Identification and Characterization of PshB, the Dicluster Ferredoxin that Harbors the Terminal Electron Acceptors F_A and F_B in *Heliobacterium modesticaldum*[†,‡]

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ABSTRACT: The Type I homodimeric photosynthetic reaction center found in anaerobic gram-positive bacteria of the genus *Heliobacteriaceae* incorporates F_A- and F_B-like iron−sulfur clusters similar to those found in Photosystem I as terminal electron acceptors. We recently isolated the PshB protein that harbors the iron−sulfur clusters from the reaction centers of *Heliobacterium modesticaldum.* Here, we report the cloning of a candidate gene and the properties of its product. Genuine PshB was dissociated from the reaction center with 1 M NaCl and purified using an affinity strategy. After acquiring its N-terminal amino acid sequence, an *fd2*-like gene encoding a 5.5-kDa dicluster ferredoxin was identified as a candidate for PshB. The Fd2-like apoprotein was expressed in *Escherichia coli* with a His tag, and the Fe/S clusters were inserted using inorganic reagents. The optical absorbance and EPR spectra of the Fd2-like holoprotein were similar to those of genuine PshB. The Fd2-like holoprotein was coeluted with P798-F_X cores on both G-75 gel filtration and Ni affinity columns. Consistent with binding, the EPR resonances at $g =$ 2.067, 1.933, and 1.890 from $[F_A/F_B]^-$ were restored after illumination at 15 K, and the long-lived, room-temperature charge recombination kinetics between $P798^+$ and $[F_A/F_B]^-$ reappeared on a laser flash. These characteristics indicate that the long-sought gene and polypeptide harboring the F_A- and F_B-like clusters in heliobacteria have been identified. The amino acid sequence of PshB indicates an entirely different mode of binding with the reaction center core than PsaC, its counterpart in Photosystem I.

Photosynthetic reaction centers (RCs[1]) are membrane-bound, protein−pigment complexes that convert light energy into a stable charge-separated state. Two types occur in nature: Type I has an Fe/S cluster as the terminal electron acceptor, and Type II has a mobile quinone as the terminal electron acceptor. Type I RCs can be further subdivided into two groups: homodimeric RCs, which are found in anaerobic bacteria of the genus *Chlorobiaceae* and *Heliobacteriaceae*, and Photosystem I (PS I), a heterodimeric RC, which is found in plants, algae, and cyanobacteria. Type II RCs can similarly be subdivided into two groups, the heterodimeric purple non-sulfur bacterial RC and Photosystem II (PS II), a heterodimeric RC, which is found in plants, algae, and cyanobacteria. X-ray crystal structures are available for the bacterial RC (*1*), PS II (*2*−*4*), and PS I (*5*, *6*), but no high-resolution structural information exists for Type I homodimeric RCs.

Heliobacteria are soil-dwelling phototrophs frequently found in rice fields (*7*). They are the only gram-positive photosynthetic bacteria (*8*) and the only spore-forming photosynthetic bacteria (*7*). They have a number of intriguing characteristics including an entirely novel pigment, bacteriochlorophyll *g* (Bchl *g*), that gives the microorganism its characteristic brown color (*9*). However, little is known about the polypeptide composition of the heliobacterial reaction center (HbRC), apart from the fact that it consists of a homodimer of the PshA protein (*10*).

The PshA homodimer incorporates all of the antenna bacteriochlorophylls as well as the primary electron donor, P798, a Bchl *g*′ homodimer (*11*, *12*); the primary electron acceptor A_0, a 8^1-OH-chlorophyll a_F monomer[2] (*13*); and the interpolypeptide electron acceptor F_X (*10*, *14*), a $[4Fe\text{-}4S]^{1+,2+}$ cluster with a ground spin state of $S = 3/2$ (*15*), see also (*16*). The PshA homodimer also contains up to two menaquinones, but their function remains unknown (*17*−*20*). To date, there is no convincing evidence for the participation of a quinone as a secondary electron acceptor in heliobacteria. The intact HbRC also contains a separate polypeptide, termed PshB (*21*), that harbors two $[4Fe\text{-}4S]^{1+,2+}$ clusters similar to F_A and F_B in PS I (*12*, *21*, *22*). However, there is no reliable data on the identity of the gene

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[‡] The sequence data in this article has been deposited in the GenBank data base (GenBank accession number EF158444).

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[1] Abbreviations: Bchl, bacteriochlorophyll; Fd2, ferredoxin from *Heliobacillus mobilis*; Fe/S, iron sulfur; HbRC, heliobacterial reaction center; PS I, Photosystem I; PS II, Photosystem II; PsaC, bound Fe/S protein in Photosystem I; PshA, photosynthetic reaction center protein in heliobacteria; PshB, bound Fe/S protein in the HbRC; RC, reaction center.

[2] The subscript refers to a farnesyl rather than phytyl tail; see ref (*13*).

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that encodes for PshB in heliobacteria. Further complicating the picture is a report that the major photosynthesis gene cluster in *Heliobacillus mobilis* does not include a gene that would code for a low molecular mass dicluster ferredoxin (*23*).

In a previous article (*21*), we showed that PshB can be removed from the reaction center complex of *Heliobacterium modesticaldum* with 1.0 M NaCl. We labeled the PshB-depleted reaction center a P798-F_X core. Upon removing PshB, the flash-induced kinetics accelerates from a ~75 ms lifetime to a ~15 ms lifetime, indicating that the former arises from the recombination of $P798^+$ with $[F_A/F_B]^-$. The latter was shown to arise from the recombination of $P798^+$ with F_X^- (*15*). We isolated PshB and succeeded in rebinding it to P798-F_X cores. The reconstituted HbRC was fully functional in terms of promoting light-induced electron transfer between P798 and F_A/F_B. In this article, we report the cloning and identification of a candidate gene for PshB from *H. modesticaldum* and the expression and properties of the recombinant PshB protein in *Escherichia coli*.

