Electron Transfer May Occur in the Chlorosome Envelope: The CsmI and CsmJ Proteins of Chlorosomes Are 2Fe-2S Ferredoxins[†,‡]

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ABSTRACT: Chlorosomes of the green sulfur bacterium *Chlorobium tepidum* have previously been shown to contain at least 10 polypeptides [Chung, S., Frank, G., Zuber, H., and Bryant, D. A. (1994) *Photosynth. Res. 41*, 261−275]. Based upon the N-terminal amino acid sequences determined for two of these proteins, the corresponding genes were isolated using degenerate oligonucleotide hybridization probes. The *csmI* and *csmJ* genes encode proteins of 244 and 225 amino acids, respectively. A third gene, denoted *csmX*, that predicts a protein of 221 amino acids with strong sequence similarity to CsmI and CsmJ, was found to be encoded immediately upstream from the *csmJ* gene. All three proteins have strong sequence similarity in their amino-terminal domains to [2Fe-2S] ferredoxins of the adrenodoxin/putidaredoxin subfamily of ferredoxins. CsmI and CsmJ were overproduced in *Escherichia coli*, and both proteins were shown by EPR spectroscopy to contain iron−sulfur clusters. The *g*-tensor and relaxation properties are consistent with their assignment as [2Fe-2S] clusters. Isolated chlorosomes were also shown to contain [2Fe-2S] clusters whose properties were similar to those of the recombinant CsmI and CsmJ proteins. Redox titration of isolated chlorosomes showed these clusters to have potentials of about −201 and +92 mV vs SHE. The former potential is similar to that measured by redox titration of the clusters in inclusion bodies of CsmJ. Possible roles for these iron−sulfur proteins in electron transport and light harvesting are discussed.

Green bacteria are phototrophic prokaryotes that contain bacteriochlorophyll (Bchl)[1] *c*, *d*, or *e* as antenna pigments in addition to smaller amounts of Bchl *a*. Two families of green bacteria are known: the Chlorobiaceae and the Chloroflexaceae. The Chlorobiaceae, or green sulfur bacteria, are obligately anaerobic, photoautotrophic eubacteria that are typically found in sulfide-rich but very low-light environments. The Chloroflexaceae, or green non-sulfur bacteria, can grow as facultative photoautotrophs or photoheterotrophs under anaerobic conditions or as respiring chemoorganotrophs under aerobic conditions (*1*). Both groups of green bacteria produce a unique light-harvesting antenna complex, the chlorosome (for reviews, see *2*, *3*). Chlorosomes are sac-like structures that are tightly appressed to the cytoplasmic surface of the cytoplasmic membrane. In green sulfur bacteria, such as *C. tepidum*, they typically have lengths of 70−180 nm and widths of 30−60 nm (*4*). The chlorosomes of green non-sulfur bacteria are smaller and usually have dimensions of 100 nm in length by 20−40 nm in width by 10−20 nm in height (*5*).

Chlorosomes predominantly contain Bchl *c*, *d*, or *e*, but lipids, proteins, carotenoids, and quinones are also components of these structures (*6*, *7*). All chlorosomes consist of a core structure composed of stacked, rodlike elements that are probably composed of the highly aggregated Bchls. Each chlorosome can contain from 10 to 30 of these rod elements, and several models for the organization of the Bchl in such aggregates have been proposed (for reviews, see *2*, *3*). The chlorosome envelope is believed to be a monolayer membrane that is mostly composed of galactolipids (*5*, *7*) but that is stabilized by proteins (*8*−*10*). Chlorosomes additionally contain a small amount of Bchl *a* that is thought to occur in the baseplate region of the chlorosome (*11*). The baseplate region of the chlorosomes of the green sulfur bacteria interacts with a paracrystalline layer with a thickness of 5−6 nm. This layer is probably comprised of the FMO protein, and the FMO protein is in turn physically connected to the reaction centers within the cytoplasmic membrane (*3*).

A remarkable feature of chlorosomes is their very low mass ratio of protein to chlorophyll. Depending upon the light intensity under which the cells were grown (*8*), this

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[‡] The nucleotide sequence of the DNA fragment encoding the *csmJ* gene has been deposited in GenBank under accession number AF060077 (protein AAC14870) and that of the DNA fragment encoding the *csmI* gene under accession number AF060079 (protein AAC14875). The sequence for the *csmX* gene has been deposited by The Institute for Genomic Research in the Microbial Genomes Blast Database as nucleotides 201009−201671 of *Cb. tepidum* contig 3499.

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[1] Abbreviations: Bchl, bacteriochlorophyll; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DTT, dithiothreitol; EPR, electron paramagnetic resonance; FMO protein, Fenna−Matthews−Olson bacteriochlorophyll *a*-binding protein; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SHE, standard hydrogen electrode.

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ratio was found to be 0.45−0.55 for highly purified chlorosomes. The chlorosomes of the green non-sulfur bacterium *Chloroflexus aurantiacus* have been reported to contain as few as four different polypeptides (*12*). However, Chung et al. (*8*) showed by SDS−PAGE and N-terminal amino acid sequence analyses that highly purified chlorosomes of the moderately thermophilic green sulfur bacterium *C. tepidum* contain at least 10 different polypeptides denoted CsmA through CsmJ. The genes encoding all of these proteins have been cloned, sequenced, and characterized (*8*−*10*, *13*; D. Bryant, V. L. Stirewalt, and E. Vassilieva, unpublished experiments). Five chlorosome proteins, CsmA, CsmB, CsmC, CsmD, and CsmE, have been overproduced in *Escherichia coli* and antisera produced against the recombinant proteins (*9*, *10*). These antisera have been used in chlorosome agglutination studies as well as immunoblotting experiments in conjunction with protease susceptibility studies. It was shown that these five proteins are at least partially exposed on the chlorosome envelope (*9*, *10*).

In this study, CsmI and CsmJ, the two largest proteins found in the chlorosomes of *C. tepidum*, were overproduced in *E. coli* and biochemically characterized. The deduced amino acid sequences of these two proteins suggested that each protein should contain a [2Fe-2S] cluster. Biochemical and spectroscopic characterization of the recombinant proteins confirmed this hypothesis. Moreover, it was shown that isolated chlorosomes also contain iron−sulfur clusters with similar spectroscopic properties and redox potentials. These findings raise the interesting possibility that, in addition to their well-established role in light energy harvesting, chlorosomes play a role in electron transport reactions in green sulfur bacteria.

MATERIALS AND METHODS

C. tepidum was kindly provided by Dr. Michael Madigan (Southern Illinois University, Carbondale, IL) and was cultivated at 48 °C in a modified version of Pfennig's medium as described (*14*). Resazurin (100 μg) was added to 2.5 L of the growth medium as a redox indicator. *Escherichia coli* strain DH5α (genotype: F$^-$ Φ80d*lacZ*Δ*M*15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*; Bethesda Research Laboratories, Gaithersburg, MD) was used for all routine recombinant DNA manipulations except protein overproduction. For most experiments, cells were grown in Luria−Bertani medium (*15*); when appropriate, the medium was supplemented with ampicillin (100 μg mL^{-1}). For protein overproduction, *E. coli* strain BL21 (DE3) (genotype: F$^-$ *ompT* *hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (λDE3); Novagen, Madison, WI) was used.

