Molecular Contacts for Chlorosome Envelope Proteins Revealed by Cross-Linking Studies with Chlorosomes from *Chlorobium tepidum*†

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ABSTRACT: Chlorosomes are unique light-harvesting antennae found in two phyla of green bacteria: *Chlorobi* and *Chloroflexi*. In the green sulfur bacterium *Chlorobium tepidum*, 10 proteins (CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, and CsmX) exist in the chlorosome envelope. Chlorosomes from the wild type and mutants lacking a single chlorosome protein were cross-linked with the zero-length cross-linker 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and analyzed by gel electrophoresis. Similar cross-linking products were observed when the time and temperature were varied or when EDC was replaced with glutaraldehyde. Specific interactions between chlorosome proteins in cross-linked products were identified by immunoblotting with polyclonal antibodies raised against recombinant chlorosome proteins. We confirmed these interactions by demonstrating that these products were missing in appropriate mutants. Confirming the location of CsmA in the paracrystalline baseplate, cross-linking showed that CsmA forms dimers, trimers, and homomultimers as large as dodecamers and that CsmA directly interacts with the Fenna−Matthews−Olson protein. Cross-linking further suggests that the precursor form of CsmA is inserted near the edges of the baseplate, where CsmA and pre-CsmA interact with CsmB and CsmF. Several chlorosome proteins, including CsmA, CsmC, CsmD, CsmH, CsmI, CsmJ, and CsmX, were shown to exist as homomultimers in the chlorosome envelope. On the basis of the structural information obtained from these cross-linking experiments, a model for the locations and interactions of the proteins of the chlorosome envelope is proposed.

Photosynthetic organisms have evolved many different light-harvesting systems whose function is to absorb light energy and to transmit that energy efficiently to photosynthetic reaction centers. Examples include the phycobilisomes of cyanobacteria and red algae, the light-harvesting I and II, caroteno-bacteriochlorophyll (BChl)[1] proteins of purple bacteria, the peridinin-chlorophyll proteins of dinoflagellates, and the caroteno-chlorophyll (Chl) *a* and *b* proteins of higher plants (*1*). The green sulfur bacteria (*Chlorobi*) and filamentous anoxygenic phototrophs (*Chloroflexi*) have evolved unique solutions to light harvesting. These organisms harbor organelles, known as chlorosomes, which can effectively harvest light energy at extraordinarily low light intensities (*2*). Chlorosomes are flattened ellipsoidal structures that contain highly aggregated BChl *c*, *d*, or *e*, a small amount of BChl *a*, carotenoids, quinones, and occasionally wax esters. A protein-stabilized, galactolipid-containing envelope encloses the chlorosome BChls (*2*−*4*). Although chlorosomes do not have strictly defined sizes, they are typically 100−200 nm long, 30−70 nm wide, and 30−40 nm thick (*5*−*7*). Unlike other light-harvesting antenna systems, in which chromophores are rigidly bound to and oriented by proteins, the BChl *c*/*d*/*e* molecules in chlorosomes are organized by intermolecular interactions among the BChl molecules. These pigment−pigment interactions include ligation by the hydroxyl group of one BChl molecule to the magnesium atom of an adjacent BChl and intermolecular hydrogen bonding and hydrophobic interactions (see refs *3* and *4*). As a result, the protein-to-chlorophyll mass ratio of chlorosomes is remarkably low. This greatly reduces the cellular energy costs to build such large arrays of pigments.

Highly purified chlorosomes of the green sulfur bacterium *Chlorobium tepidum* contain 10 polypeptides (Table 1) (*2*). Subcellular localization experiments have shown that all 10 chlorosome proteins are copurified in a constant proportion with BChl *c* and that none of these proteins are associated with other subcellular fractions of cells (*8*). The genes encoding all 10 proteins have been cloned, overexpressed, and used to produce polyclonal antisera against the recombinant proteins (*8*−*12*). The N-terminal domains of CsmI, CsmJ, and CsmX are strongly similar in sequence to adrenodoxin-type [2Fe-2S] ferredoxins (*13*). The remaining

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[1] Abbreviations: BChl, bacteriochlorophyll; Chl, chlorophyll; Csm, chlorosome protein product of a specified gene; CL, *Chlorobium* liquid (medium); DTT, dithiothreitol; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; FmoA, product of the *fmoA* gene, the apoprotein of the Fenna−Matthews−Olson protein; FMO protein, Fenna−Matthews−Olson protein, which is a BChl *a*−FmoA complex; LH, light-harvesting; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate.

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Table 1: Interactions and Locations of Chlorosome Envelope Proteins of *C. tepidum*

protein	mass (kDa)	copies per chlorosome[a]	cross-linking properties[b]	location[b]
CsmA	6.2	~2700	dimers, trimers up to dodecamers; cross-linked to pre-CsmA, CsmB, CsmF, CsmD, FmoA	baseplate; binds BChl *a* and carotenoids
pre-CsmA	8.3	~200	cross-linked to CsmA, CsmB, CsmF	edge of baseplate
CsmB	7.5	~1000	cross-linked to CsmA, pre-CsmA, CsmC, CsmD, CsmE, CsmH, CsmI, CsmJ, CsmX	everywhere except the baseplate
CsmC	14.3	~300	dimers, trimers, and tetramers; cross-linked to CsmB	cytoplasmic surface
CsmD	11.1	~300	dimers and trimers; cross-linked to CsmA and CsmB	cytoplasmic surface
CsmE	7.5	~200	cross-linked to CsmB	no evidence
CsmF	7.7	~200	cross-linked to CsmA and pre-CsmA	edge of the baseplate
CsmH	21.8	~100	cross-linked to CsmB and probably CsmA	cytoplasmic surface
CsmI	25.9	~100	cross-linked to CsmJ and CsmB	cytoplasmic surface
CsmJ	23.9	~100	cross-linked to CsmI and CsmB	cytoplasmic surface
CsmX	24.0	~5	cross-linked to CsmB	cytoplasmic surface

[a] Data taken from refs *2*, *13*, and *21*. [b] This work.

chlorosome proteins can be divided into three structural motif families: CsmA/E, CsmB/F, and CsmC/D; none of these chlorosome protein families share any significant sequence similarity with other proteins in the databases. CsmA, the smallest and most abundant chlorosome protein, covers roughly one-third of the surface of the chlorosome and forms multimeric, paracrystalline arrays (*14*, *15*). Protein extraction studies using detergents strongly suggest that CsmA binds BChl *a* in *Chlorobium*-type chlorosomes (*14*), and quantitative estimates suggest that CsmA binds a single BChl *a* molecule and one or more carotenoids (*14*, *16*). Both CsmA and CsmE (and possibly CsmJ) are produced as pre-proteins with carboxyl-terminal extensions of ~20 residues, which are proteolytically removed during chlorosome biogenesis (*9*, *10*). The C-termini of CsmI, CsmJ, and CsmX are distantly related to CsmA/E. The C-terminus of CsmH is similar to that of CsmC/D, while its N-terminus is most closely related to that of CsmB/F (*8*, *17*).

