freshly frozen cells of *H. modesticaldum* are depicted in Figure 1. The one-electron reduced EPR spectrum (Figure 1A, trace a), generated by illuminating dark-adapted cells at 20 K, is rhombic, with *g* values of 2.067, 1.933, and 1.890. These resonances are typical of a reduced Fe−S protein and appear to be derived either from a single Fe−S cluster or from two (or more) Fe−S clusters that have identical *g* values. The magnitude of the signal does not diminish after the light is turned off, indicating that charge separation with $P798^+$ is irreversible at low temperatures. The amplitude of the signal is maximal at 20 K (−2 dB microwave power) and disappears completely above 35−40 K. The relative number of spins represents 3% of that found after chemical reduction (Figure 1C, trace a), indicating that either electron transfer to the Fe−S clusters is inefficient

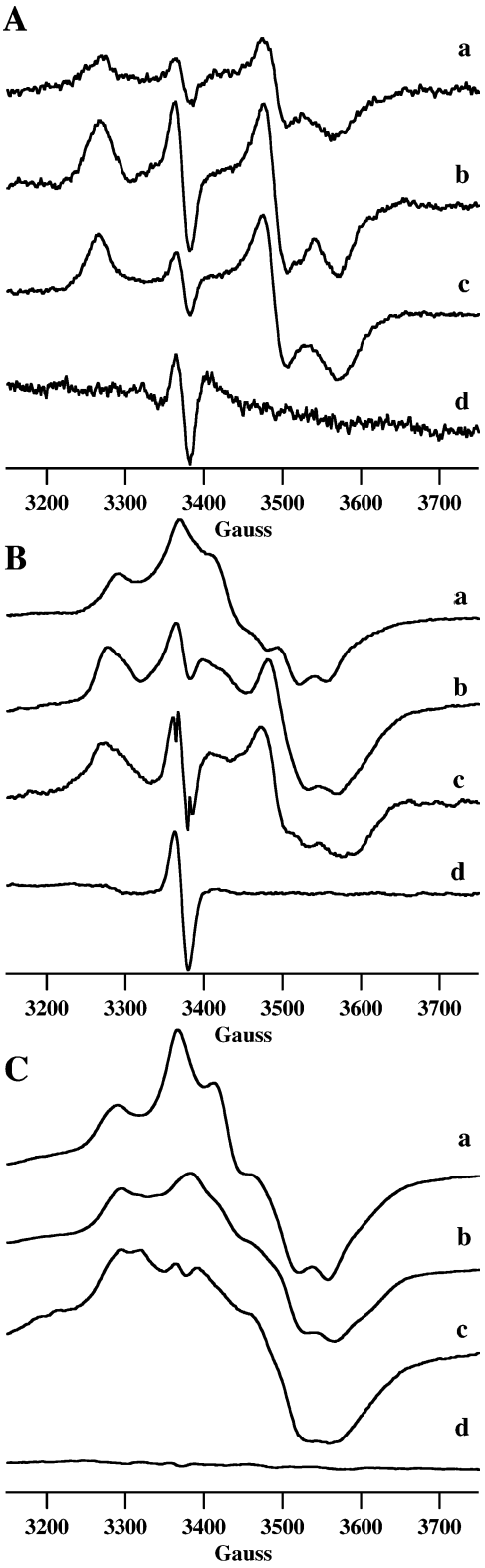

FIGURE 1: EPR spectra of whole cells, membranes, and RCs of *H. modesticaldum*. Spectrometer conditions: temperature, 20 K; power, 126 mW; microwave frequency, 9.47 GHz; receiver gain, 2×10^4. (A) Difference spectrum between a sample illuminated at 20 K and a dark-adapted sample. (B) Difference spectrum between a sample frozen in the presence of light and a dark-adapted sample. (C) Difference spectrum between a chemically reduced and an oxidized (no treatment) sample. The sample was reduced with 33 mM sodium hydrosulfite in 100 mM glycine buffer (pH 10.0): (a) whole cells, (b) isolated membranes, (c) HbRCs isolated by sucrose gradient ultracentrifugation, and (d) HbRCs purified from DEAE ion-exchange chromatography.

at 20 K or self-shielding and scatter in the whole cells prevent light from reaching the majority of the chlorophylls. The rapid spin relaxation properties, inferred from the temperature and microwave power dependencies, are more characteristic of a [4Fe-4S] cluster than a [2Fe-2S] cluster. The two-electron-reduced EPR spectrum, generated either by illuminating cells during freezing to 77 K (Figure 1B, trace a) or by reducing the cells with sodium hydrosulfite at pH 10.0 (Figure 1C, trace a), is complex, showing apparent *g* values of 2.05, 1.97, 1.93, and 1.90. Note that the light-reduced and chemically reduced spectra are virtually identical, indicating that additional Fe−S proteins in the cellular milieu do not contribute to the spectrum of the chemically reduced sample. Note also that the *g* anisotropy of the spectrum, as indicated by the low-field and high-field turning points, is narrower than that of the one-electron-reduced spectrum. The amplitude of the signal is maximal at 20 K (−2 dB microwave power) and disappears completely above 35−40 K; at all but the lowest temperatures, the resonances remain unchanged. This complex EPR spectrum is attributed to an exchange interaction that occurs between the two [4Fe-4S] clusters that are typically located 12.5 Å apart in dicluster ferredoxins. Because the Fe−S clusters can be reduced by illumination at 20 K, a temperature at which diffusion and protein motion are limited, these resonances are attributed to an Fe−S protein bound to the HbRC.