Cloning of the csmI and csmJ Genes of C. tepidum and Protein Overproduction. Based upon the N-terminal amino acid sequence of the CsmJ protein (see Results), a 128-fold degenerate oligonucleotide with the sequence 5′ ATGAT-(T/C)AT(T/C)TA(T/C)AT(T/C)AA(T/C) GA(T/C)AA (A/G)CC 3′ was synthesized as a hybridization probe. Genomic Southern blot-hybridization experiments revealed that this oligonucleotide hybridized to a 0.9 kb *Bam*HI−*Hin*dIII fragment (see Figure 1). DNA fragments of approximately this size were ligated into pBluescript KS(+) (Stratagene, La Jolla, CA), and clones containing the *csmJ* gene were isolated by colony hybridization (*16*). This DNA fragment was completely sequenced on both strands, and the nucleotide sequence has been deposited in GenBank under accession number AF060077. Based upon the N-terminal amino acid sequence of the CsmI protein (*8*), a 128-fold degenerate oligonucleotide with the sequence 5′ AT(T/C)AT(T/C)AA-(T/C)GA (T/C)AA(A/G)ACNGC 3′ was synthesized as the hybridization probe. Genomic Southern blot-hybridization experiments revealed that this oligonucleotide hybridized to a 0.84 kb *Hin*dIII fragment, which was cloned in plasmid pBluescript KS(+). However, sequence analysis revealed that the complete *csmI* gene was not encoded on this DNA fragment. Thus, an overlapping 4.4 kb *Eco*RI−*Bam*HI fragment was cloned (see Figure 1). The complete coding region (a total of 1.18 kb) of the *csmI* gene was determined on both strands, and these data have been deposited in GenBank under accession number AF060079.

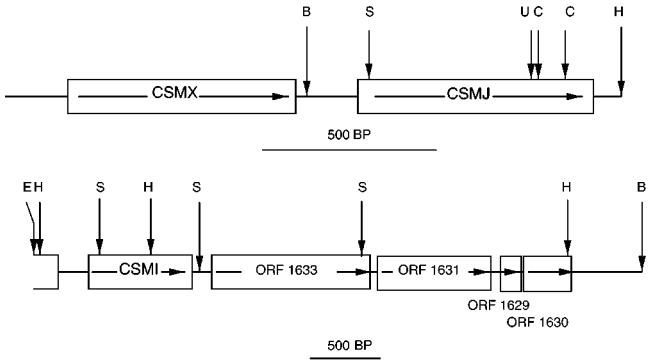

FIGURE 1: Restriction maps of the regions encoding the *csmX*, *csmJ*, and *csmI* genes of *C. tepidum*. Abbreviations for restriction enzymes: B, *Bam*HI; C, *Hin*cII; E, *Eco*RI; H, *Hin*dIII; S, *Sty*I; U, *Stu*I.

For overproduction of the CsmI and CsmJ proteins in *E. coli*, the corresponding genes were modified by mutagenic PCR to contain a *Bsp*HI site at the start codon and a *Bam*HI site downstream from the termination codon for *csmJ* or a *Blp*I site overlapping the stop codon for *csmI*. After digestion with the appropriate restriction enzymes, the PCR products were ligated to the corresponding sites in plasmid pET3d (*17*) to produce plasmids pET3d::*csmI* and pET3d::*csmJ*. The final constructs were resequenced to ensure the correctness of the PCR amplification. The resulting plasmids were transformed into *E. coli* strain BL21 (DE3), and a single colony was grown at 37 °C in NZCYM medium (*15*) containing 0.1 mg mL^{-1} ampicillin. For protein overproduction, a modified NZCYM medium was used from which the $MgSO_4$ had been omitted and 6 g L^{-1} ferric ammonium citrate was added. Target DNA expression was induced by addition of IPTG (0.5 mM) to the cultures grown at 37 °C in the late exponential phase of growth ($OD_{550\ nm}$ about 0.8). Cells were harvested after 2.5−3 h of induction and stored at −70 °C until required. The cells were disrupted by two passes through a cold French pressure cell at 4 °C at 124 MPa in the presence of 1 mM DTT, 1 mM EDTA, 1 mM PMSF, and 0.1 mg mL^{-1} DNase I (Sigma, St. Louis, MO). The inclusion bodies were recovered by centrifugation of the cell lysate at 18000*g* at 4 °C for 15 min. The resulting pellet was washed 2 times with 2 M urea, 2 mM DTT, and 0.4% (w/v) Triton X-100 for CsmJ inclusion bodies, or with 2 mM DTT and 0.3% (w/v) Triton X-100 for CsmI inclusion bodies. Washed inclusion bodies were stored at −70 °C until

required. The washed inclusion bodies were solubilized with 5.5−6.5 M urea, 300 mM Tris-HCl (pH 8.4 for CsmJ or pH 7.5 for CsmI), 0.5 mM Na-EDTA, 0.5 mM PMSF. Proteins were refolded during gel exclusion chromatography on a Sephadex G-25 column developed with a flow rate of 15 cm h^{-1} at 4 °C. The column was equilibrated with 50 mM Tris-HCl, pH 8.3, 150 mM NaCl, 5 mM DTT buffer that had been purged with nitrogen. Chemical reinsertion of iron−sulfur clusters into apoproteins was performed essentially as described by Li et al. (*18*).

Chlorosome Preparation, Chlorophyll Concentration, and Absorbance Spectroscopy. Chlorosomes were isolated on 7−47% (w/v) continuous sucrose gradients by a modification of the method of Gerola and Olson (*19*) essentially as described by Chung et al. (*8*), except that 0.5−1 mM PMSF and 2 mM DTT were present throughout the entire procedure. Chlorosomes were twice repelleted from the sucrose gradient fraction by ultracentrifugation (240000*g* for 1.5 h) to completely remove sucrose and NaSCN. The Bchl *c* concentration was determined by measuring the absorbance of acetone extracts of the chlorosomes using the specific absorption coefficient 92.6 L $(g \cdot cm)^{-1}$ (*20*).

Protein Analyses. Protein concentrations were estimated using the dye-binding method of Bradford (*21*) with bovine serum albumin as the standard. The protein composition of chlorosomes and the purification of recombinant CsmI and CsmJ were monitored by polyacrylamide gel electrophoresis in the presence of SDS using the Tris−Tricine buffer system described by Schägger and von Jagow (*22*). The stacking gel contained 4% (w/v) acrylamide, and the resolving gel contained 17% (w/v) acrylamide. Proteins were stained with Coomassie blue. Amino-terminal amino acid sequence analyses were performed as previously described (*8*−*10*).

EPR Spectroscopy and Redox Titrations. EPR studies were performed at X-band using a Bruker ECS-106 spectrometer equipped with an ER/4102 ST resonator. Cryogenic conditions were maintained with an Oxford Instruments ESR900 liquid helium cryostat, and temperature was controlled by an ITC4 temperature control unit. The microwave frequency was measured with a Hewlett-Packard 5352B frequency counter.