MATERIALS AND METHODS

Isolation of Heliobacterial Reaction Centers. A culture of *H. modesticaldum* was generously provided by Dr. Michael Madigan (Southern Illinois University). Liquid cultures of *H. modesticaldum* were grown anaerobically in PYE media as described in ref (*24*) except that resazurin, an oxygen reporter dye, was added to a final concentration of 0.001%. The media was degassed and allowed to incubate in an anaerobic chamber with an atmosphere of 10% H_2 and 90% N_2 (Coy Labs, Grass Lake, MI) until the color of the resazurin indicated that it was devoid of oxygen. The bottles were inoculated using the Hungate technique, and the culture was allowed to grow under illumination for 48 h at 43 °C. Banks of eight fluorescent bulbs provided white light illumination. All biochemical manipulations were performed anaerobically. Plasticware and glassware were placed in the anaerobic chamber 3 h prior to use and were tested with resazurin to verify that any residual oxygen had been removed. Cells grown to late-exponential phase were harvested anaerobically at 10,000*g* and resuspended in 50 mM MOPS buffer at pH 7. Whole cells were lysed by sonication, and membranes were pelleted by centrifugation at 200,000*g*. Membranes were solubilized with 1% *n*-dodecyl-β-D-maltopyranoside for 1 h; insoluble fragments were removed by centrifugation at 200,000*g*. P798-F_X cores devoid of PshB were isolated by passing detergent-solubilized membranes over a diethylaminoethyl cellulose ion-exchange column equilibrated in 50 mM MOPS at pH 7.0.

Purification of PshB, the Bound Fe/S Protein Containing F_A and F_B. Solubilized membranes were incubated with 1 M NaCl for 1 h and ultrafiltered over a 30-kDa cutoff membrane (PM 30, Millipore). The proteins that passed through the membrane were collected, and the filtrate was concentrated over a 3-kDa cutoff membrane (YM-3, Millipore) and desalted on a PD-10 column (Amersham Biosciences). After purification, the PshB protein was resolved by SDS−PAGE and blotted onto a PVDF membrane. N-terminal sequencing was carried out in the Macromolecular Core Facility of The Pennsylvania State University.

Cloning of the pshB Gene. Chromosomal DNA was isolated from *H. modesticaldum* using a previously published protocol (*25*). The DNA fragment containing the *pshB* gene was amplified using the chromosomal DNA of *H. modesticaldum* as template and PCR primers, which were designed on the basis of a sequence comparison with the Fd2 protein from *H. mobilis* (*26*). The 5′ DNA oligo was AACGGAGGTGAACATATGTATAAAATC with the generation of a *Nde* I site, and the 3′ primer was TGGTTAGGATCCATTAGGCTTTGATGGCGTC with the generation of a *Bam*HI site. The PCR fragment was purified and cloned into a pET16b expression vector after digestion with *Nde*I and *Bam*HI. The cloned DNA fragment unintentionally contained the *fd1* and *fd2* genes encoding two different ferredoxins, which were verified by DNA sequencing. For expression of the second ferredoxin-like protein (Fd2), a DNA fragment was amplified from this plasmid using the primers 5′ AGGCAGGTGAAAAGACATATGGCTTACAAA and 3′ CAAAAAACCCCTCCAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAG. The PCR fragment was cloned into a pET16b expression vector after digestion with *Bam*HI and *Nde*I and verified by DNA sequencing.

Protein Expression and Purification. The pET16bFd2 plasmid was transformed into BL21 (DE3) cells for expression. *E. coli* cells were grown in LB media to an OD_{600} of 0.6, and IPTG was added to a final concentration of 200 μM. After 6 h of growth, cells were spun down at 10,000*g* and lysed using a French Pressure cell. Unlysed cells were removed by centrifugation at 10,000*g*. The sodium chloride concentration in the cell lysate was brought to 300 mM, and imidazole was added to a final concentration of 10 mM. The cell lysate was loaded onto Ni resin that was equilibrated in 300 mM NaCl, 10 mM imidazole, and 50 mM Tris (pH 8.3), the column was washed extensively with the same buffer, and the apoprotein was eluted with 250 mM imidazole, 300 mM NaCl, and 50 mM Tris (pH 8.3).

Reconstitution of the Fe/S Clusters. The Fe/S apoprotein 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 brown, 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 and concentrated by ultrafiltration over a YM-3 membrane (*21*). After reconstitution, the Fe/S protein concentration was determined by using a previously published molar extinction coefficient (*27*).

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 recombinant protein was chemically reduced with 10 mM sodium hydrosulfite in 100 mM glycine at pH 10.0. A difference spectrum was constructed by subtracting the oxidized spectrum (no additions) from the chemically reduced spectrum. HbRC complexes were illuminated with an argon-ion laser, which was operated at 2.5 W in all-lines mode. A 3-fold beam expander was used to fill the grid of the resonator. A difference spectrum was constructed by subtracting the dark-adapted spectrum from the light-induced spectrum.