The one-electron-reduced EPR spectrum of isolated membranes (Figure 1A, trace b) is similar to that of whole cells (Figure 1A, trace a), and is nearly identical to that reported for isolated membranes from *Heliobacterium chlorum* (*21*). The relative number of spins, calculated by comparing the areas under the integrated spectra, represents 12% of that found after chemical reduction (Figure 2C, trace b), a value similar to that reported in isolated membranes from *H. chlorum* (*21*). The light-induced two-electron-reduced EPR spectrum of isolated membranes (Figure 1B, trace b) is slightly different from the chemically reduced spectrum (Figure 1C, trace b), particularly the *g* values of the high-field and low-field resonances. Considering the different *g* anisotropies of the one-electron- and two-electron-reduced spectra, the light-induced sample of the membranes appears to represent an admixture. This could come about if there were a mixed population of one-electron- and two-electron-reduced spectra due to incomplete reduction of the Fe−S clusters during the photoaccumulation protocol.

The isolation of intact HbRCs depends on the use of detergents to dissolve the membrane and render the hydrophobic membrane proteins soluble. We chose two criteria for screening the efficacy of detergents: one is the ability to isolate the HbRC on a sucrose density gradient, and the other is the retention of the intact light-induced EPR resonances of the Fe−S clusters. We found that among the variety of detergents tested (see Materials and Methods) *n*-dodecyl *β*-D-maltopyranoside was the most effective at fulfilling these criteria. HbRCs recovered from the sucrose gradients prepared under anaerobic conditions were brown in color due to the retention of Bchl *g*. However, HbRCs recovered from the sucrose gradients prepared under aerobic conditions were green in color due to the (partial) conversion (oxidation) of Bchl *g* to Bchl *a*. The light-induced Fe−S clusters were present in the brown samples that were prepared anaerobically, whereas they were missing in the green sample

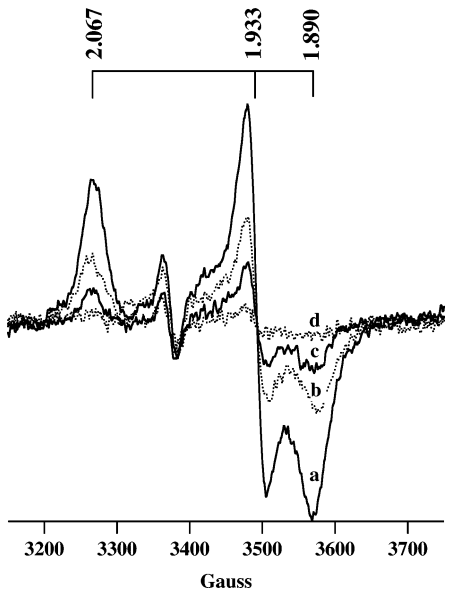

FIGURE 2: EPR spectra of salt-washed membranes from *H. modesticaldum*. The membranes were solubilized using *n*-dodecyl *β*-D-maltopyranoside and were either untreated (a) or treated with (b) 100, (c) 250, or (d) 500 mM NaCl for 5 min. The samples were then flash-frozen to 77 K and placed in the resonator at 20 K. Light-induced EPR signals were generated by illuminating dark-adapted samples at 20 K with a 2.5 W argon ion laser for 1 min. The spectra depicted are the difference between the illuminated and dark-adapted samples. Spectrometer conditions: temperature, 20 K; power, 126 mW; microwave frequency, 9.47 GHz; receiver gain, 2×10^4. The optical absorbance at 788 nm was 75 OD.

prepared aerobically. This visual indicator of color has proven to be an excellent marker for exposure of the HbRC to oxygen. The EPR spectra in the brown sample, measured under one-electron reducing conditions (Figure 1A, trace c) and two-electron reducing conditions (Figure 1B, trace c; and Figure 1C, trace c), were similar to those measured in membranes. Thus, the Fe−S clusters are judged to be intact after detergent treatment and purification by sucrose density ultracentrifugation (*21*). Interestingly, the relative number of spins represents as much as 41% of that found after chemical reduction (Figure 2C, trace c). Because both of the [4Fe-4S] clusters in the Fe−S protein are reduced by chemical reduction, whereas only one can be reduced by illumination at 20 K, nearly 82% of the terminal Fe−S cluster can be reduced in isolated HbRCs by illumination at low temperatures.

Loss of the Light-Induced EPR Signal of Fe−S Clusters at High Ionic Strengths. We found that the light-induced EPR resonances of the Fe−S clusters were present when the HbRCs were subject to chromatography by gel filtration but that they were missing when the HbRCs were passed over a DEAE ion-exchange column. Because both types of chromatography were carried out under anaerobic conditions (the HbRCs remained brown in color), we reasoned that either the Fe−S protein was sensitive to ionic strengths or the Fe−S clusters were degraded. To study this effect further, membranes that were solubilized with *n*-dodecyl *β*-D-maltopyranoside were incubated with various concentrations of NaCl for 5 min. The samples were flash-frozen to 77 K, and and the light-induced EPR spectra of the Fe−S clusters were measured at 20 K. We found that relatively low concentrations of NaCl lead to a marked reduction in the amplitude

of the light-induced Fe−S resonances. At 100 mM NaCl (Figure 2, trace b), 250 mM NaCl (Figure 2, trace c), and 500 mM NaCl (Figure 2, trace d), the amplitude of the light-induced spectrum was reduced to 46, 26, and <5% of the control (Figure 2, trace a), respectively. The relative spin concentrations were determined by comparing the peak to trough intensities of the $g = 1.925$ midfield resonance. HbRCs that were isolated by sucrose density ultracentrifugation showed an identical loss of the light-induced Fe−S resonances as the ionic strength was increased. In contrast, the light-induced Fe−S resonances of isolated membranes were resistant to loss at high ionic strengths. Thus, *n*-dodecyl β-D-maltopyranoside must be present for the effect to be observed. The intensity of the radical at $g = 2.002$ derived from $P798^+$ is generally lower when the Fe−S clusters are not observed. However, these signals cannot be used to quantify the amount of oxidized primary donor because the organic radical is saturated at the temperature and power required to measure the Fe−S clusters.

