Subcellular Localization of Chlorosome Proteins in *Chlorobium tepidum* and Characterization of Three New Chlorosome Proteins: CsmF, CsmH, and CsmX[†]

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ABSTRACT: Chlorosomes are unique light-harvesting structures found in two families of photosynthetic bacteria. In this study, three chlorosome proteins (CsmF, CsmH, and CsmX) of the green sulfur bacterium *Chlorobium tepidum* were characterized by cloning and sequencing the genes which encode them, by overproducing the respective proteins in *Escherichia coli*, and by raising polyclonal antisera to the purified proteins. Three other proteins (AtpF, CT1970, and CT2144) which were identified in chlorosome fractions have similarly been characterized. The antisera were used to establish the distribution of each protein in various cellular fractions. Ten chlorosome proteins (CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, and CsmX) copurified in a constant proportion together with bacteriochlorophyll *c*, and none of these 10 proteins was found in substantial amounts in other subcellular fractions. An antiserum to CsmH was highly effective in agglutinating chlorosomes, and antisera to CsmI, CsmJ, CsmX, and CsmA also immunoprecipitated chlorosomes to varying extents. However, an antiserum to CsmF did not agglutinate chlorosomes. The sequences of chlorosome proteins generally are not significantly similar to the sequences of other proteins in the databases. However, the N-terminal domains of three chlorosome proteins, CsmI, CsmJ, and CsmX, are related to adrenodoxin-type ferredoxins that ligate [2Fe-2S] clusters [Vassilieva, E. V., Antonkine, M. L., Zybailov, B. L., Yang, F., Jakobs, C. U., Golbeck, J. H., and Bryant, D. A. (2001) *Biochemistry 40*, 464−473]. The sequences of the C-terminal domains of these three proteins appear to be distantly related to CsmA and CsmE. The remaining chlorosome proteins can be divided into two additional structural families, CsmB/F and CsmC/D. CsmH is recovered in water-soluble form after overproduction in *E. coli*. Interestingly, this protein contains an N-terminal domain that is similar to CsmB/D, while its C-terminal domain is related to CsmC/D. The sequence relationships indicate that, although the protein composition of *Chlorobium*-type chlorosomes is superficially more complex than that of the chlorosomes of *Chloroflexus aurantiacus*, this heterogeneity is mostly produced by gene duplication and divergence among a small number of protein types.

Chlorosomes are remarkably efficient photosynthetic light-harvesting structures that are only found in two eubacterial families: the *Chlorobiaceae*, or green sulfur bacteria, and the *Chloroflexaceae*, or green filamentous bacteria (for reviews, see refs *1*−*4*). Despite the differing physiology and the very different photosynthetic reaction centers of these bacteria, their chlorosomes are nevertheless strikingly similar. When viewed by electron microscopy, these structures appear as ellipsoidal bodies which are attached to the inner side of the cytoplasmic membrane (*5*, *6*). The light-harvesting BChl[1] *c*, *d*, or *e* aggregates into rod-shaped elements that fill the chlorosome interior, while a monolayer lipid−protein envelope surrounds the entire structure. Galactolipids, wax esters, carotenoids, and quinones are also present in chlorosomes (*7*, *8*). It is generally accepted that pigment−pigment interactions, but not pigment−protein interactions, play the major roles in establishing the structure and properties of chlorosomes (*1*, *2*, *9*). Several models describing the structural features of the BChl aggregation in chlorosomes have been proposed (*1*, *2*, *10*−*12*). The roles of the protein components of chlorosomes, on the other hand, are essentially unknown, and this issue has been a subject of considerable debate (see ref *1* for review).

All chlorosomes contain a small amount of BChl *a* (*1*), although its location and function are not yet well understood. Chlorosomes of the green sulfur bacteria are attached to a paracrystalline layer [Staehelin et al. (*6*) referred to this paracrystalline layer as the "baseplate"], 5−6 nm in thickness, that in turn interacts with the cytoplasmic membrane (*6*). This layer probably contains the BChl *a*-binding FMO protein, which mediates excitation transfer from the chlorosome to the reaction center and which is physically connected

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[1] Abbreviations: BChl, bacteriochlorophyll; CTXXXX, product of open reading frame *CTXXXX* of *C. tepidum*; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FMO protein, Fenna−Matthews−Olson protein; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate.