For the recording of the X-band EPR spectra, chlorosomes were prepared by pelleting the chlorosome fraction isolated from sucrose gradients in 10 mM potassium phosphate, pH 7.2, 150 mM NaCl, 2 mM DTT, 0.5 mM PMSF. Reduced samples were prepared in an anaerobic chamber by addition of 50 μL of a freshly prepared solution of 0.5 M sodium hydrosulfite in 0.5 M CAPS buffer, pH 10.5, to 0.2 mL of the chlorosome sample stored aerobically on ice. Spectra were recorded at 30 K, at a microwave power of 1.26 mW. The EPR spectra of chlorosomes (16 mg of Bchl *c* mL^{-1}), that had been oxidized in the presence of 1 and 20 mM ferricyanide, were recorded at 125 K using chlorosomes that had been stored at −70 °C. Data acquisition parameters were the same as described by van Noort et al. (*23*). The same preparation of chlorosomes was used for a microwave power saturation study of the EPR signal under reducing conditions. The sample was prepared as described above, yielding a final Bchl *c* concentration of 16.4 mg mL^{-1}. For the EPR measurements of recombinant CsmJ and CsmI, inclusion bodies were resuspended in 50 mM CAPS buffer, pH 10.5, 2 mM DTT using a glass−Teflon homogenizer, pelleted by centrifugation, and then resuspended in the same buffer. Reduced samples were prepared as described for chlorosomes.

For the redox titration experiments with EPR detection, chlorosomes were diluted in 50 mM Tris-HCl buffer, pH 8.3, and pelleted by centrifugation at 230000*g* at 5 °C for 1.5 h. The Bchl *c* concentration in the titration mixture was 10.2 and 9.3 mg mL^{-1} in two separate experiments. Iron−sulfur clusters were titrated by addition of 5 μL aliquots of 1 M sodium hydrosulfite or potassium ferricyanide to a cocktail of redox mediators in a suspension of CsmJ inclusion bodies (A) or freshly prepared chlorosomes (B) in an anaerobic chamber at room temperature. The redox mediators used in these two titration experiments were 2,5-dihydroxylbenzoquinone (A, B), indigo creamine (A, B), benzyl viologen (A, B), riboflavin 5′-phosphate (A, B), methyl viologen (A), 1,1′-trimethylene-2,2′-dipyridinium dibromide (A), 2-methyl-1,4-naphthoquinone (B), 5-hydroxy-1,4-naphthoquinone (B), 1,4-naphthoquinone (B), and 2,3′,6-trichloroindophenol sodium salt (B). The ambient potential of the solution was measured using platinum and $Ag/AgCl_2$ electrodes and a high-impedance voltmeter. Aliquots of a sample poised at a given potential were quickly frozen in an EPR tube. Midpoint redox potentials were calculated by fitting the amplitude of the $g = 1.937$ EPR resonance versus the ambient potential of the solution to the Nernst equation for one-electron-transfer per oxidation/reduction process using a nonlinear Marquardt regression algorithm in IgorPro Version 3.14 (Wavemetrics, Inc.). The EPR measurements for the titration experiments were made at 30 K at a microwave power of 1.26 mW and modulation amplitude of 1 mT.

RESULTS

Cloning and Sequence Analysis of the csmI and csmJ Genes. Chung et al. (*8*) showed that chlorosomes of *C. tepidum* contained 10 polypeptides, and N-terminal sequences were determined for 9 of these polypeptides (Csm proteins A, B, C, D, E, F, G, H, and I). Additional SDS−PAGE electrophoretic analyses of chlorosome proteins revealed that the 26 kDa region actually contained at least two polypeptides. The N-terminal amino acid sequence of one of these polypeptides, denoted CsmH, matched that originally detected and sequenced by Chung et al. (*8*). However, the second polypeptide, denoted CsmJ, was found to have the N-terminal sequence: MIIYINDKPCNAKVGDLLLNTAKLNWAHIG. This sequence was obviously related to that of the 27 kDa protein denoted CsmI (see below and Figure 2). Using degenerate oligonucleotides as hybridization probes, the genes encoding chlorosome proteins CsmI and CsmJ were cloned and completely sequenced as described under Materials and Methods (see Figure 1).

The *csmJ* gene encodes a protein of 225 amino acids with a mass of 23 894 Da and a predicted isoelectric point of 9.31. The deduced amino acid sequence exactly matches the experimentally determined N-terminal sequence with only one exception: the nucleotide sequence predicts residue 26 to be lysine rather than tryptophan. The start codon for *csmJ* is not preceded by a strong ribosome binding sequence; however, both the *csmJ* and *csmI* genes are preceded by the sequence 5′ AATGAAAA 3′, which occurs 10−12 residues upstream from the start codon.

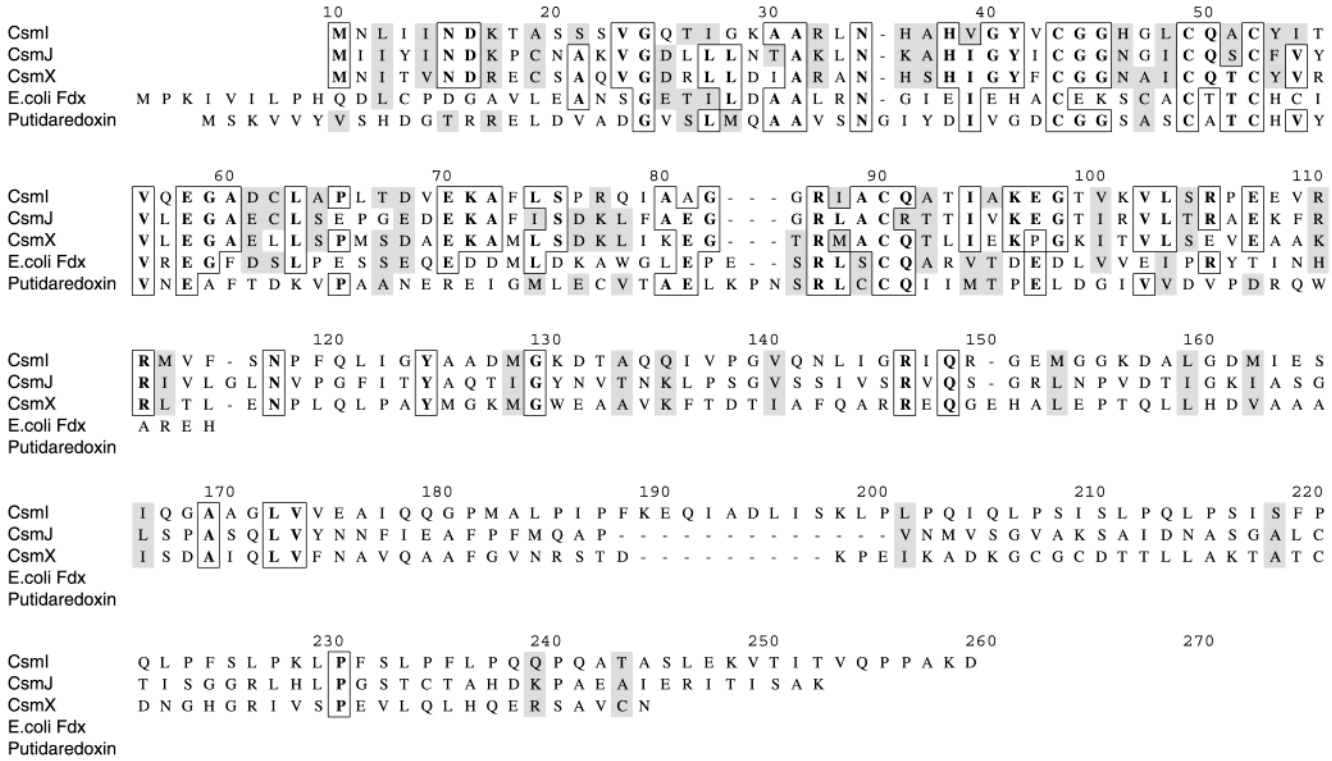

FIGURE 2: Multiple sequence alignment for CsmI, CsmJ, CsmX, putidaredoxin (*26*), and *E. coli* 2Fe-2S ferredoxin (*25*). Boxed residues are identical in three or more of the sequences, and shaded residues indicate conservative replacements. The sequence alignment was generated with ClustalW. The cysteines expected to participate in ligating the 2Fe-2S cluster occur at positions 43, 49, 52, and 90.