Properties of the HbRC Core Isolated by Ion-Exchange Chromatography. The HbRCs isolated by sucrose density ultracentrifugation were passed over a DEAE-Sepharose CL-6B ion-exchange column that had been pre-equilibrated with 50 mM MOPS buffer. The HbRCs were not retained on the column, but rather flowed through in the void volume. The EPR spectra of the DEAE-purified HbRCs showed that the EPR resonances of the bound Fe−S clusters were completely missing regardless of whether the sample was illuminated (Figure 1A, trace d; and Figure 1B, trace d) or reduced with sodium hydrosulfite (Figure 1C, trace d). The presence of the $g = 2.002$ radical derived from $P798^+$ shows that the HbRC core is intact and that light-induced charge separation occurs between P798 and an earlier electron acceptor.

To determine whether the Fe−S protein is attached to the HbRC but devoid of Fe−S clusters, we attempted reconstitution of the Fe−S clusters by treating the DEAE-purified HbRCs with 2-mercaptoethanol, ferrous ammonium sulfate, and sodium sulfide (see Materials and Methods). No EPR resonances of the Fe−S clusters were detected regardless of whether the sample was illuminated or chemically reduced with sodium hydrosulfite (data not shown). Like soluble 2[4Fe-4S] ferredoxins and to PsaC of PS I, the bound Fe−S protein in the HbRC is likely to be negatively charged at pH 7.0. If the Fe−S protein is in equilibrium between the HbRC-bound and unbound states, a fraction should be retained at any given time by the anion-exchange resin. As the HbRC passes through the column, it will be become successively depleted of the Fe−S protein until a purified HbRC core is attained. (We did not attempt to recover the isolated Fe−S protein from the ion-exchange column.) The DEAE-purified particles will henceforth be called HbRC cores.

The HbRC cores were treated with 1% SDS and subjected to SDS−PAGE analysis, and the gel was stained with Coomassie Brilliant Blue R-250 (Figure 3, left). The major band with an apparent mass of ca. 40 kDa is the PshA reaction center protein (*27*). Its predicted mass, derived from the sequence of the *pshA* gene from *Heliobacillus mobilis* is 68 kDa (*28*); thus, this hydrophobic membrane protein runs anomalously on SDS−PAGE (*28*, 29). This is a common feature of integral membrane proteins, including PsaA and PsaB of PS I, which tend to unfold incompletely

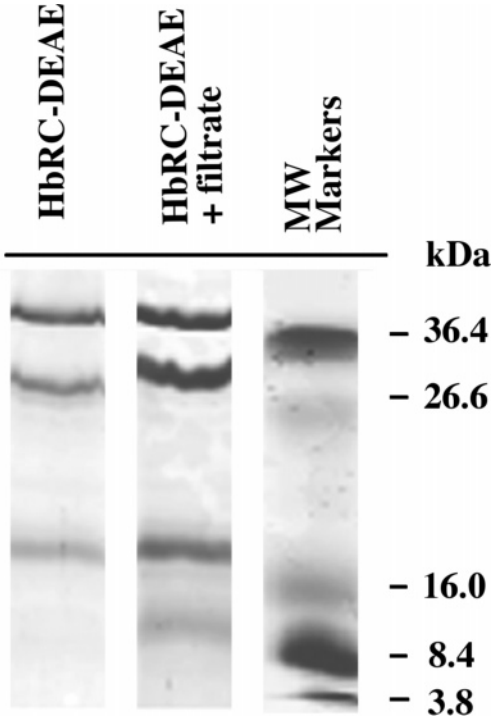

FIGURE 3: SDS−polyacrylamide gel electrophoresis of the isolated HbRC cores (left lane) and reconstituted HbRC complexes of *H. modesticaldum*. The HbRC cores were isolated by sucrose gradient ultracentrifugation and purified by DEAE ion-exchange chromatography. The reconstituted HbRC complexes were prepared by incubating PshB with HbRC cores in a 5:1 ratio and washing three times over a 30 kDa cutoff membrane. The sample lanes were loaded with the equivalent of 2 μg of Chl. The proteins were resolved in a 12% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R-250. Molecular mass markers are shown in the right lane.

and to bind larger amounts of SDS than soluble proteins. The HbRC core also contains at least two additional protein bands with apparent masses of ~30 and ~18 kDa. (Details of the polypeptide composition of the HbRC complex and the HbRC core are beyond the scope of this study and will be published elsewhere.) When the HbRC cores are subject to chromatography over a DEAE-Sepharose CL-6B column under aerobic conditions, the color turns from brown to green, but the protein composition remains identical to that shown in Figure 3 (left lane). Hence, none of the low-molecular mass polypeptides are released from the HbRC core due to exposure to oxygen.

Isolation and Characterization of the Bound Fe−S Protein by SDS−PAGE. To determine the mass of the resolved Fe−S protein, detergent-solubilized membranes were treated with 1 M NaCl for 10 min and the solution was ultrafiltered over a 30 kDa cutoff membrane. The HbRC cores, which were retained above the membrane, were desalted over a PD-10 column and stored for later use. The low-molecular mass proteins, which were present in the filtrate, were collected, concentrated over a 3 kDa ultrafiltration membrane, and desalted over a PD-10 column. The protein composition of the filtrate was analyzed by SDS−PAGE. As shown in Figure 4A, the filtrate contains one major protein band with a molecular mass of ca. 8 kDa. The band stains poorly with Coomassie Brilliant Blue R-250, but it stains intensely brown with silver. We attribute this difference to a relative lack of amino acids that bind the organic dye and to the presence