Antisera to all chlorosome proteins except CsmF are capable of agglutinating and precipitating isolated chlorosomes, confirming that some portion of these proteins (except CsmF) is exposed to the medium in suspensions of isolated chlorosomes (*8*, *11*, *12*). Protease susceptibility mapping also suggests that chlorosome proteins are exposed on the envelope surface to various extents (*11*, *12*). Protease digestion and mass spectrometry have been used to show that the N-terminus of CsmA is buried in the envelope while the C-terminus is exposed to proteases (*18*). When isolated chlorosomes are treated with detergents, the integrity of the chlorosome envelope is disrupted, chlorosome proteins become more sensitive to proteases, and chlorosome proteins are released from the envelope (*14*, *19*). CsmC, CsmD, and CsmH are very easily extracted from the chlorosome envelope by incubation with 0.01% (w/v) SDS, and at SDS concentrations of ≥0.1% (w/v), all proteins except CsmA are extracted from the chlorosome envelope. Despite the removal of most of the chlorosome envelope proteins, SDS-treated chlorosomes retain their characteristic size, shape, and spectroscopic features (*14*). "Carotenosomes", vestigial chlorosomes isolated from a *bchK* mutant that cannot synthesize BChl *c*, lack a full complement of chlorosome proteins (*20*). Carotenosomes and chlorosomes contain similar amounts of CsmA, but carotenosomes contain only a small amount of CsmD; trace amounts of CsmB, CsmE, CsmF, and CsmI; and no other chlorosome proteins (*15*, *20*). The CsmA in carotenosomes forms paracrystalline arrays that can be cross-linked in a manner identical to that for the CsmA found in SDS-treated, wild-type chlorosomes (*15*).

Except for the finding that CsmA binds BChl *a* and forms multimers (*14*, *16*), very little information is known about the organization, interactions, and functions of chlorosome envelope proteins. The main goal of the studies reported here was to probe the organization of chlorosome proteins by chemical cross-linking experiments with glutaraldehyde and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). Cross-linked proteins from wild-type chlorosomes and chlorosomes from mutants lacking a single chlorosome protein (*21*) were analyzed by SDS−polyacrylamide gel electrophoresis, and immunoblotting with antibodies against chlorosome proteins was employed to identify the interacting chlorosome proteins. On the basis of the information obtained from these cross-linking experiments, a model is proposed that describes the location, organization, and interactions of the proteins of the chlorosome envelope.

EXPERIMENTAL PROCEDURES

Chlorosome Isolation. The wild-type strain of *C. tepidum* used in these studies is WT2321 (*22*), a plating strain derived from *C. tepidum* strain ATCC 49652 (*23*). Frigaard et al. (*21*) described the construction of nine mutant strains, each lacking a single chlorosome protein except CsmA, and some properties of the chlorosomes of these mutants. The construction of double mutants lacking CsmI and CsmX and lacking CsmJ and CsmX will be described in full elsewhere (*24*). Inoculation of cultures and small-volume growth experiments were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2 (v/v). Large-volume cultures were grown in completely filled and tightly sealed 2.4 L bottles in CL medium at 45−48 °C (*25*). Chlorosomes were isolated by sucrose-gradient ultracentrifugation (*8*). Cells from 2 L cultures were incubated with 3 mg lysozyme/mL for >20 min in 50 mL of isolation buffer [10 mM Tris-HCl (pH 7.5), 2 M NaSCN, 5 mM EDTA, 1 mM PMSF, and 2 mM DTT] and disrupted at 4 °C by being passed three times through a chilled French pressure cell operated at 124 MPa. Cell debris was pelleted by centrifugation at 17000*g* for 20 min, and chlorosomes in the supernatant were then enriched by ultracentrifugation at 220000*g* for 2 h at 4 °C. Resuspended chlorosomes were loaded on the top of

continuous sucrose gradients (from 7 to 47%, w/v) for further purification (ultracentrifugation at 220000*g* for 18 h at 4 °C). The fraction containing chlorosomes was collected, diluted 4-fold with 10 mM potassium phosphate (pH 7.2) and 150 mM NaCl, and centrifuged at 240000*g* for 1.5 h. The resulting pellet was resuspended in the same buffer and pelleted again under the same conditions, and the purified chlorosomes were finally resuspended in ~1 mL of chlorosome isolation buffer containing 1 mM PMSF and 2 mM DTT. Purified chlorosomes were stored at −80 °C until they were required.

Cross-Linking of Chlorosome Proteins. Proteins were cross-linked with glutaraldehyde or 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC). For cross-linking with glutaraldehyde, chlorosome samples were diluted with buffer ~3-fold to a protein concentration of 1 mg/mL. Glutaraldehyde was added from a 2% (w/v) stock to the desired final concentration [from 0.003 to 1.5% (w/v)]. The cross-linking mixtures were shaken at room temperature for 1 h and stored at 4 °C prior to electrophoresis.

EDC can cross-link an amine to a carboxylic acid group at zero distance (i.e., salt-bridged residues are directly cross-linked without the introduction of additional atoms from the cross-linking agent). Chlorosomes were diluted 2-fold in cross-linking buffer {0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid]-NaOH (pH 5.5)} and incubated at room temperature for 10 min. One-ninth volume of 50 mM EDC was added to reach the final concentration of 5 mM, and the chlorosomes were incubated with gentle shaking at room temperature for variable times (from 5 min to 3 h). Cross-linking reactions were stopped by the addition of $^1/_{10}$ volume of 1 M ammonium acetate.