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N-terminal
sequences          AYKITD
PshB H.mod.  1   MAYKITDACTACGACMDGCCVGAIVEGK---------KYSITSDCVDCGVCADKCPVDAIIPG   55
Fd2 H.mob.   1   MYKIDASQCTGCGACVSGCYTNAIVEANG--------KYTITDDCVDCGVCQDSCPVDAIKA   54
Fd1 H.mod.   1   VVYKISDACVACGACEDACPVNAIIKG-D---------VYSITDACIDCGTCADTCPAGAISEG   55
Fd1 H. mob.  1    AYKISDACVNCGSCVDACPVGAIEKGSD---------IYCINDTCIDCGSCVDTCPAGAISEG   54
PsaC BP-1    1 MAHTVKIYDTCIGCTQCVRACPTDVLEMVPWDGCKAGQIASSPRTEDCVGCKRCETACPTDFLSIRVYLGAETTRSMGLAY 81
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FIGURE 1: Comparison of the N-terminal amino acid sequences of PshB from *H. modesticaldum* with amino acid sequences of Fd1 and Fd2 from *H. mobilis* and *H. modesticaldum* as well as PsaC from *Thermosynechococcus elongatus* BP-1.

Time-Resolved Optical Spectroscopy in the Near-IR. The kinetics of $P798^+$ reduction were measured by monitoring the flash-induced absorbance change after a laser flash. The sample was probed with a continuous measuring beam at 798 nm isolated from a 400 W tungsten−halogen lamp with a 1/4 meter monochromator. A shutter admitted the measuring beam 10 ms prior to the laser flash. The measuring 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 using a Tektronix AM502 differential amplifier and digitized using a DSA601 Tektronix digital oscilloscope. The data was sent to a Macintosh computer via an IEEE-488 bus (National Instruments). The electronic bandwidth of the detection system was 1 MHz. The sample was excited by an 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 to 16 transients were recorded and averaged. Samples were placed in a quartz anaerobic cuvette with an optical path of 10 mm. The samples were in 50 mM MOPS buffer at pH 7.0 and an absorbance of 1.0 at 788 nm. The kinetic traces were analyzed by fitting a multiexponential decay using the Marquardt least-squares algorithm (Igor Pro, Lake Oswego, OR). The HbRC concentration was determined using a previously published protocol (*15*).

RESULTS

N-Terminal Amino Acid Sequencing of Genuine PshB. The PshB protein is weakly bound to HbRCs and can be removed with 1.0 M NaCl followed by ultrafiltration over a 30-kDa cutoff membrane. To unambiguously identify PshB, an affinity technique was devised in which the ultrafiltrate was added to P798-F_X cores to reconstitute an HbRC complex. The rebinding of PshB was confirmed by the restoration of the flash-induced long-lived charge recombination kinetics between $P798^+$ and $[F_A/F_B]^-$. The HbRC complexes were separated from unbound PshB and other contaminating proteins by passage over a G-75 gel filtration column and collected. The HbRC complexes were then treated with 1% SDS, and the polypeptides were resolved on a 15% SDS−PAGE gel and stained with silver. The polypeptide composition of the HbRC complexes includes a band at $\sim$10 kDa, which was not present in P798-F_X cores (data not shown). The $\sim$10 kDa polypeptide was blotted onto a PVDF membrane, and the N-terminal sequence was determined to be AYKITD.

Cloning and Sequencing of the Gene Encoding a Dicluster Ferredoxin. The N-terminal sequence of genuine PshB is similar to the N-terminal sequences of Fd2 and Fd1, ferredoxins that were previously isolated from *H. mobilis* (*26*). Fd1 is a 5513 Da polypeptide with the N-terminal sequence AYKISDA, and Fd2 is a 5575 Da polypeptide with the N-terminal sequence MYKIDAS. Both contain two CxxCxxCxxxC binding motifs for two $[4Fe\text{-}4S]^{1+,2+}$ clusters. Figure 1 shows a comparison of the products of the *fd1* and *fd2* genes from *H. mobilis* and the products of the *fd1*-like and *fd2*-like genes from the unpublished genome sequence of *H. modesticaldum* (Blankenship,R., personal communication). The N-terminal sequence of genuine PshB is a perfect match to the deduced amino acid sequence of the Fd2-like protein in *H. modesticaldum*. The *fd2*-like gene was cloned from *H. modesticaldum* and expressed with an N-terminal His-Tag.

Comparison of Genuine PshB and the Expressed Fd2-Like Protein. The Fd2-like apoprotein of *H. modesticaldum* was expressed in *E. coli*, purified by Ni-chelation chromatography, and the iron−sulfur clusters were inserted using inorganic techniques. The optical absorbance spectra of PshB and the Fd2-like holoprotein were shown to be similar (data not shown). Both have a broad absorbance maximum at 400 nm and a ratio of 280 to 400 nm of $\sim$2.1 to 1, and both lose one-half of their absorbance in the visible region after the addition of sodium dithionite at pH 10. The low-temperature EPR spectra of PshB and the Fd2-like protein were also shown to be similar (Figure 2). Both show a complex interaction spectrum after reduction with dithionite, characteristic of bacterial dicluster ferredoxins that contain two closely spaced $[4Fe\text{-}4S]^{1+,2+}$ clusters.

Interaction of the Expressed Fd2-Like Protein with P798-F_X Cores. If the expressed Fd2-like protein is equivalent to PshB, it should bind to P798-F_X cores. In the first experiment, the His-tagged Fd2-like holoprotein was added to P798-F_X cores and subject to chromatography on a G-75 gel filtration column. The eluate containing the brown-colored reaction center was collected, and the polypeptide composition was analyzed by SDS−PAGE (Figure 3, lane 1). The band visible at $\sim$55 kDa was previously shown to be PshA (*10*, *28*). An additional band is present at $\sim$10-kDa, indicating that the Fd2-like protein binds to P798-F_X cores under the low ionic strength conditions of this experiment. (The mass of the Fd2-like protein with the added His tag is 7879 Da.) In the second experiment, the His-Tagged Fd2-like protein was first bound to a Ni-affinity column, and then the P798-F_X cores were loaded. The column was washed until the eluate was free of protein, imidazole was added to displace the bound Fd2 protein, and the polypeptide composition of the eluate was analyzed by SDS−PAGE (Figure 3, lane 2). The presence of a band at $\sim$10-kDa as well as $\sim$55 kDa indicates that P798-F_X cores bind the Fd2-like protein, thereby reconstituting the HbRC complex. It should be noted that P798-F_X cores alone are not retained on the Ni-affinity column.