The *csmI* gene encodes a protein of 244 amino acids with a mass of 25 909 Da and a predicted isoelectric point of 8.26. The deduced sequence matches the N-terminal amino acid sequence determined by Chung et al. (*8*) except at residues 13 and 33; the deduced amino acid sequence shows these residues to be serine and cysteine, respectively, rather than threonine and isoleucine. Upstream from *csmI*, the 3′ portion of a gene encoding a conserved hypothetical protein with similarity to open reading frames found in a variety of eubacteria was observed (see Figure 1). Immediately downstream from the *csmI* gene is an inverted repeat sequence followed by a run of five T residues. This sequence, which has the potential to form a G+C-rich stem−loop structure, strongly resembles Rho-independent transcription termination signals of *E. coli* and probably functions both as a transcription termination signal and as an mRNA stabilizing element (*24*).

Using the *csmI* and *csmJ* sequences as queries, searches of the partial sequence database for *C. tepidum* identified a third gene, denoted *csmX*, that is predicted to encode a protein closely related to CsmJ and CsmI (see Figure 2). The *csmX* gene is found immediately upstream from the *csmJ* gene; a spacer region of 173 bp occurs between the stop codon of *csmX* and the start codon of *csmJ* (see Figure 1). The *csmX* gene predicts a protein of 221 amino acids with a mass of 23 969 Da, and although CsmI and CsmJ have rather basic isoelectric points, CsmX is predicted to have an acidic isoelectric point (pI = 5.64). Results demonstrating that CsmX is localized in the chlorosomes will be presented elsewhere (E. Vassilieva and D. A. Bryant, unpublished experiments).

Figure 2 shows a multiple sequence alignment of CsmI, CsmJ, and CsmX as well as the [2Fe-2S] ferredoxin of *E. coli* (*25*) and the [2Fe-2S] putidaredoxin of *Pseudomonas putida* (*26*). The sequence similarity among CsmI, CsmJ, and CsmX is very high up to about the 100th residue at their amino termini. CsmI and CsmJ are about 50% identical and additionally are about 30% identical to *E. coli* ferredoxin in this same region. CsmJ and CsmX are 55% identical and 70% similar in this same region. Four cysteine residues, at positions 43, 49, 52, and 90 according to the numbering scheme in Figure 2, are conserved in all five proteins and likely represent the expected four ligands to the [2Fe-2S] clusters of CsmI and CsmJ (see below). The cysteine at position 62, which is found in both CsmI and CsmJ, is not conserved in CsmX; therefore, participation of Cys62 in the ligation of the [2Fe-2S] cluster is unlikely. These observations support the assignment of the conserved Cys at residue 90 as the most likely fourth ligand for the [2Fe-2S] cluster. CsmI, CsmJ, and CsmX show much more limited sequence identity (about 23%) in their carboxy-terminal regions. An interesting feature of the CsmI protein is the occurrence of an amino acid motif of seven residues that is repeated 4 times near the carboxy terminus of the protein. The CsmI protein contains a somewhat higher proportion of nonpolar amino acids than CsmJ (49.4% vs 43.1%). Hydropathy analyses indicated that neither CsmI nor CsmJ contains long, hydrophobic sequences of amino acids typical of integral membrane proteins found in lipid bilayer membranes. However, CsmX may contain such a motif, since the sequence from residue 158 to residue 183 is predicted to adopt a helical conformation and has an average relative hydrophilicity of about −1.4 on the Kyte−Doolittle scale.

Overproduction of CsmI and CsmJ in E. coli. E. coli strain BL21 (DE3) cells were transformed with plasmids pET3d::*csmJ* and pET3d::*csmI* for overproduction of proteins CsmJ

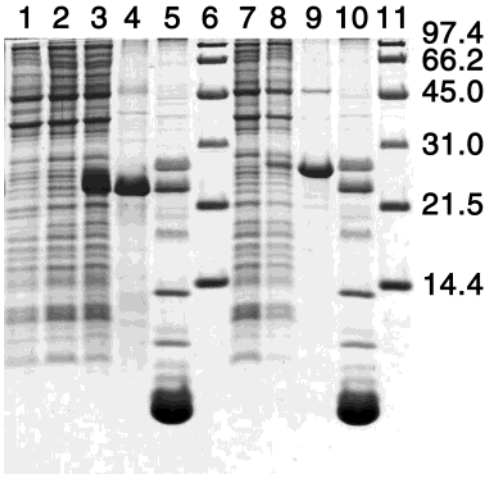

FIGURE 3: Polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate. Lane 1, whole-cell extract of *E. coli* strain BL21 (DE3) harboring control plasmid pET3d 3 h after addition of 0.5 mM IPTG; lane 2, whole-cell extract of *E. coli* strain BL21 (DE3) harboring plasmid pET3d::*csmJ* prior to induction with IPTG; lane 3, whole-cell extract of *E. coli* strain BL21 (DE3) harboring plasmid pET3d*::csmJ* after induction with 0.5 mM IPTG for 4 h; lane 4, washed CsmJ inclusion bodies; lanes 5 and 10, purified chlorosomes from *C. tepidum*; lanes 6 and 11, molecular mass standards; the masses are indicated at the right in kDa; lane 7, uninduced whole-cell extract of *E. coli* strain BL21 (DE3) harboring plasmid pET3d::*csmI*; lane 8, whole-cell extract of *E. coli* strain BL21 (DE3) harboring plasmid pET3d::*csmI* 2.5 h after induction with 0.5 mM IPTG; lane 9, washed inclusion CsmI inclusion bodies.

and CsmI, respectively. Figure 3 shows SDS−PAGE analyses of whole-cell extracts from *E. coli* strain BL21 (DE3) control cells harboring plasmid pET3d 3 h after induction with 0.5 mM IPTG (lane 1) or harboring plasmid pET3d::*csmI* before (lane 2) and after (lane 3) 4 h of induction with 0.5 mM IPTG. The recombinant CsmJ protein was recovered in the form of inclusion bodies (Figure 3, lane 4). Similarly, Figure 3 shows SDS−PAGE analyses of whole-cell extracts from *E. coli* strain BL21 (DE3) cells harboring plasmid pET3d::*csmI* before (lane 7) and after 2.5 h of induction with 0.5 mM IPTG (lane 8). As can be seen by comparing lanes 3 and 8 in Figure 3, the maximal yield of CsmJ (∼10 mg L^{-1}) was significantly greater than that of CsmI (∼3.3 mg L^{-1}). The electrophoretic migration of the recombinant CsmI protein (M_r = 27 000) matched that of the largest polypeptide of isolated chlorosomes (Figure 3, lanes 8−10), while the migration of the recombinant CsmJ protein (M_r = 25 000) matched that of the second largest polypeptide (Figure 3, lanes 4−6). N-Terminal amino acid sequence analysis of each recombinant protein confirmed that the sequences were identical to those determined for these proteins as isolated from chlorosomes. In contrast to results obtained with CsmA and CsmE (*8*, *10*), these observations suggest that no processing of these polypeptides occurs during chlorosome biogenesis.