SDS−PAGE and Immunoblotting. The cross-linked protein products were separated by SDS−polyacrylamide gel electrophoresis (*26*) and detected by silver staining or immunoblotting. The stacking gel was 2.6% C and 4 or 6% T, and the resolving gel was 3.3% C and 16 or 8% T depending on the resolution requirements, where T specifies the total concentration of acrylamide and bisacrylamide (w/v) and C specifies the percentage of bisacrylamide (w/w), as defined by Schägger and von Jagow (*26*). Chlorosome samples (60−200 μg of BChl *c*) were extracted with 1.4 mL of acetone at −20 °C overnight, and proteins were collected by centrifugation and dissolved in sample buffer [0.1 M Tris-HCl (pH 6.8), 24% (v/v) glycerol, 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, and 0.02% (w/v) bromophenol blue]. The samples were heated at 50 °C for 5 min or boiled for 1 min before being loaded onto gels. Samples containing ~15 μg of BChl *c* were loaded for silver stain, and samples containing ~60−200 μg of BChl *c* (with or without acetone extraction) were loaded for immunodetection. Gels were stained with silver as described by Blum et al. (*27*). For immunoblotting, proteins were transferred to 0.45 μm nitrocellulose membranes (Schleicher and Schuell, Keene, NH) or 0.45 μm "Immunobilon-P" PVDF membranes (Millipore, Billerica, MA) using a semi-dry transfer cell (Bio-Rad, Richmond, CA). Membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline [50 mM Tris-HCl (pH 8.0) and 100 mM NaCl] and incubated with rabbit antibodies against recombinant chlorosome proteins. The dilutions of the antibodies against chlorosome proteins were as follows: anti-pre-CsmA, 1:2000; anti-CsmB, 1:180; anti-CsmC, 1:1000; anti-CsmD, 1:800; anti-CsmE, 1:180; anti-CsmF, 1:1000; anti-CsmH, 1:3000; anti-CsmI, 1:5000; anti-CsmJ, 1:2500; anti-CsmX, 1:1000. Membranes were later incubated with goat anti-(rabbit IgG) antibodies (Sigma, St. Louis, MO) conjugated with horseradish peroxidase at a dilution of 1:7000. Immunoreactions were detected by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ). It should be noted that the antibodies used to detect CsmA and pre-CsmA were produced with pre-CsmA as the immunizing antigen. These antibodies appear to have their highest titer and avidity for the carboxy terminus of pre-CsmA.

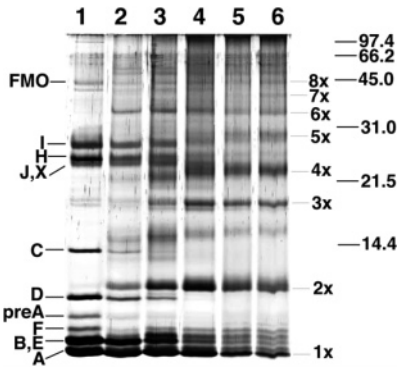

FIGURE 1: SDS−PAGE analysis of wild-type chlorosomes cross-linked with EDC for 1 h at various temperatures. Proteins were separated by SDS−PAGE (16%) and detected by silver staining: lane 1, untreated chlorosomes; and lanes 2−6, chlorosomes cross-linked at 0, 25, 37, 50, and 70 °C, respectively, for 1 h. Chlorosome proteins are identified at the left, and masses of marker proteins are given at the far right. The electrophoretic mobilities of monomers (1×), dimers (2×), trimers (3×), and higher multimers of CsmA are also indicated at the right.

RESULTS

Cross-Linking of Chlorosome Proteins. Figure 1 shows an SDS−PAGE analysis of chlorosome proteins cross-linked for 1 h with 5 mM EDC at temperatures from 0 to 70 °C. As can be seen from the figure, some cross-linking of chlorosome proteins occurred after 1 h at 0 °C, but optimal cross-linking occurred at 37 and 50 °C. Bands corresponding to pre-CsmA, CsmF, CsmC, and CsmD decreased in intensity and were already undetectable after 1 h at 25 °C, while new bands appeared at 12, 16, and 18 kDa. Although trace amounts of CsmI, CsmJ, CsmX, and CsmH can still be observed after cross-linking for 1 h at 50 °C, these proteins could no longer be detected by silver staining after cross-linking for 1 h at 70 °C.

In a previously published study (*14*), chlorosomes were cross-linked with EDC at 25 °C for time intervals ranging from 5 min to 3 h. Interestingly, the results from that study and those shown in Figure 1 can nearly be overlaid, as they exhibit extremely similar patterns with respect to the disappearance of proteins and the appearance of cross-linked products. Only one exception to this conclusion was noted: the cross-linking observed at high temperatures [50 and 70 °C for 1 h (Figure 1, lanes 5 and 6)] was not as complete as when cross-linking was performed for a longer time at a lower temperature (2−3 h at 25 °C). This could indicate that EDC is less stable and decomposes at higher temperatures, or it could indicate that competing side reactions occur more

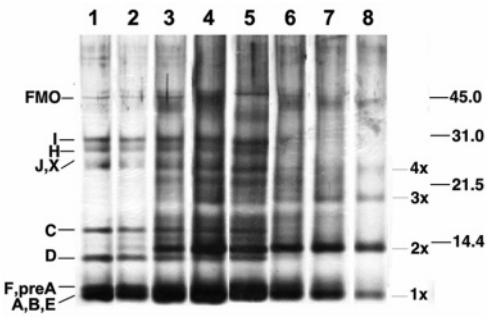

FIGURE 2: Wild-type chlorosomes cross-linked at room temperature for 1 h with various concentrations of glutaraldehyde. Proteins were separated by SDS−PAGE (8%) and detected by silver staining: lane 1, untreated chlorosomes; and lanes 2−8, chlorosomes cross-linked with glutaraldehyde at concentrations of 0.02, 0.04, 0.08, 0.1, 0.2, 0.4, and 0.8% (w/v), respectively. Chlorosome proteins are identified at the left, and masses of marker proteins are given at the far right. The electrophoretic mobilities of monomers (1×), dimers (2×), trimers (3×), and tetramers (4×) of CsmA are also indicated at the right.