Time-Resolved Optical Kinetics of P798-F_X Cores with the Expressed Fd2-Like Protein. To determine whether the reconstituted HbRC complex is functionally active at room

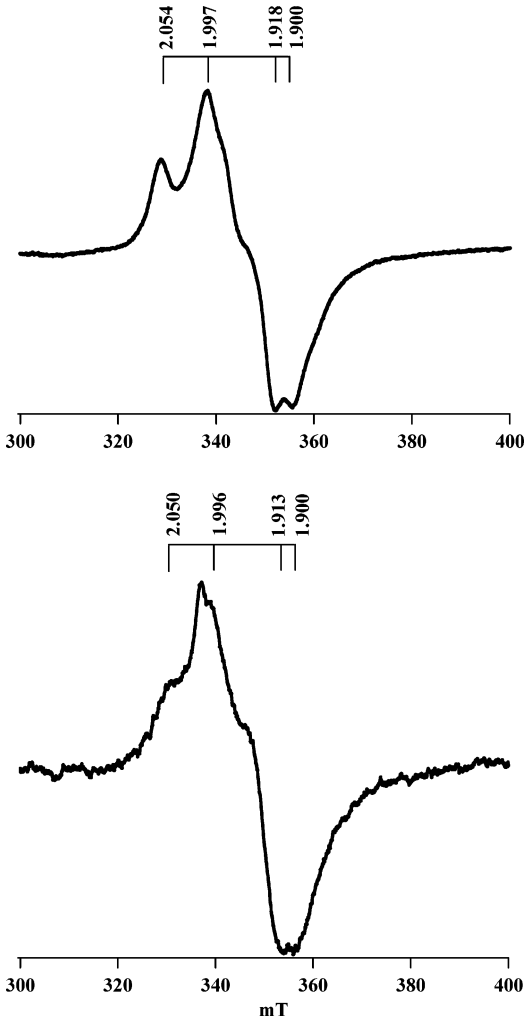

FIGURE 2: Low-temperature EPR spectra of PshB (top) and the Fd2-like protein (bottom). The samples were treated with 33 mM dithionite in 100 mM glycine (pH 10) to reduce the iron−sulfur clusters. The spectrometer conditions were as follows: temperature, 20 K; power, 126 mW; microwave frequency, 9.47 GHz; receiver gain, 2×10^4; and modulation amplitude, 10 G at 100 kHz.

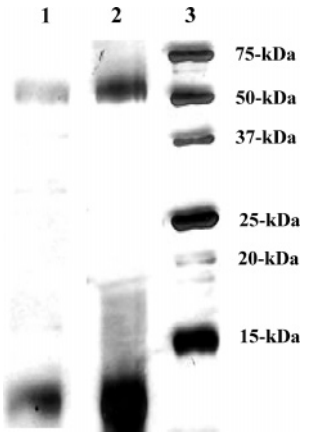

FIGURE 3: SDS−PAGE analysis of P798-F_X cores co-purified with His-tagged Fd2-like protein. Lane 1, P798-F_X cores + Fd2-like protein after purification on a G-75 gel filtration column; lane 2, P798-F_X cores + Fd2-like protein after elution from the Ni affinity column with 250 mM imidazole; lane 3, molecular weight markers.

temperature, the kinetics between $P798^+$ and $[F_A/F_B]^-$ were measured using time-resolved optical spectroscopy. Freshly isolated HbRC complexes were shown previously to have biphasic kinetics; an ~15-ms kinetic phase originates from

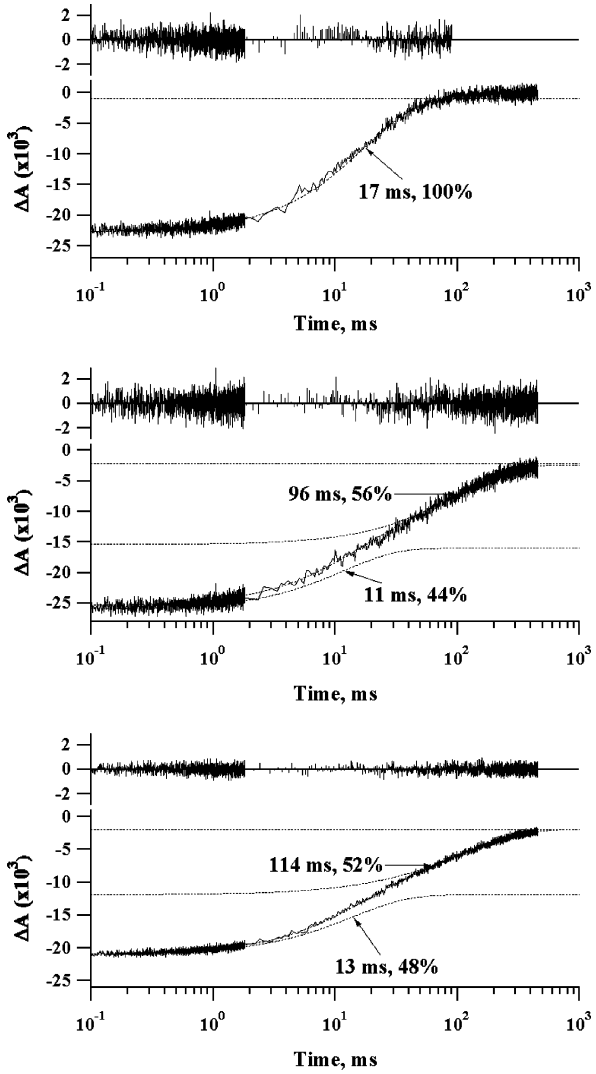

FIGURE 4: Flash-induced absorption changes at 798 nm of P798-F_X cores purified by DEAE ion-exchange chromatography (top), P798-F_X cores with a 5-fold molar excess of genuine PshB (middle), and P798-F_X cores with a 10-fold molar excess of reconstituted Fd2-like protein (bottom). The experimental data represents the average of 8 scans except for the Fd2-like protein (bottom), which is the average of 16 scans. The data are fitted using the Marquardt algorithm; the residuals (difference between experimental and fit curves) are shown in the upper panel.