Consistent with the postulated relationship of the CsmI and CsmJ proteins to the [2Fe-2S] ferredoxin of *E. coli*, the inclusion body fractions for both proteins were brown-colored. Even after unfolding of the inclusion bodies in urea solutions and subsequent refolding during gel exclusion chromatography, the proteins retained a pale pinkish-brown coloration. After removal of the urea, both proteins and especially CsmI were unstable in solution and tended to

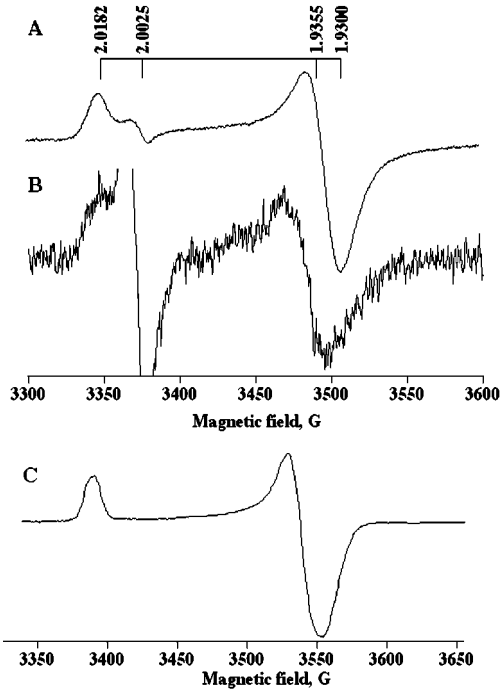

FIGURE 4: EPR spectrum of chemically reduced inclusion bodies of CsmJ (A) and CsmI (B). The inclusion bodies were suspended in 50 mM CAPS buffer, pH 10.5, 2 mM DTT and reduced by addition of sodium hydrosulfite to a final concentration of 50 mM. EPR conditions (A): temperature, 14 K; microwave power, 1.26 mW; microwave frequency, 9.4500 GHz. (B) The spectrum shown is an average of three spectra: 14 K at microwave power 0.1 mW, and 15 and 25 K at microwave power 1.26 mW; microwave frequency, 9.4498 GHz; modulation amplitude, 10 G; magnetic field, 3130−3830 G (3300−3600 G depicted). (C) EPR spectrum of reduced ferredoxin from *E. coli* measured at a microwave frequency 9.6140 GHz and temperature of 10.2 K, redrawn from the data of Ta and Vickery (*25*).

precipitate. The recombinant CsmJ protein, at concentrations up to 4 mg mL^{-1}, could be stored on ice without precipitation for a few days in 50 mM Tris-HCl, pH 8.3, 150 mM NaCl, 5 mM DTT buffer that had been purged with nitrogen. However, at room temperature, very rapid precipitation of the protein occurred from such solutions. The recombinant CsmI protein was much less soluble and adsorbed as a brown-colored film on the walls of plastic test tubes even from solutions that contained 6.4 M urea.

EPR Properties of the Recombinant CsmJ and CsmI Proteins. Figure 4A shows the X-band EPR spectrum of inclusion bodies of the recombinant CsmJ protein reduced at pH 10.5 in the presence of sodium hydrosulfite. The chemically reduced sample exhibits a nearly axial EPR spectrum with principal *g*-values of 2.018, 1.936, and 1.930 (g_{av} of 1.961) and line widths of 12, 18, and 22 G as determined by numerical simulation. A derivative-shaped EPR feature with a *g*-value of 2.0025 and a line width of ca. 11 G, as determined by visual inspection, was also observed. The *g*-values and line widths of the nearly axial spectrum are similar to those of the [2Fe-2S] ferredoxin isolated from *E. coli* (see Figure 4C). The derivative-shaped spectrum is most likely derived from an organic radical of unknown origin. A matrix of temperature and microwave power levels was carried out to ensure that signal intensities were determined in the Curie law region, i.e., that the doubly integrated signal intensity is proportional to the square root

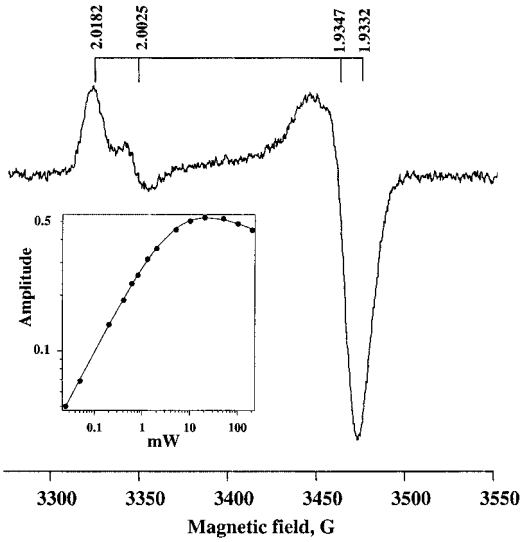

FIGURE 5: EPR spectrum of purified chlorosomes from *C. tepidum*. The chlorosomes were diluted in 100 mM CAPS buffer, pH 10.5, 1 mM DTT, to a final Bchl *c* concentration of 11.4 mg mL^{-1} and reduced by addition of sodium hydrosulfite to a final concentration 50 mM. EPR conditions: temperature 30 K, microwave power 1.26 mW, microwave frequency 9.3837 GHz. The inset shows the relative amplitude of the $g = 1.933$ resonance as a function of microwave power. The $P_{1/2}$ is calculated to be 5 mW at 30 K.

of the microwave power. Double integration of simulated spectra under nonsaturating conditions of microwave power shows that the ratio of spins for the nearly axial iron−sulfur spectrum relative to the organic radical species was ca. 110:1. The EPR spectrum of the CsmJ protein after resolubilization of the inclusion bodies in urea and chemical reinsertion of the iron−sulfur clusters (*18*) was identical to the nearly axial spectrum depicted in Figure 4A. Because of the limited solubility of the CsmI protein, it was only possible to obtain EPR spectra of inclusion bodies of this protein (Figure 4B). Although the S/N is low, the *g*-tensors (i.e., the shape and the measured *g*-values and line widths) are similar to those of the CsmJ protein (Figure 4B, solid line). However, the ratio of spins of the spectrum of the iron−sulfur cluster relative to the organic radical species was much lower (about 7:1). The signal intensity of the nearly axial iron−sulfur spectrum of CsmJ was maximal at 30 K, but the resonances were still detectable, although considerably broadened, at temperatures as high as 100 K. Since EPR signals from [4Fe-4S] clusters are rarely detectable at temperatures above 40 K, the g_{av} and the relaxation properties provide strong support for the proposal that the CsmJ protein ligates a [2Fe-2S] cluster.