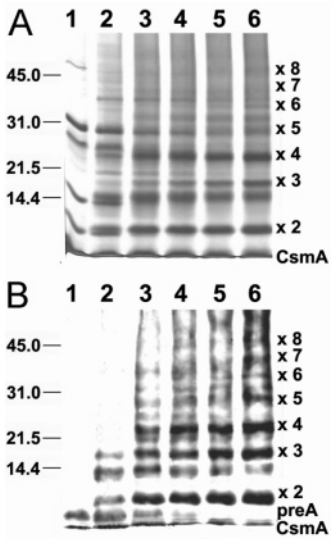

FIGURE 3: SDS−PAGE analysis (A) and immunoblotting (B) of chlorosomes from wild-type *C. tepidum* cross-linked with EDC for various times at room temperature. Proteins and cross-linking products were separated by SDS−PAGE (8%) and detected by silver staining (A) or immunoblotting with anti-pre-CsmA antibodies (B): lane 1, untreated chlorosomes; and lanes 2−6, chlorosomes cross-linked with EDC for 5 min, 0.5 h, 1 h, 2 h, and 3 h, respectively. The positions of molecular mass markers are given at the left, and the mobilities of monomers (CsmA), dimers (2×), trimers (3×), and higher multimers of CsmA are indicated at the right.

readily at higher temperatures (or both). New protein species at 12, 16, 18, 22, and 30 kDa (and even more) appeared in both studies; the pattern of appearance suggested that larger cross-linking products were formed at higher temperatures or at longer times. Interestingly, many of the cross-linking products did not have well-defined sizes, and some species migrated more slowly as time or temperature increased (e.g., the 16 kDa species in Figure 1). The reason for this apparent decrease in electrophoretic mobility could be the formation of intrachain cross-links or an increasing level of chemical modification of the polypeptides. The EDC-derived, cross-linking intermediate *O*-acylisourea can rearrange to form *N*-acylurea derivatives of proteins (*28*), which can have altered electrophoretic properties. For these same reasons, the multiple polypeptide bands around 7 kDa in lanes 5 and 6 of Figure 1 are likely to arise from chemical modification of CsmA and CsmB.

Figure 2 shows the SDS−PAGE analysis of chlorosome envelope proteins cross-linked for 1 h at room temperature with different concentrations of glutaraldehyde [from 0.02 to 0.8% (w/v)]. Although the resolution is not as high as in Figure 1 because of the lower acrylamide concentration used, some of the same general features can be observed. For example, cross-linking products of 12, 16, 18, and 22 kDa are observed with both cross-linking reagents. The CsmA and CsmB protein levels decreased with an increase in glutaraldehyde concentration, and at the highest concentration that was tested [0.8% (w/v)], cross-linking products at 12, 18, and 24 kDa had appeared. Although the results in Figures 1 and 2 were obtained with different chemical reagents, the overall pattern of protein disappearance and appearance of cross-linking products was very similar to those for EDC. Thus, the cross-linking pattern is not dependent upon the chemical nature of the cross-linking reagent but rather appears to depend on the structure, location, and organization of the chlorosome envelope proteins. Because cross-linking with EDC appeared to produce more specific cross-linking and less smearing on SDS−PAGE, only chlorosome proteins cross-linked with EDC were analyzed by immunoblotting.

Organization of CsmA. Previous cross-linking experiments using EDC with SDS-treated chlorosomes (*14*) or carotenosomes (*15*) provided strong evidence that CsmA forms homomultimers that comprise the paracrystalline baseplate substructure of the chlorosome. Figure 3 shows an SDS−PAGE analysis as well as an immunoblot probed with anti-CsmA antibodies for wild-type chlorosomes that had been cross-linked with EDC at room temperature for times ranging from 5 min to 3 h. The pattern of cross-linking products is highly complex due to the presence of both CsmA and pre-CsmA and the many multimers that can be formed from these proteins. Nevertheless, the results clearly demonstrate that CsmA (and pre-CsmA) forms homomultimers even when other chlorosome proteins have not been extracted by SDS treatment or eliminated by mutation.

Figure 4 shows the results of immunoblots from the high-resolution electrophoretic analysis of EDC cross-linking products for several strains as revealed with antibodies against both CsmA and CsmF (Figure 4A). The purpose of these experiments was to identify interactions between CsmA and pre-CsmA as well as the interactions of these two proteins with other low-molecular mass chlorosome proteins. The 14.5 kDa product observed in all chlorosome samples analyzed with anti-CsmA is probably a heterodimer of pre-CsmA and CsmA (Figure 4A, left). Differences could be observed in the cross-linking patterns for some of the mutant chlorosomes. For example, products with apparent masses of 15.7 and 13.6 kDa were missing for chlorosomes from the *csmB* mutant (Figure 4A, left, lane *csmB*), and the 16 and 13.9 kDa products were missing for chlorosomes from the *csmF* mutant (data not shown). The latter products could also be detected by anti-CsmF antibodies, which cross-react with both CsmB and CsmF, after EDC cross-linking of chlorosomes from the wild type or single mutants (Figure 4A, left, lane *csmF*). The observed cross-linking patterns and the products missing from the chlorosomes of the mutants strongly imply that both CsmA and pre-CsmA can be cross-