the charge recombination between $P798^+$ and F_X, and an ~75 ms kinetic phase originates from the charge recombination between $P798^+$ and $[F_A/F_B]^-$ (*21*). The ratio between the two kinetic phases is approximately 1:1 in both detergent-solubilized membranes and freshly isolated HbRC complexes (*21*). The P798-F_X cores used in the reconstitution studies (Figure 4, top) show a monoexponential charge recombination of ~15 ms, indicating that PshB has been completely removed by anion exchange chromatography. When genuine PshB is added to the P798-F_X cores in 5-fold molar excess, a long-lived kinetic phase with a lifetime of 96 ms appears, corresponding to 56% of the total amplitude (Figure 4, middle). When the Fd2-like protein is added to the P798-F_X cores in 10-fold molar excess, a long-lived kinetic phase with a lifetime of ~114 ms appears, corresponding to 52% of the total amplitude (Figure 4, bottom). This percentage can be raised to ~85% by adding a 30-fold molar excess of the Fd2-like protein; however, when the mixture is subject to gel filtration to remove the unbound proteins, the contribution

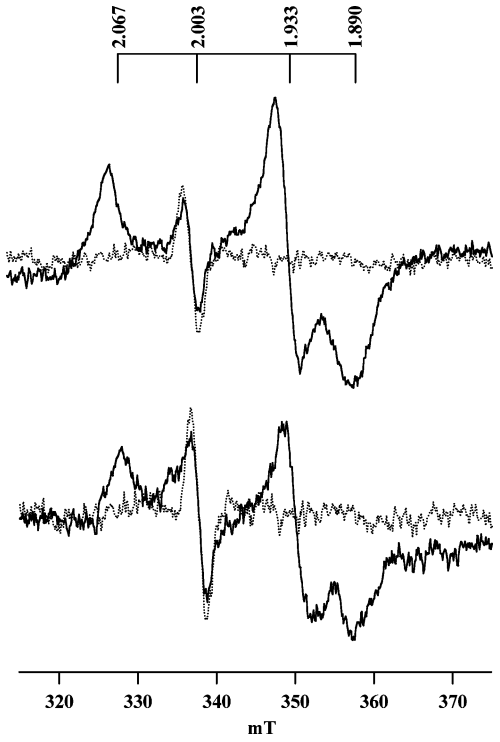

FIGURE 5: Light-induced EPR difference spectra of P798-F_X cores purified by DEAE ion-exchange chromatography reconstituted with a 5-fold molar excess of genuine PshB (top) and reconstituted with a 10-fold molar excess of reconstituted Fd2-like protein (bottom). The EPR spectrum of P798-F_X complexes prior to the addition of PshB and the Fd2-like protein are depicted as dotted lines (top and bottom). The spectrometer conditions were as follows: temperature, 20 K; power, 126 mW; microwave frequency, 9.47 GHz; receiver gain, 2×10^4; and modulation amplitude, 10 G at 100 kHz. The optical absorbance at 788 nm of the sample was 45.

of the slow kinetic phase returns to ~50% of the total absorption change (data not shown). We found that an ~2-fold larger molar excess of the Fd2-like protein relative to genuine PshB is required to achieve the same level of reconstitution.

Low-Temperature EPR Spectrum of P798-F_X Cores with the Expressed Fd2-Like Protein. To determine whether the reconstituted HbRC complex is functionally active at low temperature, the EPR spectrum of the terminal Fe/S clusters was measured after illumination at 15 K. Freshly isolated HbRCs, were shown previously to have a rhombic set of resonances at $g = 2.067$, 1.933, and 1.890, similar to those in whole cells (*21*). When PshB is removed, these resonances disappear, and only a derivative-shaped resonance at $g = 2.003$ is present from $P798^+$ (Figure 5, top and bottom, broken line). The electron acceptor under these conditions is iron−sulfur cluster F_X; however, it is in a ground spin state of $S = 3/2$, and is only visible downfield as a relatively weak doublet between $g = 4$ and 6 (*15*). When genuine PshB is incubated with the HbRC cores in 5-fold molar excess, a rhombic set of resonances at $g = 2.067$, 1.933, and 1.890 is seen after illumination at 15 K (Figure 5, top, solid line). When the expressed Fd2-like protein is incubated with the HbRC cores in 10-fold molar excess, an identical rhombic set of resonances at $g = 2.067$, 1.933, and 1.890 is seen after illumination at 15 K (Figure 5, bottom, solid line). Because the experiment was carried out at low temperature, no diffusion is possible, consistent with the proposal that the Fd2-like protein is bound to the P798-F_X cores.