EPR Properties of Isolated Chlorosomes. The demonstration that the recombinant CsmI and CsmJ proteins ligate [2Fe-2S] clusters prompted a search for the presence of intact iron−sulfur proteins in isolated chlorosomes. Figure 5 shows the EPR spectrum of highly purified chlorosomes from *C. tepidum* under strongly reducing conditions, and the inset shows the power saturation profile at the temperature optimum of 30 K. The chemically reduced chlorosomes demonstrate a nearly axial EPR spectrum, with principal *g*-values of 2.018, 1.935, and 1.933 (g_{av} of 1.962) and line widths of 11, 18, and 19 G, which is virtually identical to that found for the recombinant CsmI and CsmJ. The $P_{1/2}$ of 5 mW (Figure 5, inset) is consistent with the identification

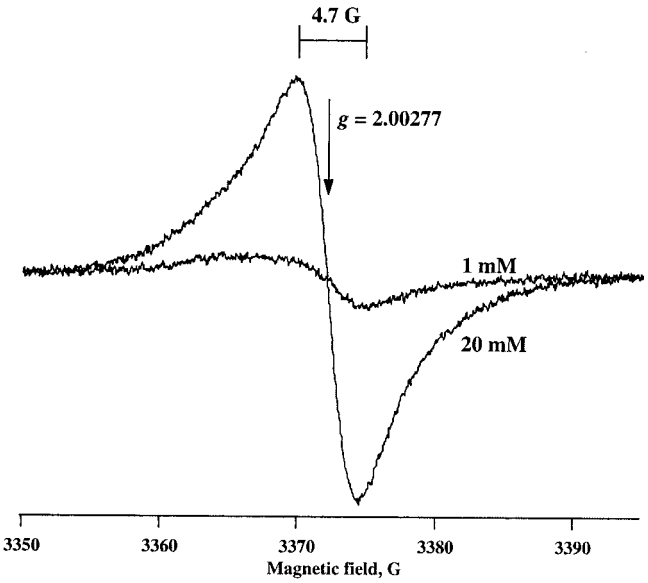

FIGURE 6: EPR spectra of *C. tepidum* chlorosomes, 16 mg of Bchl *c* mL^{-1}, in phosphate buffer, in the presence of 1 and 20 mM ferricyanide at 125 K. Data acquisition parameters were the same as specified by van Noort et al. (*23*): temperature 125 K, microwave power 2.0 mW, modulation frequency 0.9 GHz.

of an iron−sulfur cluster. This characteristic EPR spectrum was obtained for chlorosomes which had been isolated and stored under aerobic conditions or under anaerobic conditions. These results suggest that the [2Fe-2S] clusters found in chlorosomes are not sensitive to the strong chaotrope sodium isothiocyanate (used in the isolation procedure) or to exposure to oxygen. Finally, treatment of chlorosomes with proteases, followed by reisolation of the treated chlorosomes by centrifugation, did not cause any substantial modification of the EPR spectrum of the isolated chlorosomes (data not shown).

van Noort et al. (*23*) previously reported that chlorosomes of *C. tepidum* exhibit a derivative-shaped, narrow EPR spectrum under oxidizing conditions. Figure 6 shows the EPR spectrum of purified chlorosomes from *C. tepidum* after oxidation with sodium ferricyanide. The observed EPR spectrum is virtually identical to that reported earlier (*23*) and is obviously unrelated to the spectra of the iron−sulfur clusters described here.

Midpoint Potentials of the Iron−Sulfur Clusters in Recombinant CsmJ and Isolated Chlorosomes. A redox titration of the [2Fe-2S] cluster found in the CsmJ inclusion bodies was performed, and the midpoint potential of this cluster was found to be −194 mV vs SHE (Figure 7A). Redox titration of the iron−sulfur clusters found in isolated chlorosomes yielded two midpoint potential values: $E_{m1} = -201$ mV and $E_{m2} = +92$ mV vs SHE (Figure 7B). The values obtained for CsmJ and E_{m1} for isolated chlorosomes are identical within experimental error. This observation suggests that the redox group responsible for E_{m1} in chlorosomes may belong to CsmJ. The very limited solubility of CsmI and the low signal-to-noise ratio of the EPR spectra for CsmI inclusion bodies did not allow titration of the [2Fe-2S] cluster associated with this protein. The +92 mV midpoint potential observed in chlorosomes must belong to either CsmI or CsmX, since no other chlorosome protein contains cysteine-containing motifs that could ligate an iron−sulfur cluster (*8*−*10*, *13*).

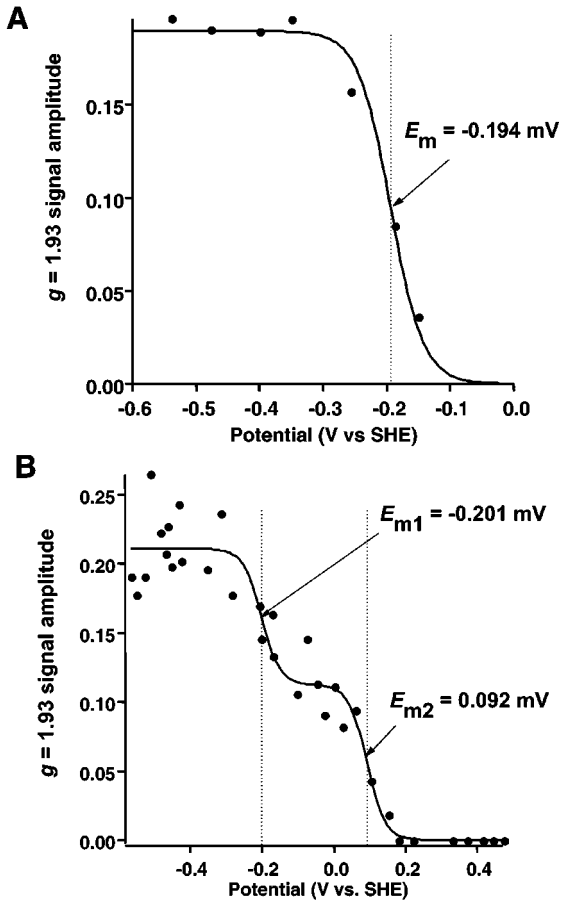

FIGURE 7: Potentiometric titration of CsmJ inclusion bodies (A) and isolated chlorosomes (B). The amplitude of the $g = 1.93$ EPR signal has been plotted against the potential of the solution as measured against the standard hydrogen electrode.

DISCUSSION

Chlorosomes of *C. tepidum* have now been shown to contain 10 polypeptides, and the genes encoding these proteins have been cloned and characterized (*8*−*10, 13*; C. Jakobs, E. Vassilieva, V. L. Stirewalt, and D. A. Bryant, unpublished experiments). Unlike other chlorosome proteins except CsmX, both CsmI and CsmJ have amino-terminal domains with strong sequence similarity to a subfamily of ferredoxins that contain [2Fe-2S] clusters. The data obtained here with recombinant proteins produced in *E. coli* demonstrate that these proteins are indeed iron−sulfur proteins, and the properties of the [2Fe-2S] clusters in the recombinant proteins are similar to those of the iron−sulfur clusters detected in isolated chlorosomes.

Two major classes of [2Fe-2S] ferredoxins have been described, and these classes have been established based upon the spacing of the ligating cysteine residues and the EPR properties of their iron−sulfur clusters (*27, 28*). The first class, exemplified by the soluble ferredoxin of spinach chloroplasts, contains the conserved motif C-X_4-C-X_2-C-X_{29}-C. These proteins, which usually function as secondary electron carriers, are broadly distributed in nature and are found in plants, eubacteria, and archaea. Most ferredoxins of this group are acidic proteins with molecular masses of less than 16 kDa. These proteins typically have extremely negative midpoint potentials (around −300 to −450 mV), and their reduced clusters exhibit characteristic, rhombic EPR spectra with g_{av} around 1.96 (*29*).