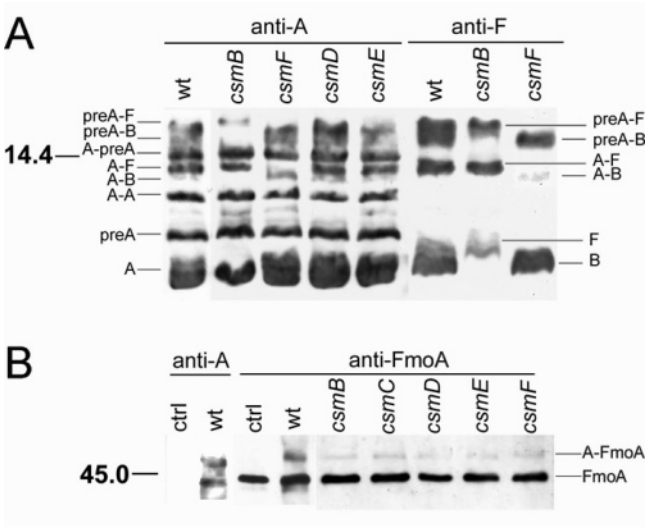

FIGURE 4: Immunoblotting analysis of cross-linking products produced for chlorosomes from the wild type (wt) and the indicated mutants. (A) Chlorosomes from the wild type and multiple mutants were cross-linked with EDC at room temperature for 5 min. Proteins were separated by SDS−PAGE (16%), and the products were detected with antibodies against pre-CsmA (left, anti-A) and CsmF (right, anti-F). (B) Chlorosomes from the wild type and various mutants were cross-linked with EDC at room temperature for 5 min. Proteins were separated by SDS−PAGE (8%), and the products were detected with antibodies to pre-CsmA (anti-A) and the Fenna−Matthews−Olson protein (anti-FmoA). The lanes labeled "ctrl" contained untreated chlorosomes from the wild type. The mobilities of molecular mass markers are indicated at the left, and the identities of various proteins and cross-linking products are given at the left and right in each panel.

linked to the structurally related proteins CsmB and CsmF. Antibodies to CsmA and CsmF also detected a complex pattern of products with masses greater than 20 kDa between CsmA, pre-CsmA, CsmB, and CsmF (data not shown). It was not possible to establish the composition of most of these cross-linking products.

Most chlorosome preparations contain a small amount of contaminating Fenna−Matthews−Olson protein. As shown in Figure 4B, antibodies to FmoA detect a cross-linking product of ~52 kDa in chlorosomes and in mutants lacking CsmB, CsmC, CsmD, CsmE, and CsmF. The size of this product, as well as the detection of a similarly sized product with anti-CsmA antibodies (Figure 4B, left), indicates that CsmA and the contaminating FmoA can be chemically cross-linked with EDC and that these proteins thus interact through salt bridging.

Organization of CsmC and CsmD. Figure 5 shows the EDC-cross-linked products involving CsmC in chlorosomes of the wild type and various mutants. As shown in the left part of Figure 5, products of 29, 43, and 52 kDa were observed for all mutants, suggesting that these products are dimers, trimers, and tetramers of CsmC, respectively, and that no other low-molecular mass chlorosome proteins occur in these multimers. Thus, like CsmA, CsmC forms homomultimers. Products with apparent masses of 22 and 36 kDa were not observed for chlorosomes from the *csmB* mutant but were still detected among the cross-linked products from chlorosomes of the *csmD*, *csmE*, and *csmF* mutants. These data indicate that CsmB interacts with and can be cross-linked to CsmC (Figure 5). Longer exposure of the immunoblots revealed that a product with a mass of ~25 kDa could

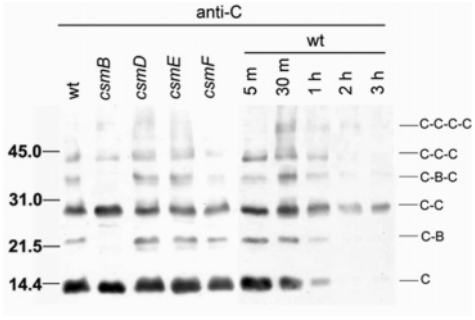

FIGURE 5: Immunoblot analysis of cross-linking products detected with antibodies to CsmC. Chlorosomes from the wild type (wt) and the mutant strains indicated were cross-linked with EDC at room temperature for 5 min (left) or various times (right). Proteins were separated by SDS−PAGE (8%), and the products were detected with antibodies against CsmC (anti-C). The electrophoretic mobilities of molecular mass markers are indicated at the left, and the identities of proteins and cross-linking products are given at the right.

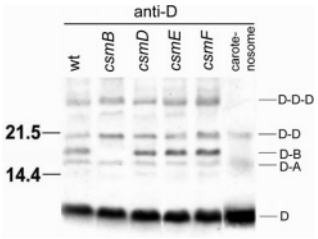

FIGURE 6: Immunoblot analysis of cross-linking products detected with antibodies to CsmD. Chlorosomes from the wild type (wt), the mutant strains, and carotenosomes from the *bchK* mutant were cross-linked with EDC at room temperature. Proteins were separated by SDS−PAGE (8%), and the products were detected with antibodies against CsmD (anti-D). The electrophoretic mobilities of molecular mass markers are indicated at the left, and the identities of proteins and cross-linking products are given at the right.

be detected among the cross-linking products and that this product was specifically missing for the *csmD* mutant (data not shown). Although this product was produced in low yield, it nevertheless suggests that CsmC and CsmD may interact in the envelope.

The cross-linking products for chlorosomes from the wild type and the *csmB*, *csmC*, *csmE*, and *csmF* mutants were also probed with antibodies against CsmD (Figure 6). In addition to CsmD that had not been cross-linked, products at approximately 17, 21.5, and 33 kDa were observed for all chlorosome samples, and the 17 and 21.5 kDa products were also detected in carotenosomes. These data suggest that CsmD forms homomultimers up to at least trimers and that CsmD interacts with CsmA in both chlorosomes and carotenosomes. A product with an estimated mass of ~18 kDa was not detected in the *csmB* mutant; this indicates that CsmD can interact with CsmB to form heterodimers. One interesting property separates CsmD from the other chlorosome proteins. After cross-linking, the samples are usually dissolved in 1.4 mL of acetone to extract pigments (mainly BChl *c*) and pellet the proteins. This step often helps to give better resolution and band shapes during SDS−PAGE and usually did not seem to affect protein amounts. However, without acetone extraction, nearly no CsmD from chlorosomes (less than 10%) could be detected by immunoblotting, even if the samples were boiled for 5 min before being loaded. For CsmD in carotenosomes, acetone extraction was

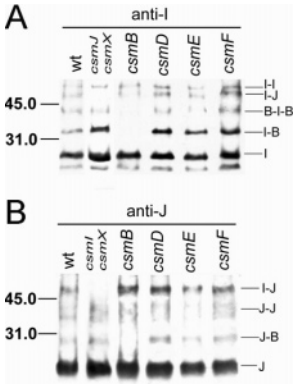

FIGURE 7: Immunoblot analysis of cross-linking products detected with antibodies to CsmI and CsmJ. Chlorosomes from the wild type (wt) and the mutant strains were cross-linked with EDC for 5 min at room temperature. Proteins were separated by SDS−PAGE (8%), and the products were detected with antibodies against CsmI (A, anti-I) and CsmJ (B, anti-J). The electrophoretic mobilities of molecular mass markers are indicated at the left, and the identities of proteins and cross-linking products are given at the right.

not necessary. Thus, it appears that some acetone-soluble, lipophilic substance in the chlorosomes interferes with the electrophoresis of CsmD.