DISCUSSION

The primary aim of this work was to identify the *pshB* gene in *H. modesticaldum* and to determine the amino acid sequence and partial characterization of its product. To date, the only protein that has been shown to be a bona fide component of the HbRC complex is PshA (*10*). A homodimer of PshA binds the Bchl *g* antenna molecules, the primary donor P798, the primary acceptor A_0, and the [4Fe-4S]$^{1+,2+}$ cluster F_X (*10*, *15*). The number of Bchl *g* antenna molecules in the HbRC is between 28 by HPLC analysis of pigments (*29*) and 22 by a comparison of chlorophyll and iron concentrations (*15*). It has been long known that F_A- and F_B-like iron−sulfur clusters function as terminal electron acceptors (*12*, *22*); however, a dicluster ferredoxin protein similar to PsaC was not present in purified HbRC preparations (*11*, *30*). Recently, we succeeded in isolating an HbRC complex from *H. modesticaldum* that contains PshB, the polypeptide that harbors the F_A and F_B clusters (*21*). Our strategy to identify its gene focused on isolating the genuine PshB protein and determining its N-terminal amino acid sequence. We were assisted by the report that two ferredoxins, Fd1 and Fd2, had been isolated previously from *H. mobilis* and their gene sequences determined (*26*). We constructed primers on the basis of the N-terminal and C-terminal amino acid sequence of *fd2* from *H. mobilis*, and we cloned the counterparts of *fd1* and *fd2* from *H. modesticaldum*. The deduced N-terminal amino acid sequence of the Fd2-like protein from *H. modesticaldum* perfectly matched the N-terminal amino acid sequence of genuine PshB. We cloned the corresponding *fd2* gene from *H. modesticaldum* into an expression vector, expressed the protein with a His-affinity tag, and showed that the product binds to P798-F_X cores. The reconstituted HbRC was found to be fully functional in promoting light-induced electron transfer between P798 and F_A/F_B. A 2-fold higher concentration of the expressed Fd2-like protein is required to achieve a level of reconstitution comparable to that of genuine PshB. This could be due to known differences between the two proteins: the expressed Fd2-like protein contains a 2.5 kDa affinity tag consisting of 21 residues, including 10 consecutive His residues, on the N-terminus, and it terminates with the residues KA instead of IPG (the preceding 51 amino acids, however, are identical). The alteration at the C-terminus is because the primers were constructed using the sequence of the *fd2* gene from *H. mobilis* in the absence of the C-terminal amino acid sequence of genuine PshB. These additional amino acids at the N-terminus and changes at the C-terminus could result in a different binding affinity than that of genuine PshB. Nevertheless, the properties of the expressed Fd2-like protein indicate that the long-sought gene that codes for the F_A/F_B polypeptide in heliobacterial reaction centers has been identified.

The Fd2-like protein in *H. modesticaldum* (hereafter termed PshB) has a predicted molecular weight of 5440 (54 amino acids) and is, therefore, considerably smaller than PsaC, which has a molecular weight of 8829 (81 amino acids). Both are acidic proteins with estimated pIs of 4.06 and 5.5, respectively. Both contain two traditional CxxCxx-CxxxC binding motifs for the [4Fe-4S]$^{1+,2+}$ clusters; how-

ever, in PsaC, a Pro follows the terminal Cys residue in both Fe/S cluster binding motifs, whereas in PshB, a Cys follows the terminal Cys in the N-terminal Fe/S cluster binding motif (Figure 1). Although it is not uncommon for a dicluster ferredoxin to contain a residue other than Pro in this position, a Cys is highly unusual. It is not known if the Cys−Cys motif has any structural or functional significance. A database search shows that PshB is a member of a family of a large number of bacterial dicluster ferredoxins that are highly acidic and have nearly the same mass and number of amino acids. A number of high-resolution X-ray and NMR structures are available for several bacterial dicluster ferredoxins (pdb entries 1FDX (now replaced with 1DUR), 1CLF, 1FDN, 2FDN, 1BLU, 1FCA, 5FD1, 1BC6, 1BQX, 1RGV) (*31*−*41*), including PsaC (pdb entry 1K0T) (*42*). In bacterial dicluster ferredoxins as well as PsaC, both [4Fe-4S]$^{1+,2+}$ cluster binding motifs and the clusters themselves exhibit a local pseudo-C_2-symmetry. All show a very similar set of protein folds characterized by a two-stranded antiparallel β-sheet arrangement of N- and C-termini and the two [4Fe-4S]$^{1+,2+}$ clusters separated by a single α-helical turn. These central structural features are common to all dicluster ferredoxins characterized thus far, and they can be expected to be present in the highly similar Fd2-like protein from *H. modesticaldum*. Conspicuously absent is the insertion of 8 residues in the middle of the loop connecting the two consensus iron−sulfur binding motifs and the C-terminal extension of 14 or 15 residues present in PsaC. The amino acids in the loop insertion region of PsaC are considered to be involved in ferredoxin/flavodoxin binding (*43*, *44*) and those in the C-terminus in binding to the PsaA/PsaB core and to other stromal subunits (*45*). Most significantly, the two Arg and one Lys residues that bind PsaC to the PsaA/PsaB heterodimer of PS I are not present in the Fd2-like protein from *H. modesticaldum;* all are replaced by neutral residues (Figure 1). This indicates a different mode of binding with the P798-F_X core, but details await a high-resolution crystal structure of the HbRC replete with PshB.