CsmJ	**CGGNGICQSCF-X_{33}-C**
CsmI	**CGGHGLCQACY-X_{33}-C**
CsmX	**CGGNAICQTCY-X_{33}-C**
Putidaredoxin	**CGGSASCATCH-X_{36}-C**
R. capsulatus Fdx VI	**CGGACACSTCH-X_{36}-C**
E. coli Fdx	**CEKSCACTTCH-X_{34}-C**
Adrenodoxin	**CEGTLACSTCH-X_{35}-C**
Consensus	**CggxxxCxtCx-X_{33-36}-C**

FIGURE 8: Sequence alignment of the cysteine motif implicated in binding the 2Fe-2S clusters in CsmJ, CsmI, CsmX, putidaredoxin (*26*), *E. coli* ferredoxin (*25*), *R. capsulatus* Fdx VI (*31*), and human adrenodoxin (*30*).

The second major class of [2Fe-2S] ferredoxins includes vertebrate ferredoxins (bovine adrenodoxin, chicken adrenodoxin, human ferredoxin/adrenodoxin) (*30*), as well as the ferredoxin of *E. coli* (*25*), ferredoxin VI of *Rhodobacter capsulatus* (*31*), and putidaredoxin of *P. putida* (*26*). These proteins share the cluster binding motif C-X_5-C-X_2-C-X_{35-37}-C (see Figure 8). This motif is identical to that found in CsmI, CsmJ, and CsmX except that the chlorosome proteins only have 34 residues between the third and fourth cluster-liganding cysteine residues (see Figure 8). In addition to the conserved cysteine motif, a pair of glycine residues is found between the first and second cysteines of the binding motif in many of the proteins (see Figure 8). The histidine residue that follows the third cysteine of many of the proteins of this subclass is replaced by either phenylalanine or tyrosine in the chlorosome proteins.

Besides the obvious similarity of their cysteine motifs to those found in proteins containing [2Fe-2S] clusters, there are several other lines of evidence that CsmI and CsmJ contain [2Fe-2S] clusters. Under reducing conditions, purified CsmJ in solution, inclusion bodies for both proteins, and highly purified chlorosomes exhibited characteristic, nearly axial EPR spectra with $g_{||} \approx 2.02$ and $g_{\perp} \approx 1.94$. Virtually identical spectra have been obtained for both native and recombinant *E. coli* ferredoxin (*32, 25*), for putidaredoxin (*33*), for bovine (*34*) and human (*35, 36*) adrenodoxin, and for ferredoxin 1 of *Sphyngomonas* sp. RW1 (*37*). All of these ferredoxins, as well as CsmI and CsmJ, yield a high-field feature with $g_{\perp} \approx 1.94$. In contrast, higher plant-type [2Fe-2S] ferredoxins exhibit rhombic EPR spectra with principal *g*-values of approximately 2.05, 1.96, and 1.89 (g_{av} of 1.97) (*38*). In some of the EPR studies presented here, inclusion bodies produced in *E. coli* were used because of the instability of the refolded proteins in solution. Validation of this approach is provided by the observation that the same principal *g*-values were obtained for CsmJ in preparations of inclusion bodies, renatured protein in solution, and isolated chlorosomes. The EPR signals from the iron−sulfur clusters in CsmI, CsmJ, and chlorosomes showed a g_{av} of 1.96, were maximal at about 30 K, and remained visible at temperatures up to 100 K. In contrast, EPR signals from [4Fe-4S] clusters are rarely detectable above about 40 K, and they are typically saturated at a power higher than 100 mW. The midpoint potentials of low-potential [4Fe-4S] clusters are typically in the range −250 to −650 mV (*39*). These properties allow us to conclude that the iron−sulfur clusters in the CsmI and CsmJ proteins are [2Fe-2S] clusters.

Unlike the low-potential clusters of the soluble 2[4Fe-4S] ferredoxins (*40*) and of the membrane-bound reaction centers

(*41*) of *Chlorobium* sp., the [2Fe-2S] clusters in the chlorosome proteins appear to be quite stable. The clusters clearly survive prolonged exposure to both oxygen and 2 M sodium isothiocyanate, and the clusters in inclusion bodies of CsmI and CsmJ were also stable to treatment with 6 M urea. When CsmJ inclusion bodies had been stored on ice for about a month, the sample color changed from brown to gray, suggesting that a loss of the iron−sulfur clusters had occurred. Similar stability properties have been described for other related proteins, including ferredoxin VI of *R. capsulatus* (*31*) and ferredoxin 1 of *Sphingomonas* sp. RW1 (*37*). Redox titration of bovine adrenodoxin in the presence of 4 M urea yielded the same midpoint potential as without urea (−270 mV) when the same mediators were used (*42*). Optical spectroscopic studies of native CsmI and CsmJ in chlorosomes were not possible because of the strong absorption by Bchl and were also complicated for the recombinant proteins due to their instability in solution. However, the absorption spectra of the recombinant CsmJ and CsmI proteins exhibited a broad absorption band in the blue region. Absorption peaks were detected at 412 and 460 nm in oxidized minus dithionite-reduced spectra of CsmJ in 1 M urea (not shown). These changes are similar to changes observed at 324, 414, and 460 nm for the ferredoxin I and ferredoxin VI of *R. capsulatus* (*31*). Although the redox potential measured for the CsmJ inclusion bodies was slightly higher than the typical range of about −240 to −460 mV observed for [2Fe-2S] clusters (*29, 43*), the observed potential of −194 mV is virtually identical to that for putidaredoxin upon binding to P-450$_{cam}$ (*44*). In putidaredoxin, a 43 mV shift of the potential due to the binding implies that the redox behavior of the cluster is affected by environmental factors (*44*−*46*).

In a study of bovine adrenodoxin mutants, it was shown that the hydroxyl group of the threonine residue (T54) preceding the third cysteine of the binding motif (see Figure 8) is essential for cluster stability in this protein (*34, 47*). When T54 was mutated to alanine (the same residue as in native CsmI), bovine adrenodoxin was destabilized, and Fe and S were readily lost. Other than the cluster-ligating cysteines, the hydroxyl-bearing side chain in this position is one of the most highly conserved features of all [2Fe-2S] proteins (*47*). In CsmX tyrosine also precedes the third ligating cysteine, and in CsmJ the side chain of serine can provide a hydroxyl. Since CsmI contains alanine at this position, the low signal-to-noise ratio in EPR spectra of CsmI may reflect an inherent instability of the iron−sulfur cluster in this protein.

EPR studies of highly purified chlorosomes show that iron−sulfur clusters with properties similar to those observed for inclusion bodies of CsmI and CsmJ are also observable in these light-harvesting structures. In previous studies of the EPR properties of isolated chlorosomes, radicals were detected under oxidizing conditions. Such radicals were first reported for chlorosomes of *Cf. aurantiacus* treated with 10 mM ferricyanide, and under these conditions a derivative-shaped EPR signal with *g*-value of 2.0026 and a line width of 2.3 G was observed at 77 K (*48*). The *g*-value and line width of this radical, which has been interpreted as a Bchl *c* radical, have been discussed in terms of its delocalization over a large number of Bchl *c* molecules and as an indication of the number of Bchl monomers that are found in organization units of these molecules within the chlorosome (*49*). More recently, chlorosomes of *C. tepidum* were also investigated by EPR under oxidizing conditions. When chlorosomes were treated with ferricyanide, a derivative-shaped EPR signal was observed with a *g*-value of 2.0025 and line width of 5 G at 125 K (*23*) and with $g = 2.0024$ and a line width of 8 G at 6 K (*50*). This radical was again assigned to oxidized Bchl *c*, because monomeric oxidized Bchl *c* also yields a radical of similar *g*-value. A similar but much smaller signal was detected in chlorosomes at ambient redox potential (about +200 mV), and this signal disappeared after reduction with dithionite (*23*). All of these results were reproduced in this study. None of these EPR signals appear to be related to the axial signals around $g = 1.94$ that are derived from iron−sulfur clusters.