Interactions among CsmI, CsmJ, and CsmB. Figure 7 shows cross-linking products detected with anti-CsmI (Figure 7A) and anti-CsmJ (Figure 7B) antibodies for EDC-cross-linked chlorosomes isolated from the wild type and several mutant strains. An ∼50 kDa cross-linking product was detected for wild-type chlorosomes with both anti-CsmI and anti-CsmJ antibodies; this observation suggests that this product is a heterodimer of CsmI (26 kDa) and CsmJ (24 kDa). This product was not observed after cross-linking of chlorosome proteins of a mutant lacking CsmJ and CsmX, although CsmI homodimers (∼52 kDa) were still detected (Figure 7A). Likewise, the 50 kDa CsmI−CsmJ heterodimer was not observed after cross-linking of chlorosome proteins from a mutant lacking CsmI and CsmX, although a broad product band interpreted to be CsmJ homodimers (and related degradation products) was still detected (Figure 7B). This product is probably smaller than translation of the gene would predict because of C-terminal processing of the polypeptide. In chlorosomes from the *csmB* mutant, the disappearance of ∼33 and ∼40 kDa products detected by anti-CsmI and the disappearance of a ∼29 kDa product detected by anti-CsmJ indicate that CsmB can be cross-linked to both CsmI and CsmJ.

Interactions for CsmE, CsmH, and CsmX. The cross-linking behavior of the remaining chlorosome envelope proteins was also studied. It was very difficult to identify cross-linking products formed with CsmE because of the low specificity and titer of the anti-CsmE antibodies. However, immunoblotting experiments for EDC-cross-linked chlorosomes from the wild-type and mutant strains suggested that a 12 kDa dimer of CsmB and CsmE could be formed (data not shown). Since no other cross-linking products that could be detected with other antibodies were specifically missing from the *csmE* mutant, interactions between CsmE and other proteins may not occur or, more likely, proteins interacting with CsmE do not do so by salt bridges that would allow cross-linking by EDC.

Figure 8 shows the cross-linking results for CsmH for chlorosomes from the wild type and several mutants. The

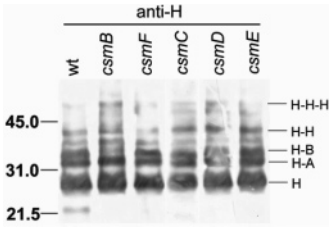

FIGURE 8: Immunoblot analysis of cross-linking products detected with antibodies to CsmH. Chlorosomes from the wild type (wt) and the mutant strains were cross-linked with EDC for 5 min at room temperature. Proteins were separated by SDS−PAGE (8%), and the cross-linked products were detected with antibodies against CsmH (anti-H). The electrophoretic mobilities of molecular mass markers are indicated at the left, and the identities of proteins and cross-linking products are given at the right.

formation of products at ∼22, ∼44, and ∼60 kDa in all chlorosome samples suggests that CsmH can form homomultimers at least as large as trimers. Moreover, the observed cross-linking products strongly suggest that CsmH can be cross-linked to CsmA (32 kDa) as well as CsmB (33 kDa) (Figure 8). Although Frigaard et al. (*21*) had suggested that CsmH might interact with CsmC because CsmH levels are much lower in a *csmC* mutant, no evidence for direct interactions between CsmH and CsmC was observed. Like CsmI and CsmJ, the products of cross-linking of chlorosomes with EDC suggest that CsmX can form homodimers and interact with CsmB to form heterodimers of ∼35 kDa (data not shown).

DISCUSSION

EDC {1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide} belongs to the carbodiimide family of cross-linking reagents, and it promotes covalent bond formation between carboxylate and primary amino groups without the introduction of any atoms from the cross-linker. Hence, EDC is often called a "zero-length" cross-linker. Inter-polypeptide cross-linking with EDC usually occurs when the free carboxylate and amino groups occur on separate polypeptides. First, EDC activates the carboxyl group by forming a highly reactive *O*-acylisourea intermediate. For cross-linking to occur, a primary amino group must be present at an appropriate reaction distance, i.e., within a few angstroms of the activated carboxylate group. A nucleophilic attack by the amino group results in the formation of a new amide bond with complete elimination of the activating moiety (*28*). Competing reactions affecting the efficiency of cross-linking include rearrangement of the activated carboxylate to form of a stable *N*-acylurea derivative and hydrolysis to regenerate the original protein (*28*).

The chlorosome envelope of green sulfur bacteria is an asymmetric membrane containing galactolipids with the galactosyl moieties exposed on the outer surface (*8*, *29*). The farnesyl tails of the BChl *c* molecules within the chlorosome probably comprise the inner leaflet of this membrane. The chlorosome envelope is not as thick, and does not have the same freeze-fracture behavior, as the typical phospholipid bilayer membranes that surround cells (*5*, *6*). Although several studies have concluded that chlorosome proteins are embedded within the chlorosome envelope, the hydrophobicity profiles of the chlorosome proteins provide no evidence