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REFERENCES

1. Deisenhofer, J., Epp, O., Miki, K., Huber, R., and Michel, H. (1984) X-ray structure analysis of a membrane protein complex. Electron density map at 3 Å resolution and a model of the chromophores of the photosynthetic reaction center from *Rhodopseudomonas viridis*, *J. Mol. Biol. 180*, 385−398.
2. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II, *Nature 438*, 1040−1044.
3. Iwata, S., and Barber, J. (2004) Structure of photosystem II and molecular architecture of the oxygen-evolving centre,. *Curr. Opin. Struct. Biol. 14*, 447−453.
4. Zouni, A., Witt, H. T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) Crystal structure of photosystem II from Synechococcus elongatus at 3.8 Å resolution, *Nature 409*, 739−743.
5. Jordan, P., Fromme, P., Witt, H. T., Klukas, O., Saenger, W., and Krauss, N. (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution, *Nature 411*, 909−917.
6. Ben-Shem, A., Frolow, F., and Nelson, N. (2003) Crystal structure of plant photosystem I, *Nature 426*, 630−635.
7. Madigan, M. T., and Ormerod, J. G. (1995) *Anoxygenic Photosynthetic Bacteria* (Blankenship, R. E., Madigan, M. T., and Bauer, C. E., Eds.), pp 17−30, Kluwer Academic Publishers, Dordrecht, The Netherlands.
8. Woese, C. R., Debrunner-Vossbrinck, B. A., Oyaizu, H., Stackebrandt, E., and Ludwig, W. (1985) Gram-positive bacteria: possible photosynthetic ancestry, *Science 229*, 762−765.
9. Gest, H., Favringer, J. L. (1983) *Heliobacterium chlorum*, an anoxygenic brownish-green photosynthetic bacterium containing a "new" form of bacteriochlorophyll, *Arch. Microbiol. 136*, 11−16.
10. Liebl, U., Mockensturm-Wilson, M., Trost, J. T., Brune, D. C., Blankenship, R. E., and Vermaas, W. (1993) Single core polypeptide in the reaction center of the photosynthetic bacterium *Heliobacillus mobilis*: structural implications and relations to other photosystems, *Proc. Natl. Acad. Sci. U.S.A. 90*, 7124−7128.
11. Trost, J. T., and Blankenship, R. E. (1989) Isolation of a photoactive photosynthetic reaction center-core antenna complex from *Heliobacillus mobilis*, *Biochemistry 28*, 9898−9904.
12. Prince, R., Blankenship, R. E, Gest, H. (1985) Thermodynamic properties of the photochemical reaction center of *Heliobacterium chlorum*, *Biochim. Biophys. Acta 810*, 377−384.
13. van de Meent, E. J., Kobayashi, M., Erkelens, C., van Veelen, D. A., and Amesz, J. (1991) Identification of 8′-hydroxychlorophyll *a* as a functional reaction center pigment in heliobacteria, *Biochim. Biophys. Acta 1058*, 356−362.
14. Kleinherenbrink, F. A. M., Chiou, H. C., LoBrutto, R., and Blankenship, R. E. (1994) Spectroscopic evidence for the presence of an iron-sulfur center similar to F_X of Photosystem I *in Heliobacillus mobilis*, *Photosynth. Res. 41*, 115−123.
15. Heinnickel, M., Agalarov, R., Svensen, N., Krebs, C., and Golbeck, J. H. (2006) Identification of F_X in the heliobacterial reaction center as a [4Fe-4S] cluster with an $S = 3/2$ ground spin state, *Biochemistry 45*, 6756−6764.
16. Miyamoto, R., Iwaki, M., Mino, H., Harada, J., Itoh, S., and Oh-Oka, H. (2006) ESR signal of the iron-sulfur center F_X and its function in the homodimeric reaction center of *Heliobacterium modesticaldum*, *Biochemistry 45*, 6306−6316.
17. Muhiuddin, I. P., Rigby, S. E., Evans, M. C., Amesz, J., and Heathcote, P. (1999) ENDOR and special TRIPLE resonance spectroscopy of photoaccumulated semiquinone electron acceptors in the reaction centers of green sulfur bacteria and heliobacteria, *Biochemistry 38*, 7159−7167.
18. Brettel, K., Leibl, W., and Leibl, U. (1998) Electron transfer in the heliobacterial reaction center: Evidence against a quinone-type electron acceptor functioning analogous to A(1) in photosystem I, *Biochim. Biophys. Acta 1363*, 175−181.
19. Kleinherenbrink, F. A., Ikegami, I., Hiraishi, A., Otte, S. C. M., and Amesz, J. (1993) Electron transfer in menaquinone depleted membranes of *Heliobacterium chlorum*, *Biochim. Biophys. Acta 1142*, 69−73.
20. van der Est, A., Hager-Braun, C., Leibl, W., Hauska, G., and Stehlik, D. (1998) Transient electron paramagnetic resonance spectroscopy on green-sulfur bacteria and heliobacteria at two microwave frequencies, *Biochim. Biophys. Acta 1409*, 87−98.
21. Heinnickel, M., Shen, G., Agalarov, R., and Golbeck, J. H. (2005) Resolution and reconstitution of a bound Fe-S protein from the photosynthetic reaction center of *Heliobacterium modesticaldum*, *Biochemistry 44*, 9950−9960.
22. Nitschke, W., Sétif, P., Liebl, U., Feiler, U., and Rutherford, A. W. (1990) Reaction center photochemistry of *Heliobacterium chlorum*, *Biochemistry 29*, 11079−11088.
23. Xiong, J., Inoue, K., and Bauer, C. E. (1998) Tracking molecular evolution of photosynthesis by characterization of a major photosynthesis gene cluster from *Heliobacillus mobilis*, *Proc. Natl. Acad. Sci. U.S.A. 95*, 14851−14856.
24. Kimble, L. K., Mandelco, L., Woese C. R., Madigan, M. T. (1995) *Heliobacterium modesticaldum*, sp. nov., a thermophilic heliobacterium of hot springs and volcanic soils, *Arch. Microbiol. 163*, 259−267.
25. Murphy, R. C., Gasparich, G. E., Bryant, D. A., and Porter, R. D. (1990) Nucleotide sequence and further characterization of the *Synechococcus* sp. strain PCC 7002 *recA* gene: complementation of a cyanobacterial *recA* mutation by the *Escherichia coli recA* gene, *J. Bacteriol. 172*, 967−976.
26. Hatano, A., Seo, D., Kitashima, M., Sakurai, H., and Inoue, K. (2004) In *24th International Congress on Photosynthesis* (van der Est, A., and Bruce, D., Eds.), Montreal, Canada.