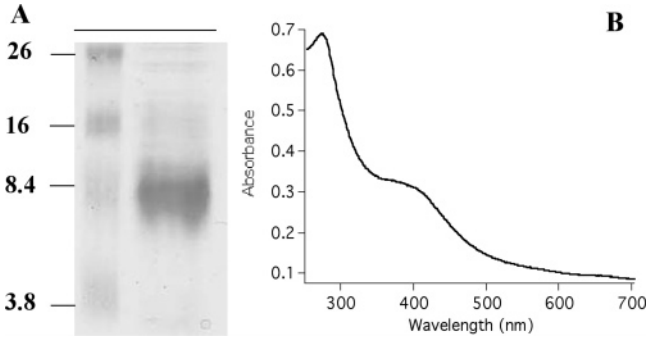

FIGURE 4: Protein composition and optical absorption spectrum of the filtrate from 250 to 700 nm of salt-washed HbRCs. (A) SDS−PAGE followed by staining with silver. Molecular mass standards were in the left lane, and the filtrate was in the right lane. The molecular masses of the standards are depicted. (B) Optical absorption spectrum of the filtrate containing the oxidized PshB protein.

of a large number of cysteines that are capable of binding silver. On the basis of mass alone, the bound Fe−S protein in Heliobacteriaceae is therefore more similar to PsaC of PS I than to PscB of Chlorobiaecea. We suggest that the bound Fe−S protein in the RC of heliobacteria be named PshB and the gene be named *pshB* according to the recommendations in ref *30*. This would be consistent with the nomenclature used in the RC of *Chlorobium*, another homodimeric type I RC, in which the bound Fe−S protein is termed PscB (*31*).

Characterization of PshB by Optical and EPR Spectroscopy. The optical spectrum of the oxidized PshB protein shows a relatively featureless absorption in the blue region with a pronounced shoulder at 410 nm (Figure 4B). The relative lack of absorbance from 500 to 700 nm is characteristic of ferredoxins that contain [4Fe-4S] rather than [2Fe-2S] clusters. The addition of sodium hydrosulfite results in a loss of approximately one-half of the absorbance between 350 and 450 nm (not shown), a feature also characteristic of dicluster ferredoxins.

Figure 5A (trace a) shows the EPR spectrum of the concentrated filtrate after incubation with 33 mM sodium hydrosulfite at pH 10.0. The spectrum closely resembles that seen in whole cells, membranes, and HbRC complexes after chemical reduction (compare with traces a−c of Figure 1C). To determine whether any damage had occurred to the Fe−S clusters, the filtrate was incubated with 2-mercaptoethanol, ferrous ammonium sulfate, and sodium sulfide, for 18 h (see Materials and Methods). As shown in Figure 5A (trace b), the EPR spectrum was identical except that the amplitude of the signal was nearly 5 times larger. This shows not only that the majority of the Fe−S clusters in the isolated PshB protein are denatured but also that it is possible to reconstitute the Fe−S clusters in the PshB apoprotein. Figure 5B compares the EPR spectrum of the reconstituted, concentrated filtrate after reduction with 10 mM sodium hydrosulfite at pH 7.0 and 10.0, where the redox potential of the aqueous solution is fixed by the H^+/H_2 couple and should be −420 and −600 mV, respectively. The EPR spectrum of the chemically reduced sample at pH 7.0 resembles the one-electron light-induced spectrum (compare traces a−c of Figure 1A), and the spectrum of the chemically reduced sample at pH 10.0 resembles the two-electron photoaccumulated (compare traces a−c of Figure 1B) or chemically

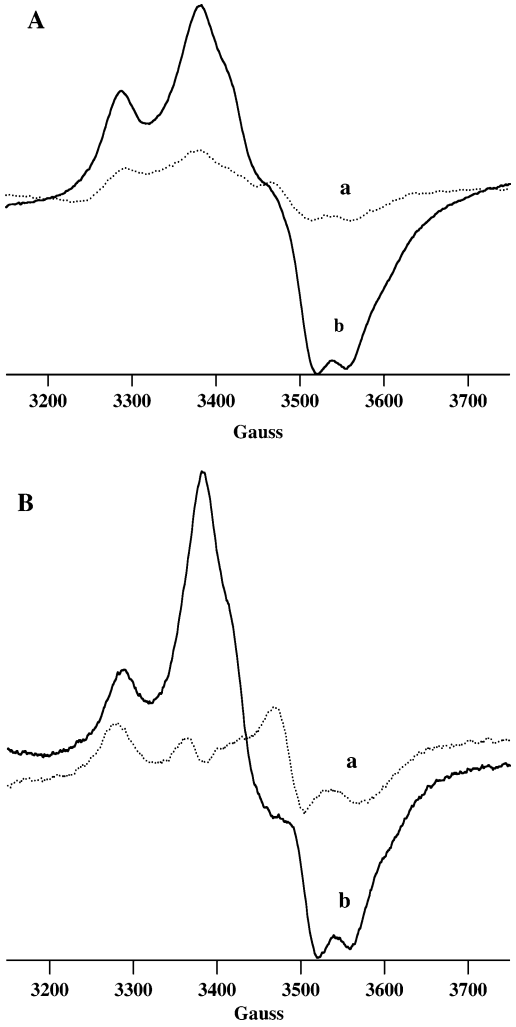

FIGURE 5: EPR spectra of the PshB-containing filtrate from salt-washed HbRCs. (A) EPR spectrum before (a) and after (b) reconstitution of the Fe−S clusters with 2-mercaptoethanol, Fe-$(NH_4)_2(SO_4)_2$, and Na_2S. The samples were incubated in 33 mM sodium hydrosulfite in 100 mM glycine buffer (pH 10). (B) EPR spectrum of the reconstituted filtrate at pH 7.0 (a) and 10.0 (b). The samples were reduced with 10 mM sodium hydrosulfite.

reduced spectra of whole cells, membranes, and HbRC complexes (compare traces a−c of Figure 1C). This result indicates that the two Fe−S clusters in the heliobacterial Fe−S protein are probably more widely separated in redox potential than are F_A and F_B in PsaC of PS I (see also ref *21*).