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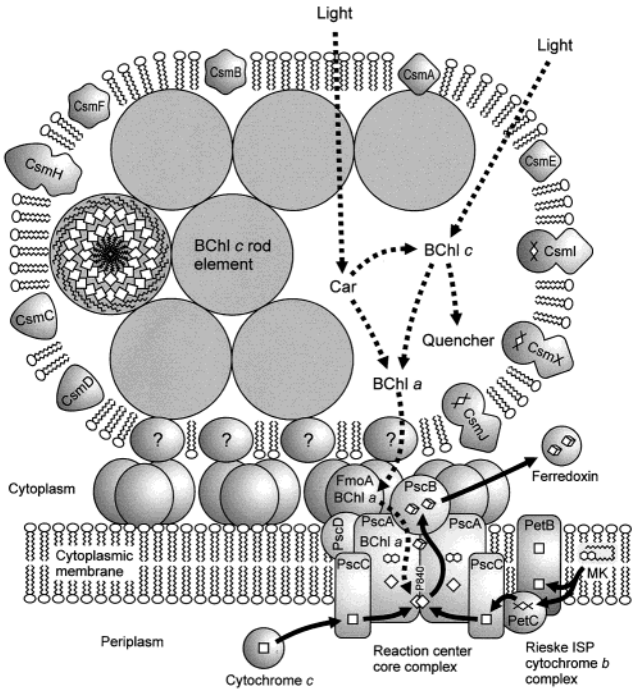

FIGURE 1: Simplified model showing excitation energy transfer (dotted arrows) and electron transfer (solid arrows) in the photosynthetic apparatus of the green sulfur bacterium *C. tepidum*. Proteins and chlorosome rod elements are shown in gray, and some cofactors are indicated as white symbols. The BChl *a*-binding FMO protein is shown as a trimer. The white diamonds within a single rod element represent individual BChl *c* molecules. The cubes represent [4Fe-4S] clusters in the reaction center and in the soluble ferredoxin. The rhomboids in CsmI, CsmJ, and CsmX represent [2Fe-2S] clusters. The white squares in cytochrome *c* and in the PscC subunits of the reaction center represent *c* heme groups. The rhomboids in the PscA subunits represent BChl *a* (the double rhomboid represents the P840 special pair) and the primary acceptor Chl a_{670} (single rhomboids). Menaquinone molecules in the reaction center are represented by double hexagons. The shapes of the proteins in the chlorosome surface monolayer correspond to the four structural motif families (see the text for details). Further details concerning this model can be found in refs *1*−*4*.

to the type 1 reaction centers in the cytoplasmic membrane (*2*, *13*). A model showing the interactions among chlorosomes, the FMO protein, and reaction centers is presented in Figure 1. It should be noted that the exact locations of the proteins in the chlorosome envelope are not known.

Chlorobium tepidum is the only characterized thermophile of the genus *Chlorobium* and was originally isolated from sulfide-rich, slightly acidic hot springs on the northern island of New Zealand (*14*). The complete genomic sequence of *C. tepidum* has recently been determined (*40*), and natural transformation of this organism has been optimized (*15*). Previous studies employing SDS−PAGE and N-terminal amino acid sequencing showed that highly purified chlorosomes of *C. tepidum* contain 10 polypeptides (*16*). The genes encoding CsmA, CsmB, CsmC, CsmD, CsmE, CsmI, and CsmJ were subsequently cloned and characterized, and the findings from these studies have been reported (*16*−*20*). Five chlorosome proteins (CsmA, CsmB, CsmC, CsmD, and CsmE) have been overproduced in *Escherichia coli*, and the purified recombinant proteins were used to produce polyclonal antisera. The antisera were used in chlorosome agglutination studies as well as in immunoblotting studies in conjunction with protease treatments of isolated chlorosomes to show that these five proteins are components of the chlorosome envelope (*18*, *19*). The complete genome sequence of *C. tepidum* allowed the identification of one additional chlorosome protein, denoted CsmX, based on its high degree of sequence similarity to CsmJ and CsmI (*20*).