27. Sweeney, W. V., and Rabinowitz, J. C. (1980) Proteins containing 4Fe-4S clusters: an overview, *Annu. Rev. Biochem. 49*, 139−161.
28. Trost, J., Brune, D., and Blankenship, R. (1992) Protein sequences and redox titrations indicate that the electron acceptors in reaction centers from heliobacteria are similar to photosystem-I, *Photosynth. Res. 32*, 11−22.
29. Kobayashi, M., van de Meent, E. J., Erkelens, C., Amesz, J., Ikegami, I., and Watanabe, T. (1991) Bacteriochlorophyll *g* epimer as a possible reaciton center component of heliobacteria, *Biochim. Biophys. Acta 1057*, 89−96.
30. van de Meent, E. J., Kleinherenbrink, F. A. M., and Amesz, J. (1990) Purification and properties of an antennae-reaction center complex from heliobacteria, *Biochim. Biophys. Acta 1015*, 223−230.
31. Adman, E. T., Sieker, L. C., and Jensen, L. H. (1973) The structure of a bacterial ferredoxin, *J. Biol. Chem. 248*, 3987−3996.
32. Adman, E. T., Sieker, L. C., and Jensen, L. H. (1976) Structure of *Peptococcus aerogenes* ferredoxin. Refinement at 2 Å resolution, *J. Biol. Chem. 251*, 3801−3806.
33. Bertini, I., Donaire, A., Feinberg, B. A., Luchinat, C., Piccioli, M., and Yuan, H. P. (1995) Solution structure of the oxidized 2[4Fe-4S] ferredoxin from *Clostridium pasteurianum*, *Eur. J. Biochem. 232*, 192−205.
34. Duee, E. D., Fanchon, E., Vicat, J., Sieker, L. C., Meyer, J., and Moulis, J. M. (1994) Refined crystal structure of the 2[4Fe-4S] ferredoxin from *Clostridium acid urici* at 1.84 Å resolution, *J. Mol. Biol. 243*, 683−695.
35. Dauter, Z., Wilson, K. S., Sieker, L. C., Meyer, J., and Moulis, J. M. (1997) Atomic resolution (0.94 Å) structure of *Clostridium acid urici* ferredoxin. Detailed geometry of [4Fe-4S] clusters in a protein, *Biochemistry 36*, 16065−16073.
36. Moulis, J. M., Sieker, L. C., Wilson, K. S., and Dauter, Z. (1996) Crystal structure of the 2[4Fe-4S] ferredoxin from *Chromatium vinosum*: Evolutionary and mechanistic inferences for [3/4Fe-4S] ferredoxins, *Protein Sci. 5*, 1765−1775.
37. Tranqui, D., and Jesior, J. C. (1995) Structure of the ferredoxin from *Clostridium acidi urici*: model at 1.8 Å resolution, *Acta. Crystallogr., Sect. D 51*, 155−159.
38. Stout, C. D. (1993) Crystal structures of oxidized and reduced *Azotobacter vinelandii* ferredoxin at pH-8 and pH-6, *J. Biol. Chem. 268*, 25920−25927.
39. Aono, S., Bentrop, D., Bertini, I., Donaire, A., Luchinat, C., Niikura, Y., and Rosato, A. (1998) Solution structure of the oxidized Fe7S8 ferredoxin from the thermophilic bacterium *Bacillus schlegelii* by ^{1}H NMR spectroscopy, *Biochemistry 37*, 9812−9826.
40. Aono, S., Bentrop, D., Bertini, I., Cosenza, G., and Luchinat, C. (1998) Solution structure of an artificial Fe8S8 ferredoxin: the D13C variant of *Bacillus schlegelii* Fe7S8 ferredoxin, *Eur. J. Biochem. 258*, 502−514.
41. Unciuleac, M., Boll, M., Warkentin, E., and Ermler, U. (2004) Crystallization of 4-hydroxybenzoyl-CoA reductase and the structure of its electron donor ferredoxin, *Acta Crystallogr., Sect. D 60*, 388−391.
42. Antonkine, M. L., Liu, G., Bentrop, D., Bryant, D. A., Bertini, I., Luchinat, C., Golbeck, J. H., and Stehlik, D. (2002) Solution structure of the unbound, oxidized Photosystem I subunit PsaC, containing [4Fe-4S] clusters F_A and F_B: a conformational change occurs upon binding to Photosystem I, *J. Biol. Inorg. Chem. 7*, 461−472.
43. Fischer, N., Hippler, M., Sétif, P., Jacquot, J. P., and Rochaix, J. D. (1998) The PsaC subunit of photosystem I provides an essential lysine residue for fast electron transfer to ferredoxin, *EMBO J. 17*, 849−858.
44. Fischer, N., Sétif, P., and Rochaix, J. D. (1999) Site-directed mutagenesis of the PsaC subunit of photosystem I. F(b) is the cluster interacting with soluble ferredoxin, *J. Biol. Chem.* 274, 23333−23340.
45. Antonkine, M. L., Jordan, P., Fromme, P., Krauss, N., Golbeck, J. H., and Stehlik, D. (2003) Assembly of protein subunits within the stromal ridge of Photosystem I. Structural changes between unbound and sequentially PS I-bound polypeptides and correlated changes of the magnetic properties of the terminal iron sulfur clusters, *J. Mol. Biol. 327*, 671−697.

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