Reconstitution of the HbRC Core with the Resolved Fe−S Protein. Our goal in this section was to determine whether the isolated PshB protein can be rebound to purified HbRC cores. In the first set of experiments, detergent-treated membranes were treated with 1 M NaCl and ultrafiltered over a 30 kDa membrane; the retentate, which contains the HbRC cores, and the filtrate, which contains the PshB protein, were collected. As shown in Figure 6A, the amplitude of the light-induced EPR signal generated by illumination of the HbRC cores at 20 K was ca. 5-fold lower than in the intact HbRC complexes (compare trace a to trace b). As shown earlier, this loss is due to the loss of the PshB protein. When the isolated PshB protein is added to the HbRC cores in a 3-fold molar excess, the magnitude of the light-induced EPR signal increases, but the degree of

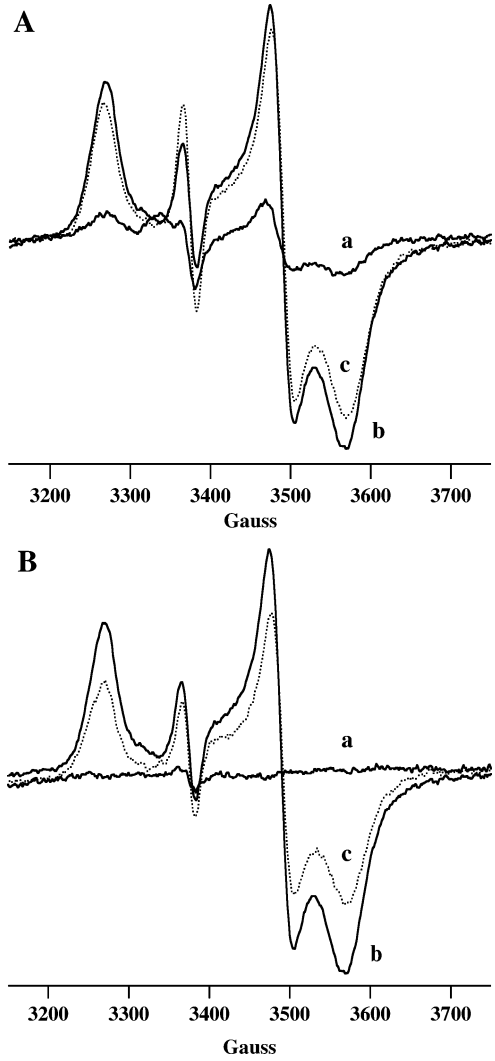

FIGURE 6: EPR spectra of reconstituted HbRC complexes from HbRC cores and the PshB protein. (A) Difference spectrum between a sample illuminated at 20 K and a dark-adapted sample. (a) The HbRC core was prepared by addition of 1 M NaCl followed by ultrafiltration and concentration over a YM-30 membrane. (b) The reconstituted HbRC complex was prepared by adding a 3-fold molar excess of PshB protein to the HbRC cores. (c) The intact HbRC complex was isolated by sucrose density ultracentrifugation. (B) Difference spectrum between a sample illuminated at 20 K and a dark-adapted sample. (a) The HbRC cores after DEAE ion-exchange chromatography, (b) the reconstituted HbRC complex prepared by adding a 7-fold molar excess of the PshB protein to the HbRC cores purified by DEAE ion-exchange chromatography, and (c) the HbRC complex isolated by sucrose gradient ultracentrifugation. Light-induced EPR signals were generated by illuminating dark-adapted samples at 20 K with a 2.5 W argon ion laser for 1 min. The spectra depicted are the difference spectra between the illuminated and dark-adapted samples. The optical absorbance at 788 nm was 75 OD.

recovery varied from preparation to preparation (not shown). However, when the PshB protein was pretreated with 2-mercaptoethanol, ferrous ammonium sulfate, and sodium sulfide for 18 h to reconstitute the Fe−S clusters, the amplitude of the light-induced EPR signal recovered to a value similar to that of the intact HbRC complex (Figure 6A, trace c). Note that a light-induced organic radical, derived from $P798^+$, is visible at a *g* value of 2.003 in all samples.

In the second set of experiments, a DEAE-purified HbRC core was treated similarly. As shown in Figure 6B, the light-induced EPR signal generated by illumination of the HbRC cores at 20 K (trace a) or by chemical reduction with sodium hydrosulfite (see trace d of Figure 1C) was completely devoid of resonances from Fe−S clusters. After incubation of the DEAE-purified HbRC cores with a 7-fold excess of the PshB protein, the amplitude of the EPR signals from the Fe−S clusters generally increased. Again, when the PshB protein was pretreated with 2-mercaptoethanol, ferrous ammonium sulfate, and sodium sulfide for 18 h to reconstitute the Fe−S clusters, the amplitude of the EPR signals increased to a value that was 70% of that of the intact HbRC complex (compare traces c and b). The addition of a larger molar excess of the reconstituted PshB protein to the HbRC core did not increase the amplitude of the light-induced signal. Thus, a small population of the HbRC cores may be irreversibly damaged either by salt treatment or by being passed through the ion-exchange column. Note again that a light-induced organic radical is also visible at a *g* value of 2.002 in all samples. The reconstituted HbRCs were washed three times by ultrafiltration over a 30 kDa cutoff membrane to remove excess PshB, and the retentate was analyzed by SDS−polyacrylamide gel electrophoresis. As shown in the middle lane of Figure 3, a new protein band at ca. 8 kDa is present in addition to those seen in HbRC cores. These reconstitution experiments show that the HbRC core is largely intact after removal of the PshB protein and that the latter can be rebound, leading to the recovery of the light-induced EPR signals of the terminal Fe−S clusters.