In the study reported here, the characterization of the remaining chlorosome proteins, as well as of three additional proteins that were identified in chlorosome-containing fractions, is presented. The genes encoding CsmF, CsmH, CsmX, and the three copurifying proteins (AtpF, CT1970, and CT2144), which had previously been assigned the provisional names CsmG, CsmL, and CsmW, respectively, were identified. Recombinant proteins were overproduced and used to raise antisera. The distribution of chlorosome proteins in various *C. tepidum* cell fractions was determined using these protein-specific antisera, and from these analyses, it is shown that 10 chlorosome proteins copurify in a constant proportion together with BChl *c*. Ten proteins (CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, and CsmX) appear to be localized in the chlorosome envelope. Finally, the common structural motifs in chlorosome proteins of *C. tepidum* and *Chloroflexus aurantiacus* were identified. The data indicate that the diversity of these chlorosome proteins probably arose by gene duplication and divergence from a small number of ancestral protein sequences.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. C. tepidum strain ATCC 49652 (*14*) was kindly provided by M. Madigan (Southern Illinois University, Carbondale, IL) and was cultivated at 48 °C in 2.4 L bottles in CL medium (*15*) with the addition of 0.5 g of sodium thioglycolate per liter or in a modified version of Pfennig's medium (*14*). Modifications were the addition of 0.5 g of sodium thioglycolate per liter of medium, increasing the amount of $Na_2S{\cdot}9H_2O$ from 0.6 to 0.96 g/L, substituting sodium acetate for ammonium acetate, and omitting $VoSO_4{\cdot}2H_2O$ from the trace element solution. Resazurin (0.035 mg/L of medium) was added to the growth medium as a redox indicator. Illumination was provided by either one or two 100 W incandescent lamps placed 40 cm from the surface of the bottle. *E. coli* strains DH5α [genotype F^- Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_K^-, m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1* from Bethesda Research Laboratories, Gaithersburg, MD] and ElectroMAX DH10B [genotype F^- *mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) *ϕ*80d*lac*ZΔM15Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G from Gibco BRL Products, Gaithersburg, MD] were used for all routine recombinant DNA manipulations except protein overproduction. For most experiments, cells were grown in Luria-Bertani medium (*21*) at 37 °C; when appropriate, the medium was supplemented with ampicillin (100 μg/mL) or kanamycin (30 μg/mL). For overproduction of all proteins except CT2144, *E. coli* strain BL21(DE3) [genotype F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3) from Novagen, Madison, WI] was used. Strain BRL(DE3) [genotype F^-*ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* Δ(*srl-recA*)306*::* Tn*10*(TcR)(DE3) from Novagen] was used for CT2144 overproduction.

Cloning and Overexpression of the Genes for Chlorosome and Chlorosome-Associated Proteins. Cloning of *csmI* and

Table 1: Cloning, Overproduction, and Purification of Recombinant *C. tepidum* Proteins for Antibody Preparation