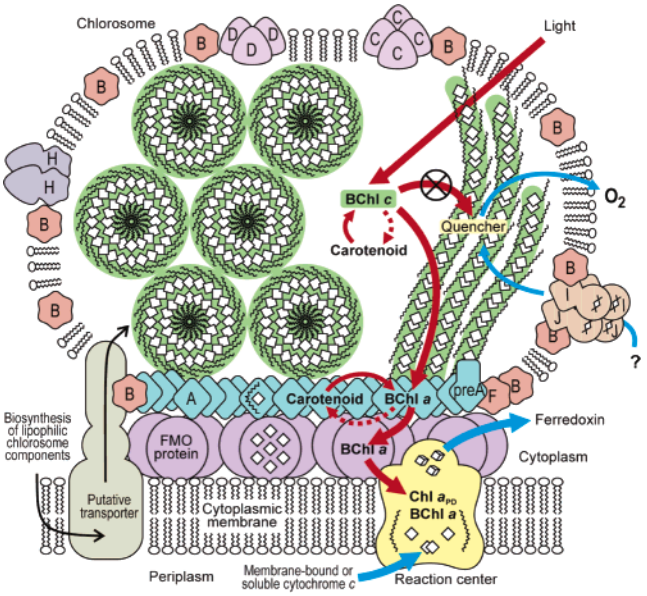

FIGURE 9: Proposed model of protein organization on the chlorosome envelope. BChl *c* aggregates are shown in the rod model (left side) (*35*) and the more recently proposed lamellar model (right side) (*36*). Chlorosome proteins are denoted with single letters (e.g., B represents CsmB). The thick red arrows indicate the paths for excitation energy transfer, and the blue arrows indicate possible electron transfer reactions. The quencher is likely to be chlorobiumquinone. For additional details, see the text and refs *2*−*4*.

for the presence of hydrophobic transmembrane α-helices that are the principal structural element of proteins that are embedded in the cytoplasmic membrane (*11*, *12*).

The chlorosome envelope is a highly unusual structure that might resemble the outer membrane of Gram-negative bacteria more than it resembles the cytoplasmic membrane or other lipid bilayer membranes. The outer leaflet of the outer membrane of Gram-negative bacteria is composed of lipopolysaccharide, while the inner leaflet is composed of phospholipids that are compositionally similar to the cytoplasmic membrane (*30*). In chlorosomes, galactolipids form the outer leaflet of an envelope structure, in which the sugar moieties of the lipids face the solvent (*8*, *29*). It is presumed that the hydrophobic tails of the BChl *c*/*d*/*e* molecules in the interior in the chlorosome assume the role of inner leaflet lipids by interacting with the hydrophobic tails of these galactolipids (see Figure 9). The envelope is likely to be stabilized by the chlorosome proteins, which are embedded in the envelope but do not appear to bind BChl *c*/*d*/*e* molecules. Since chlorosome proteins do not appear in the cytoplasmic membrane and other proteins do not accumulate in the chlorosome envelope (*8*), the protein targeting mechanisms for delivering proteins to the chlorosome envelope and the cytoplasmic membrane must be clearly distinct. The targeting and insertion of proteins into the cytoplasmic membrane of bacteria is a complex, energy-dependent process, and multiple pathways and several protein complexes are devoted to this process (*30*−*32*). Recently, it has been shown that a multiprotein complex in the outer membrane participates in the insertion of β-barrel proteins into the outer membrane, but it is currently believed that no energy source is required (or available) for the assembly of such proteins (*30*). The mechanism by which chlorosome proteins are inserted or spontaneously assemble into the chlorosome envelope is unknown.

Although there are no data demonstrating that galactolipids and proteins within the chlorosome envelope are capable of lateral diffusion, the cross-linking results presented here are generally not consistent with random protein cross-linking produced through protein diffusion. Membrane fluidity normally increases with an increase in temperature (*33*). If collisional cross-linking were occurring, more random products should be produced at higher temperatures and after longer time periods. However, essentially the same patterns of cross-linking were observed at high or low temperatures (Figure 1), after long or short time periods (Figure 3), and using chemically distinct cross-linkers (Figures 1 and 2). For the majority of the proteins, including CsmA, CsmC, CsmD, CsmH, CsmI, CsmJ, and CsmX, homomultimers were among the most predominant cross-linking products (see Table 1). Considering the very low abundance of some of these proteins in the chlorosome envelope (*14*), it is extremely unlikely that homomultimers could be produced by chance. In contrast, homomultimers of the second most abundant protein, CsmB, were not detected. Since CsmB is not believed to form a repeating structure like CsmA, it is difficult to explain why homomultimers were not detected for this very abundant protein if random collisions were responsible for the cross-linking pattern.

CsmA is the most abundant chlorosome protein, comprising nearly one-half of the total protein and covering roughly 20−35% of the envelope surface of chlorosomes in *C. tepidum* (*2*, *14*). CsmA binds BChl *a* and is an important intermediate in light energy transfer from BChl *c* to the reaction centers in both *C. tepidum* and *Chloroflexus aurantiacus* (*14*, *16*, *34*). CsmA forms paracrystalline arrays within the chlorosome baseplate and thus is unlikely to be mobile, since such mobility would greatly reduce energy transfer efficiency while likely promoting the formation of potentially dangerous BChl *a* triplet states.

Although proteins other than CsmA are easily extracted from the chlorosome envelope by detergents, including SDS, Lubrol PX, and Triton X-100, CsmA is only released from chlorosomes by detergents in combination with a low concentration of 1-hexanol (*14*). Moreover, in isolated chlorosomes, only the C-terminus of CsmA is sensitive to proteases, suggesting that CsmA is probably compactly organized with its N-terminus buried in the envelope (*18*). Cross-linking experiments with wild-type chlorosomes showed that CsmA (and pre-CsmA) is cross-linked to form multimers. Very similar cross-linking patterns were also detected with SDS-treated chlorosomes and carotenosomes; these data indicate that CsmA maintains its quaternary structure even in the absence of all other chlorosome proteins or BChl *c* (*14*, *15*). Previous fractionation studies with chlorosomes from *Cfx. aurantiacus* had shown that the baseplate is comprised of CsmA, BChl *a*, and carotenoids (*16*, *34*). Similarly, CsmA in *Chlorobium* spp. likewise binds BChl *a* and carotenoids (*14*, *15*), implying that CsmA forms the chlorosome baseplate, which is appressed to the FmoA layer, in green sulfur bacteria. Consistent with this idea, the contaminating FmoA in chlorosome preparations could be cross-linked to CsmA and detected with both anti-CsmA and anti-FmoA antibodies. This result suggests that the FmoA protein interacts rather strongly with CsmA and that it may be partially inserted into the baseplate region of the envelope.