Time-Resolved Optical Spectroscopy of Resolved and Reconstituted HbRC Complexes. The charge recombination kinetics between the primary donor, $P798^+$, and the bound Fe−S cluster acceptors were measured using time-resolved optical spectroscopy in the near IR. Our primary goal was to correlate the lifetimes of the charge-separated states in the HbRC complexes and HbRC cores with the presence and absence of the PshB protein. As shown in Figure 7A, the charge recombination kinetics in the HbRC complex are biphasic, with lifetimes of 75 and 15 ms. These will henceforth be called the slow and fast kinetic components, respectively. In the first set of experiments, detergent-treated membranes were incubated with 1 M NaCl, and the retentate, which contains the HbRC cores, and the filtrate, which contains the PshB protein, were collected. The PshB protein was treated with 2-mercaptoethanol, ferrous ammonium sulfate, and sodium sulfide for 18 h to reconstitute any damaged or lost Fe−S clusters (see Materials and Methods). The flash-induced absorbance change in the retentate that contains the HbRC cores (Figure 7B) shows a significant loss in amplitude of the slow kinetic component, which now accounts for 27% of the signal, and an equivalent gain in amplitude of the slow kinetic component, which now accounts for 67% of the total amplitude. There are no significant kinetic events on the microsecond time scale. When the filtrate is added to the HbRC cores in a 10-fold molar excess, the slow kinetic component is partially regained and the fast kinetic component is partially lost (Figure 7C). Note that the total absorbance change is diminished by 18% after salt washing and by 32% after rebinding of PshB.

In the second set of experiments, DEAE-purified HbRC cores were used in the reconstitution experiments. As shown in Figure 8B, only the fast kinetic component is retained,

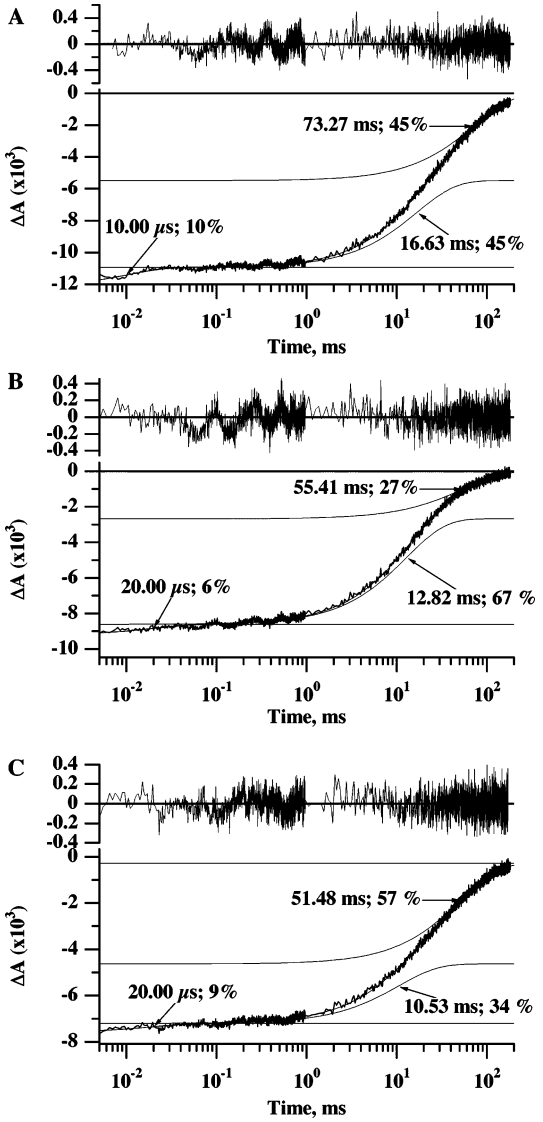

FIGURE 7: Flash-induced absorption changes of $P798^+$ reduction detected at 798 nm. (A) Decay kinetics of the HbRC complexes isolated by sucrose density ultracentrifugation. (B) Decay kinetics of the HbRC cores after addition of 1 M NaCl followed by ultrafiltration and concentration over a YM-30 membrane. (C) Decay kinetics after incubation of the HbRC cores isolated by 1 M NaCl washing with a 10-fold molar excess of the PshB-containing filtrate. The optical absorbance at 788 nm was 0.5 OD.

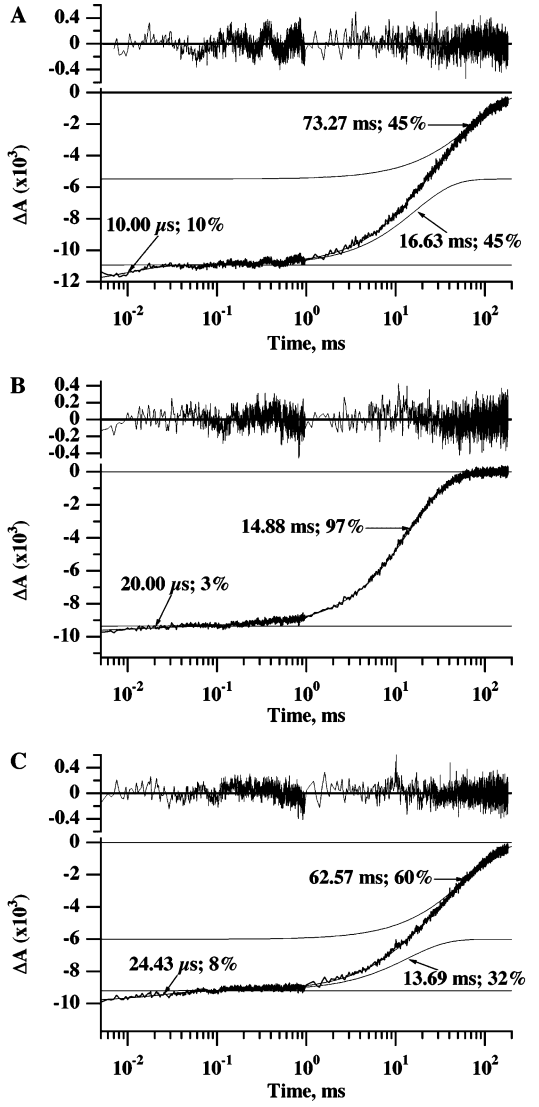

FIGURE 8: Flash-induced absorption changes of $P798^+$ reduction detected at 798 nm. (A) Decay kinetics of the HbRC complexes isolated by sucrose density ultracentrifugation. (B) Decay kinetics of the HbRC cores purified by DEAE ion-exchange chromatography. **(**C) Decay kinetics after incubation of the HbRC cores purified from DEAE ion-exchange chromatography with a 10-fold molar excess of the PshB-containing filtrate. The optical absorbance at 788 nm was 0.5 OD.