expression plasmid and cloning sites	primers[a]	mutated residues[b]	purification[c]	yield (mg/L)
pET30::*atpF*$_{trunc}$[f]	sense, 5′ CTTT**C***A**T**G*ATCGTTCTCGTTATTCTCAG (*Bsp*HI)	Leu29 → Met[g]	homogenate (D) →	7.4
*Nco*I and *Bam*HI	antisense complementary, 5′ GCACTTGACAT**G**G**A**TCCGAGGTT (*Bam*HI)		Ni (D) → PAGE	
pET32a::*csmA*	sense, 5′ CCAAAAGGAGGAGT**C***ATG*AGTGGAGGA (*Bsp*HI)	Lys58 → Ser	homogenate (D) →	10.6
*Nco*I and *Blp*I	antisense complementary first,[i] 5′ CGGTTTGCTTA**GC**TGGCAAACTTCGAT		Ni (D) → PAGE	
	antisense complementary second, 5′ CGGTTTGC*T***C**AGCTGGCAAAC (*Blp*I)			
pET32a::*csmF*	sense, 5′ ACTGACCA**CC***ATG*GCAAACGAATCA (*Nco*I)	none	homogenate (D) →	4
*Nco*I and *Bam*HI	antisense complementary, 5′ CGGTATATCAG**G**ATCCGCATGA (*Bam*HI)		Ni (D) → PAGE	
pET3d::*csmH*	sense, 5′ ATCAAACCA**CC***ATG*GCTACCGAAGAA (*Nco*I)	none	SF[d] → PAGE	1.6
*Nco*I and *Bam*HI	antisense complementary, 5′ CGAATGCCTT**G**GATC**C**GTTCTATTCA (*Bam*HI)			
pET3d::*csmI*	sense, 5′ TTTTTACGG**T**C*ATG*AATCTCATTATC (*Bsp*HI)	Asp244 → Ala	washed inclusion	1.2
*Nco*I and *Blp*I	antisense complementary, 5′ ATGCCC*GCTCAG***C**CTTTAGCC (*Blp*I)		bodies[h] → PAGE	
pET3d::*csmJ*	sense, 5′ ATCTCACCG**T**C*ATG*ATTATCTACATC (*Bsp*HI)	none	washed inclusion	4
*Nco*I and *Bam*HI	antisense complementary, 5′ GAAAGCCGAG**G**G**A**TCCCCGGAGC (*Bam*HI)		bodies[h] → PAGE	
pET32a::*csmX*	sense, 5′ **T**CATACAGT**C***ATG*AACATCACCGT (*Bsp*HI)	Val209 → Ala	homogenate (D) →	2.3
*Nco*I and *Bam*HI	antisense complementary,[e] 5′ GATGAC**G**CTCAG**C**AATAAGTGC		Ni (D) → PAGE	
pET32a::*CT1970*	sense, 5′ AGGAAATAAC**C***ATG*GCACTCAC (*Nco*I)	none	homogenate (D) →	27
*Nco*I and *Bam*HI	antisense complementary, 5′ AAACTGACGGA**TC**CAATCGCCTTC (*Bam*HI)		Ni (D), two runs	
pET32a::*CT2144*	sense, 5′ GCTCATGTTCTGATAAAC**C***ATG***G**AAAAATCAA (*Nco*I)	Lys2 → Glu;	washed inclusion	0.35
*Nco*I and *Blp*I	antisense complementary, 5′ CGTATTGACAATGCC**G***CTCAG***C**AGCTGTA (*Blp*I)	Phe204 → Cys	bodies[j] → Ni (D) → PAGE	

[a] Mutated bases are highlighted, and introduced restriction sites are underlined; start and stop codons are italicized. [b] Residues altered in the recombinant proteins as compared to the corresponding sequences of the wild-type proteins based on the genome sequencing project (*40*). [c] Abbreviations: PAGE, preparative SDS−PAGE; homogenate (D), homogenate of the *E. coli* cells prepared with 6 M urea (under denaturing conditions) and clarified by low-speed centrifugation; Ni (D), His-tagged proteins were purified using His-binding resin in the presence of 6 M urea according to the recommendations of the manufacturer (Novagen). [d] SF−rCsmH was purified by PAGE from the 32% saturation ammonium sulfate precipitate of the soluble fraction of the clarified whole-cell extract. [e] The *Blp*I site was created 136 bp downstream of the *csmX* stop codon by mutagenic PCR, but for cloning into plasmid pET32a, a naturally occurring *Bam*HI site 22 nucleotides downstream of the *csmX* stop codon was used instead. [f] Construct pET3d/AtpF$_{trunc}$ was also made, but the expression level was too low. [g] Met1 in AtpF$_{trunc}$ is Leu29 in the AtpF sequence. [h] Inclusion bodies formed by rCsmI and rCsmJ were prepared as described by Vassilieva et al. (*20*). [i] The first PCR was performed with the first 3′ primer using cDNA as a template, and then the product of that PCR was used as a template for the second PCR with the second antisense primer. [j] Inclusion bodies formed by the fusion protein product of the construct pET32a::CT2144 were washed with 1 M urea and 0.1% (v/v) Triton X-100.