More than 20 years ago, Knaff and Malkin reported that *C. limicola f. thiosulfatophilum* contained two membrane-associated iron−sulfur centers with a *g*-value of 1.94 and midpoint potentials of −25 and −175 mV (*51*). In those studies, EPR spectra and redox titrations were obtained with *Cb. limicola* "chromatophores", which were probably membrane fragments with attached chlorosomes. It is highly likely that the $g = 1.94$ EPR signals detected in that work originated from the [2Fe-2S] clusters associated with CsmI, CsmJ, and CsmX of the *Cb. limicola* chlorosomes. The observed difference between the estimated potential for the E_{m2} in that study and in this one likely arises from the fact that the titration in the earlier study was not carried to sufficiently high potentials to completely oxidize the responsible [2Fe-2S] cluster. Alternatively, it is possible that the potentials are somewhat different due to differences between the two species studied. Since *Chlorobium* sp. fix carbon dioxide by the reverse TCA cycle reactions (*52*), the intracellular midpoint potential in these strictly anaerobic organisms would probably be at least as low as −400 mV to allow the terminal electron acceptors from the reaction centers, the soluble 2[4Fe-4S] bacterial-type ferredoxins, to deliver electrons to the pyruvate synthase and 2-oxoglutarate synthase enzymes of the reverse TCA cycle (*53*). Iron−sulfur clusters with midpoint potentials higher than about −400 mV should therefore always be in the reduced state, unless they are structurally compartmentalized in the cell or unless the rate of oxidation of these clusters greatly exceeds their rate of reduction. The latter situation is not impossible, and it should be noted that, during the redox titrations, longer times were required for redox equilibration after dithionite addition to chlorosome preparations than for the CsmJ inclusion bodies.

Immunoprecipitation experiments with polyclonal antibodies raised against the recombinant CsmI and CsmJ proteins effectively agglutinate isolated chlorosomes (E. Vassilieva and D. A. Bryant, unpublished experiments). This provides strong evidence that at least some portions of these proteins are exposed to solvent. Moreover, the CsmI and CsmJ proteins can be effectively extracted by detergent treatments that leave most of the Bchl *c* aggregates intact (*54*). The presence in the chlorosome envelope of these two proteins that contain redox centers begs the question of their potential function(s). The relatively high redox potentials for these [2Fe-2S] proteins suggest that they probably do not play a role in the transfer of electrons from the reaction center acceptors to the soluble 2[4Fe-4S] bacterial ferredoxins that

are required for the reductive steps of the reverse TCA cycle. One possibility is that the iron−sulfur clusters associated with CsmI, CsmJ, and CsmX play some role in cyclic electron transport. The midpoint potential of CsmJ is more reducing, while that tentatively assigned to either CsmI or CsmX is more oxidizing, than the midpoint potential for menaquinone, which serves as the mobile electron carrier in the membranes of *Chlorobium* sp. (*55*). However, since chlorosomes are probably not in direct contact with the cytoplasmic membrane, but rather make contact with the membrane surface via an intervening layer of the Bchl *a*-binding FMO protein, it is not clear how these potential electron transport centers would function in any cyclic electron transport scheme unless additional, intervening electron transport proteins were also employed.

The most likely function for the redox centers in CsmI, CsmJ, and presumably CsmX is that these iron−sulfur proteins play a role in the control of excitation energy transfer from the chlorosomes to the reaction center under conditions of oxidative stress. In the antenna system of green sulfur bacteria, but not in the chlorosomes of the green filamentous bacterium *Cf. aurantiacus*, redox potential appears to regulate the efficiency of energy transfer in both the chlorosome and the FMO protein (*2*). This phenomenon apparently involves a direct chemical titration of redox-active groups in the chlorosome antenna, and the effect has been observed in whole cells, isolated membranes, and purified chlorosomes. When in its oxidized form, the redox-active species efficiently quenches excited states in the antenna system and thereby reduces the overall energy transfer efficiency from nearly 100% to less than 10%. Redox titrations of the fluorescence quenching of isolated chlorosomes gave a midpoint potential of −146 mV at pH 7.0 with a pH dependence of −59 mV per pH unit (*56*, *57*). It has been proposed that quenchers are formed in chlorosomes at high redox potentials and that these quenchers provide a very rapid nonradiative decay pathway that competes with energy transfer from Bchl *c* to Bchl *a* (*57*, *58*).

Chlorosomes of both *C. tepidum* and *Cf. aurantiacus* contain menaquinone, but chlorosomes of *C. tepidum* additionally contain chlorobiumquinone at a high molar ratio to Bchl *c* (Bchl *c*:chlorobiumquinone = 10:1.0) (*6*, *59*). Based upon these and other observations, it has been proposed that oxidized chlorobiumquinone is the quencher of chlorosome energy transfer (*58*, *59*). The midpoint potential of chlorobiumquinone is about +35 mV (*55*), and the semiquinone form of chlorobiumquinone could have a midpoint potential close to the −146 mV potential measured by Blankenship and co-workers for the quenching species (*57*). These redox potentials lie between those determined here and suggest that the iron−sulfur proteins of the chlorosome envelope could play a role in the oxidation or reduction of the redox-active quencher, perhaps chlorobiumquinone.

Although green sulfur bacteria are obligate anaerobic photoautotrophs, it is possible that these organisms encounter oxic conditions in their natural growth habitats. Evidence that this may be the case is provided by the observations that *Chlorobium* sp. contains superoxide dismutase, an alkylhydroperoxide reductase, and a cytochrome *bd*-type quinol oxidase (*60*; K. Ketchum, J. Eisen, and D. A. Bryant, unpublished observations). Reactive oxygen species are the basis of O_2 toxicity in all living organisms, and such species are typically formed by the reaction of O_2 with cellular reductants including reduced iron−sulfur proteins, reduced flavoproteins, and thiols. Since highly reducing ferredoxins are abundant proteins in *Chlorobium* sp. cells, an effective means of protecting cells against reactive oxygen species would be to decrease the rate of reduction of these iron−sulfur proteins. Because the iron−sulfur clusters in CsmI and CsmJ are stable in the presence of oxygen, and because the EPR properties of chlorosomes maintained under anaerobic conditions were identical to those for chlorosomes prepared in the presence of oxygen, these proteins are not expected to be targets for cluster interconversion and oxidation-induced conformational changes of the type observed for the transcriptional activator protein FNR (*61*). However, the chlorosomal iron−sulfur proteins could play roles in sensing the redox status of the cell in a manner similar to that observed for the transcriptional activator SoxR of *E. coli* (*62*). Finally, as noted above, CsmI, CsmJ, and/or CsmX could play a direct role in the oxidation or reduction of the quenching species formed in the chlorosome. Since *Chlorobium* sp. can be manipulated genetically (*63*), future work will be directed toward establishing the roles of CsmI, CsmJ, and CsmX through insertional inactivation and site-directed mutagenesis approaches.

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