and it accounts for 97% of the total amplitude (compare Figure 8A). This matches the total loss of the light-induced EPR signals of the Fe−S signals in the DEAE-purified HbRC core (see trace a of Figure 6B). When the filtrate was added back to the HbRC core, a significant fraction of the slow kinetic component is restored at the expense of the fast kinetic component. Note that the total absorbance change is diminished by 14% after purification by DEAE ion-exchange chromatography and after rebinding of the PshB protein. The slightly lower amplitude of both the optical (Figures 7 and 8) and EPR (Figure 6) signals after reconstitution may be due to damage to an early acceptor incurred during the salt wash and/or DEAE ion-exchange chromatographic steps.

DISCUSSION

The primary aim of this study was to initiate a detailed characterization of the cofactors and proteins that comprise the photosynthetic RC in Heliobactericae. We chose to study *H. modesticaldum*, a mild thermophile that grows optimally at 50−52 °C, because the RC from this organism would likely be more stable than one from a mesophile. The HbRC is a type I (Fe−S) RC with a homodimeric core; it does not (to our knowledge) harbor a tightly bound cytochrome, and it does not contain chlorosomes, the FMO protein, or any other type of extended antenna system as is found in species of *Chlorobium* (*31*). Given the absence of these structures, the HbRC should be ideal for study of a prototypical homodimeric RC. However, our knowledge of the HbRC is incomplete, particularly with respect to the polypeptide composition and the full complement of electron acceptors. To paraphrase Amesz (*10*), with the exception of the primary charge separation steps, limited progress has been made in the last several years on the HbRC, with respect to either its polypeptide composition, the components of the electron acceptor chain, or the kinetics of forward and backward

electron transfer. What is known with certainty concerns primarily the initial charge-separated state. The primary electron donor, $P798^+$, is a dimer of Bchl *g*′ (the 13^2 epimer that now appears to be a common feature of all type I reaction centers), and as might be expected in a homodimeric RC, the cation is delocalized over both Bchl *g* molecules (*22*, *26*). The primary electron acceptor is Chl a_{670}, an 8^1-hydroxy Chl *a* molecule. Charge separation between P798 and A_0 occurs within 25 ps of a flash, but this value may instead represent energy transfer from the thermally populated antenna; the actual time of charge separation is estimated to be within 1 or 2 ps (*32*, *33*). Forward electron transfer from A_0^- occurs in 600 ps at room temperature (*34*), and if blocked, the resulting charge recombination between $P798^+$ and A_0^- occurs in 17 ns.

There are only a few reliable reports that describe the properties of the bound Fe−S clusters in heliobacteria. There is optical evidence for an F_X-like cluster in isolated membranes (*20*) but not for F_A- or F_B-like clusters. Conversely, there exists EPR evidence for a light-induced Fe−S cluster (*22*) as well as a more detailed report of EPR resonances comparable to F_A and F_B of PS I (*21*) but not for an F_X-like cluster (*20*, 27). No single methodology reveals the presence of all three Fe−S clusters in either cells, membranes, or isolated HbRCs. Taken at face value, these data can be fit into a model such that the Fe−S clusters in the HbRC are equivalent to F_X, F_B, and F_A in PS I. The issue, however, is further complicated by a report that the major photosynthesis gene cluster from *H. mobilis* does not contain a gene that encodes a PsaC (or PscB)-like protein (*23*). There also have been few, if any, reports of a PsaC (or PscB)-like protein in membranes or HbRCs, either as a band on a SDS−polyacrylamide electrophoresis gel or as a purified protein. No doubt, the lack of evidence for many of the components of the electron transport chain is due to the sensitivity of the terminal Fe−S clusters to oxygen and, as we have found in this study, an instability of the Fe−S protein to moderate ionic strength in the presence of detergent. It is therefore likely that the combined deleterious effect of oxygen, detergent, and ionic strength is the reason that the PshB protein has not been previously reported. It should be noted that the conditions under which the HbRC complex was purified in ref *27* probably resulted in the removal of the PshB protein.

We therefore considered it timely to investigate the detailed properties of the bound Fe−S clusters in heliobacteria, paying particular attention to the need to maintain anaerobic conditions throughout the purification procedure. Our studies indicate that PshB, the bound Fe−S protein in the HbRC, has a mass of ca. 8 kDa and that it contains two [4Fe-4S] clusters. The PshB protein in the HbRC is therefore more similar to PsaC of PS I than to PscB of the *Chlorobium* RC. The interaction spectrum observed at low redox potentials or after photoaccumulation is also characteristic of ferredoxins that contain two closely spaced [4Fe-4S] clusters, including PsaC of PS I. However, the line widths of the individual *g* components of the reduced Fe−S clusters in PshB are considerably broader than the resonances of F_A^- or F_B^- in PS I. Indeed, the *g* values and the line widths of the resonances in the bound and unbound forms of the reduced PshB protein are quite similar, and compare favorably with those of unbound PsaC. When PsaC docks onto the F_X core in PS I, the spectroscopic properties of F_A and F_B change, and line widths of the individual *g* components become narrower (*35*). The addition of PsaD leads to further narrowing of the individual *g* components whereby they become similar to those of the wild type. In contrast, when PshB docks to the HbRC core, the magnetic properties of the cluster(s) remain relatively unaltered. We do not know if the HbRC has a subunit comparable to PsaD; however, the salt treatment removes primarily the 8 kDa Fe−S protein, PshB. The HbRC does contain several additional, prominent polypeptides with molecular masses ranging from 15 to 25 kDa, but these proteins are tightly associated with the PshA homodimer and are not removed by salt washing. They can be removed when membranes are solubilized using Triton X-100 (data not shown), and they are also apparently removed using the detergent Deriphat 160c, which results in a HbRC that contains only a single subunit, the PshA protein (*27*).