csmJ is described elsewhere (*20*). For cloning and overexpression of the *csmA*, *csmF*, *csmH*, *csmX*, *atpF*, *CT1970*, and *CT2144* genes in *E. coli*, the corresponding genes were modified by PCR to contain restriction sites that were compatible with the cloning sites in pET expression plasmids (*22*; Novagen). An *Nco*I or *Bsp*HI restriction site was introduced at the start codon, and a *Blp*I or *Bam*HI site was introduced either directly at the stop codon or immediately downstream from it, respectively (see Table 1). Cloning into the *Nco*I site resulted in production of N-terminal fusions of the corresponding chlorosome proteins with the His/S tags in pET30c(+) or with thioredoxin/His/S tags in pET32a-(+). In the case of AtpF, the construct pET32a(+)*::atpF*$_{trunc}$ was made which lacked the sequence encoding the first 28 amino acid residues of the AtpF protein. All plasmid constructs were sequenced to ensure that no sequence changes other than those intentionally introduced had occurred during the PCR amplification procedure.

The recombinant proteins were overproduced in *E. coli* at 37 °C in NZCYM or LB medium (*21*) supplemented with the appropriate antibiotic. A single colony was grown at 37 °C in the medium containing 0.1 mg of ampicillin/mL or 0.03 mg of kanamycin/mL. Target DNA expression was induced by addition of IPTG [0.5 mM if using the pET3d-(+) vector or 1 mM if using the pET30c(+) or pET32a(+) vector] to cultures with an OD_{550} of ∼0.8. The cell mass was harvested 2.5−3.5 h after IPTG induction by centrifugation and stored at −70 °C until it was required.

Purification of Recombinant Proteins and Antigen Preparation for Antibody Production. CsmH, CsmI, and CsmJ were overproduced without fusion tags; AtpF$_{trunc}$ was overproduced with a His/S fusion tag, and CsmA, CsmF, CsmX, CT1970, and CT2144 were overproduced with a thioredoxin/His/S fusion tag. The protein yield and purification procedures for each recombinant protein are briefly summarized in Table 1. *E. coli* cells containing recombinant proteins were disrupted by two passes through a chilled French pressure cell at 124 MPa in the presence of 1 mM PMSF and 0.1 mg of DNAse I/mL. Histidine-tagged proteins (CsmA, CsmF, CsmX, AtpF$_{trunc}$, CT1970, and CT2144) were purified by Ni^{2+} affinity chromatography using His-bind resin (Novagen) under denaturing conditions in the presence of 6 M urea according to the manufacturer's instructions (TB0554197, Novagen). CsmH was purified from the 32% ammonium sulfate precipitate from the soluble fraction of the cell homogenate prepared with buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2 M NaCl. CsmI and CsmJ were purified from washed inclusion bodies as described elsewhere (*20*; see Table 1).

After two separations on the His-bind column, the purity of CT1970 was ≥95% according to SDS−PAGE (data not shown); therefore, the second eluate was used as the immunizing antigen after buffer exchange, concentration, and lyophilization. For all other recombinant proteins, the final purification step was preparative SDS−PAGE. The recombinant protein-enriched soluble *E. coli* fraction or the washed inclusion bodies were incubated at 100 °C in SDS sample buffer [2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.05% (w/v) bromophenol blue] for 4 min and electrophoresed using 3 mm thick slab gels made of a 12−15% T and 2.6% C acrylamide separating gel and a 4% T and 2.6% C acrylamide

stacking gel (*23*). By staining side strips of the gels with Coomassie Blue, the bands of interest were visualized, and the desired proteins were then excised from the unstained gel. The proteins were extracted from the minced gel strips in three changes of buffer [20 mM NH_4HCO_3 and 0.05% (w/v) SDS; for extraction of recombinant CsmI, CsmJ, CsmX, and $AtpF_{trunc}$, 1 mM β-mercaptoethanol was added to the first change of the buffer] for 24−36 h with mixing at 6 °C. The eluted proteins were concentrated by ultrafiltration in a stirred, pressurized cell using a 10 000 molecular weight cutoff (MWCO) membrane, and lyophilized. Antisera were produced in female, New Zealand white rabbits; 0.3−0.5 mg of the antigen in 0.1% (w/v) SDS and complete Freund's adjuvant was used per rabbit per injection. Antisera were produced in the Antibody Core Facility of the University of Nebraska (Lincoln, NE) and in the Centralized Biological Laboratory of The Pennsylvania State University. The antiserum developed against the thioredoxin/His/S tag−CsmA fusion protein had a much higher titer than the antiserum previously made against CsmA only (*19*).