Frigaard et al. (*21*) reported the construction and characterization of nine mutant strains of *C. tepidum*, each lacking a single chlorosome protein. These mutants were invaluable in the identification of cross-linking products in the studies reported here. In general, the cross-linking products observed for chlorosomes from the wild-type and mutant strains were identical except for cross-linking products that included the protein encoded by the mutated gene. In the studies presented here, cross-linking between CsmA and pre-CsmA and between pre-CsmA and CsmB or CsmF was demonstrated (Table 1). Pre-CsmA is the precursor of CsmA and contains a hydrophilic carboxyl-terminal extension of 20 amino acids (*9*). The presence of pre-CsmA in chlorosomes indicates that the cleavage of the carboxyl-terminal amino acids probably occurs after the insertion of pre-CsmA into the chlorosome envelope. Cross-linking between CsmA and pre-CsmA likely occurs at the edges of the paracrystalline CsmA baseplate, where we propose that pre-CsmA may initially be inserted into the chlorosome envelope. CsmB is the second most abundant chlorosome protein in *C. tepidum*; the sequence of CsmF is highly similar to that of CsmB (63%), but CsmF is present in a much smaller amount (*8*, *11*). Analyses of CsmB and CsmF indicate that these polypeptides are slightly hydrophobic throughout nearly their entire lengths (*8*, *11*). After cross-linking for only 5 min, the majority of CsmF was cross-linked, and as detected by anti-CsmF antibodies, CsmF was cross-linked with an approximately equal frequency to CsmA and pre-CsmA (Figure 4A, right, lane *csmB*). Since there is actually much less pre-CsmA than CsmA in chlorosomes, and given the suggested peripheral location of pre-CsmA around the baseplate, these observations imply that CsmF is also located near the edges of the baseplate. Similarly, CsmB was much more readily cross-linked to pre-CsmA than to CsmA (see Figure 4A, right, lane *csmF*); this result also suggests that pre-CsmA and CsmB interact along the edges of the baseplate.

Antisera to CsmC and CsmD readily agglutinate chlorosomes (*8*), and these two proteins are the most accessible to proteases (*12*) and the most easily released from chlorosomes by detergent treatments (*14*). These results suggest that significant portions of the CsmC and CsmD proteins are exposed at the surface of the chlorosome envelope. In the cross-linking experiments, CsmC and CsmD were rapidly cross-linked to form several homo- and heteromultimers (Table 1). Chlorosomes from a *csmC* mutant were ~25% smaller than those of the wild type (*21*); this suggests that CsmC and its multimers might somehow be correlated with the elongation of chlorosomes and/or their BChl *c* aggregates. CsmC is almost certainly excluded from the baseplate region, so it must be located on the cytoplasm-facing lateral surfaces of the envelope opposite the baseplate. CsmC (and possibly CsmH) might play roles in organizing the BChl *c*−*e* aggregates that could therefore promote elongation of chlorosomes (*24*).

Except for the amino and carboxyl termini, CsmD is hydrophobic throughout its entire length (*12*). Trypsin digestion suggested that both the termini are exposed at the chlorosome surface (*12*). CsmD dimers and trimers were detected in chlorosomes (Table 1), and dimers were also detected in carotenosomes (*24*). CsmD is the second-most abundant protein in the flattened carotenosomes isolated from a *C. tepidum bchK* mutant (*15*). Thus, CsmD seems to be located in that portion of the chlorosome envelope that faces the cytoplasm. However, a survey of the genomes of 10 green sulfur bacteria shows that most green sulfur bacteria do not encode a *csmD* gene; thus, CsmD is not a component of the chlorosomes of most other green sulfur bacteria except for *Prosthecochloris aestuarii* (*24*).

CsmH is also very easily extracted from the chlorosome envelope by detergents (*19*), and antibodies to CsmH agglutinate chlorosomes very efficiently (*8*). These data indicate that CsmH is exposed at the chlorosome envelope surface (*8*). Cross-linking indicates that CsmH forms homomultimers that interact with both CsmA and CsmB. Since *csmH* mutants also affect chlorosome size (*21*) and since CsmH is depleted in chlorosomes from a *csmC* mutant, it seems that CsmC and CsmH together must play important roles in determining the size and shape of chlorosomes. Consistent with this possibility, all sequenced genomes of green sulfur bacteria encode *csmC* and *csmH* genes (*24*).

CsmI, CsmJ, and CsmX are [2Fe-2S] proteins associated with the chlorosome envelope (*13*). Mutational analyses to be reported in full elsewhere show that CsmI and CsmJ play important roles in the redox regulation of electron transfer in chlorosomes (*24*). CsmI and CsmJ are present at 10-fold higher levels than CsmX in chlorosomes of *C. tepidum* (*2*, *8*, *13*). Although many green sulfur bacteria contain all three Fe−S proteins, several organisms apparently lack the gene encoding at least one of these proteins (*24*). The cross-linking experiments reported here show that CsmI and CsmJ form both heterodimers and homodimers, and this observation suggests that the two proteins may exist as a $CsmI_2$−$CsmJ_2$ heterotetramer. All three Fe−S proteins could also be cross-linked to CsmB (Table 1). Thus, while CsmB does not appear to form homomultimers, CsmB can be cross-linked to nearly all the other proteins. This result indicates that CsmB, the second-most abundant protein in chlorosomes of *C. tepidum*, is probably distributed over much of the chlorosome envelope except the baseplate. Figure 9 summarizes the conclusions drawn from the cross-linking experiments and presents an overall model for the localization of chlorosome proteins.

A comparative analysis of the genomes of green sulfur bacteria shows that the following chlorosome proteins are universally present: CsmA, CsmB, CsmC, CsmE, CsmH, and two Fe−S proteins, which may be most similar to either CsmI, CsmJ, or CsmX. In studies of nine mutants each lacking a single chlorosome protein (except CsmA), no significant changes in BChl *c* content or gross chlorosome organization were observed, and the mutants generally had growth rates similar to that of the wild type (*21*). These observations indicate that chlorosomes are extremely robust structures, which can tolerate major changes in protein composition. Gene duplication and divergence certainly account for the sequence relationships among the four protein motif families for chlorosomes, and this could lead to structural and functional substitution when a single protein is eliminated. Double and triple mutants, in which all chlorosome proteins belonging to different motif classes have been eliminated, have been constructed to test whether this is the case and to provide further information about the functions of the proteins of the chlorosome envelope (*24*; H. Li and D. A. Bryant, manuscript in preparation).

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