There are other notable differences between the properties of the HbRC and PS I. First, the relatively small number of antenna chlorophylls in the HbRC (reported to be ca. 35−40 per P798 in the HbRC vs 100 per P700 in PS I) required that we use a higher intensity of light to achieve the maximum amplitude of the Fe−S resonances. This requirement is due to either the relatively small number of Bchl *g* molecules that are associated with the HbRC (*26*) or the reported inefficiency of electron transfer to the Fe−S clusters at low temperatures (*21*). Second, illumination of the HbRCs at 20 K results in the generation of a single rhombic signal, whereas in PS I, the same protocol results in the generation of two rhombic signals, F_A^- and F_B^-, in a ca. 3:1 ratio. One possible explanation is that because of the expected high degree of symmetry of the HbRC, the two [4Fe-4S] clusters in PshB have identical EPR spectra and cannot be distinguished when only one is reduced in a given PshB protein. Another possibility is that the two [4Fe-4S] clusters have dissimilar EPR spectra, but that the two Fe−S clusters have sufficiently different redox potentials such that the equilibrium favors population of only the high-potential Fe−S cluster. The assessment that the two Fe−S clusters have widely different redox potentials is supported by an EPR study of a sample that was chemically reduced at different pH values. The addition of sodium hydrosulfite to PshB at pH 7.0, where the solution potential is −420 mV, leads to the chemical reduction of a single Fe−S cluster, whereas the same treatment at pH 10.0, where the solution potential is ca. −600 mV, leads to the chemical reduction of both Fe−S clusters. Were the two Fe−S clusters are identical, there should have been a complex interaction spectrum present at both pH 7.0 and 10.0. A complete redox titration and a detailed comparison of the redox potentials of the Fe−S clusters when PshB is bound and unbound to the HbRC core will be reported elsewhere. Lastly, the PshB protein associates with the PshA homodimer in the HbRC in a manner unlike that of PsaC with the PsaA−PsaB heterodimer in PS I. The facile removal of the bound Fe−S protein in HbRCs with 1 M NaCl is in striking contrast to the need for 7.8 M urea or high concentrations of chaotropic agents (i.e., 2 M $NaClO_4$) to remove PsaC from PS I. The salt-based removal of the bound Fe−S protein from the HbRC leaves a population of the Fe−S clusters intact, whereas the chao-

trope-based removal of PsaC from PS I destroys the F_A and F_B clusters.

Time-resolved optical spectroscopy performed on the HbRC complex and the HbRC core allows us to assign with relative certainty the kinetic phases derived from charge recombination with $P798^+$. The partial elimination of the 75 ms kinetic component uncovered by time-resolved optical spectroscopy after salt washing is in agreement with the partial loss of the light-induced Fe−S resonances by EPR spectroscopy. Indeed, the total elimination of the 75 ms kinetic component after DEAE ion-exchange chromatography is in complete agreement with the EPR data. These results provide a compelling argument for assigning the slow, 75 ms kinetic component to charge recombination between P798 and the terminal Fe−S cluster bound to PshB. The HbRC core prepared by DEAE ion-exchange chromatography shows a single monophasic, 15 ms lifetime component by time-resolved optical spectroscopy that we attribute to the recombination of $P798^+$ with an earlier electron acceptor. The identity of this acceptor, however, is not obvious from the spectroscopic results. It is unlikely to be A_0 because if forward electron transfer from A_0^- to the next electron acceptor is blocked, charge recombination between $P798^+$ and A_0^- occurs in 17 ns. If the fast, 15 ms kinetic component in the HbRC core represents charge recombination from a quinone, then the charge-separated state between $P798^+$ and Q^- would be stable for periods 2−3 orders of magnitude longer than that of the comparable charge-separated state in PS I. If it represents charge recombination from F_X, then the charge-separated state between $P798^+$ and F_X would be stable for periods up to 15 times longer than that of the comparable charge-separated state in PS I. The 15 ms lifetime, however, is strikingly similar to the 14 ms (aerobic) or 20 ms (anaerobic) decays measured in a HbRC core isolated from *H. mobilis* membranes after they had been treated with 6 M urea. The spectrum of this electron acceptor, extracted by taking the difference of the flash-induced absorbance changes in the presence and absence of benzyl viologen, showed a bleaching in the blue between 400 and 500 nm attributed to Fe−S cluster F_X (*20*). This agrees with the circumstantial evidence for the existence of F_X from the FPCLGPAYGGTC sequence in the PshA protein that likely corresponds to the FPCDGPCRGGTC binding site for F_X in PS I (*28*). Nevertheless, we were unable to measure an EPR spectrum of an Fe−S cluster in the $g = 2$ region after photoaccumulation of the HbRC core in the presence of sodium hydrosulfite at pH 10.0 (see also ref *20*). This could be because the F_X cluster has unusual relaxation properties or an unusual ground spin state; both would make detection by EPR difficult. Further work is directed toward obtaining spectroscopic evidence for the existence of F_X and in the purified HbRC core.

CONCLUSIONS

Heliobacteria contain a bound Fe−S protein that is more similar to PsaC in PS I than to PscB in *Chlorobium*. The bound Fe−S protein can be removed from the HbRC by treatment with 1 M NaCl in the presence of *n*-dodecyl β-D-maltopyranoside. The ability to restore functional electron transfer to the terminal Fe−S clusters on rebinding the resolved Fe−S protein to HbRC cores indicates that the presumed F_X cluster is relatively stable in the purified HbRC core. The 75 ms kinetic phase after a saturating laser flash in the HbRC complex is from recombination of a terminal Fe−S cluster with $P798^+$, and the 15 ms kinetic phase in the HbRC core is from recombination with an earlier acceptor, probably F_X. We suggest that the bound Fe−S protein present in the HbRC be designated PshB.

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