Chlorosome Isolation. Chlorosomes were isolated by a modification of the method of Gerola and Olson (*24*), and aliquots were taken at all purification stages and stored at −20 °C. The harvested cells were incubated with lysozyme (3 mg/mL) in isolation buffer [10 mM Tris-HCl (pH 7.5), 2 M NaSCN, 5 mM EDTA, 1 mM PMSF, and 2 mM DTT] at room temperature for 20 min and then disrupted by three passes through a French pressure cell operated at 124 MPa and 4 °C. The cell homogenate was clarified by low-speed centrifugation (17000*g* for 20 min at 4 °C), and a chlorosome-enriched fraction was separated from the soluble proteins by ultracentrifugation (220000*g* for 2 h at 4 °C). The resulting pellet was resuspended in the isolation buffer and further purified on 7 to 47% (w/v) continuous sucrose gradients prepared with the isolation buffer. The gradients were centrifuged at 220000*g* for 18 h at 4 °C. A viscous, brownish pellet that probably contains cell wall fragments and cytoplasmic membranes was recovered from the bottom of the tube. The dark green, BChl *c*-containing chlorosome fraction was collected from the sucrose gradient, diluted four times with phosphate/saline buffer [10 mM potassium phosphate (pH 7.2) and 150 mM NaCl], and centrifuged at 240000*g* for 1.5 h at 4 °C. The resulting pellet was resuspended in the same buffer, and the chlorosomes were pelleted again by centrifugation at 220000*g* for 1.5 h at 4 °C. The final pellet consisted of a loose fraction (designated light chlorosomes) on the top of a firm pellet (designated heavy chlorosomes). Approximately 10% of the total BChl *c* was typically recovered in the light chlorosome fraction. This fraction (Figure 2, lane 6) contained many more proteins than the heavy chlorosome fraction (Figure 2, lane 7) and correspondingly had a higher protein:BChl *c* ratio (see the Results). The pellets were resuspended in a minimal volume of the phosphate/saline buffer supplemented with 1 mM PMSF and 2 mM DTT. Chlorosomes prepared in this manner were aliquoted and stored at −80 °C.

Chlorophyll and Protein Measurements. BChl *c* concentrations were determined from the absorption of acetone or methanol extracts of chlorosomes and cell fractions using a specific absorption coefficient of 92.6 or 86 L g^{-1} cm^{-1}, respectively (*25*). For the protein assays, the proteins were precipitated from the cellular fractions with cold acetone

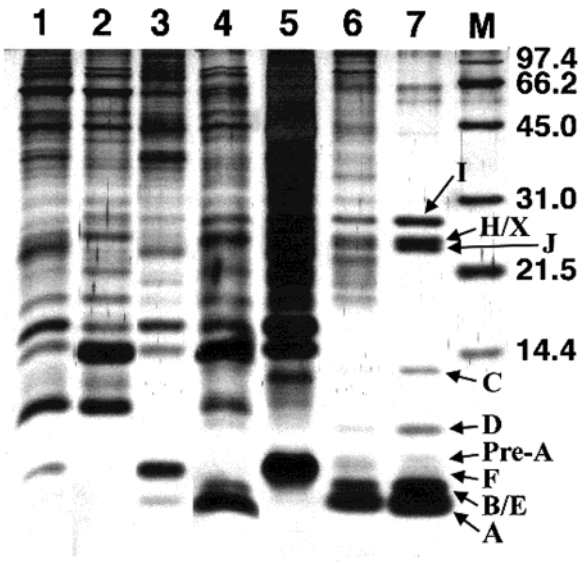

FIGURE 2: Analysis of the fractions collected during the chlorosome purification procedure by SDS−PAGE and by using silver staining to detect the proteins: lane 1, whole-cell extract (3 μg of protein); lane 2, soluble protein fraction (supernatant from the first ultracentrifugation) (4 μg of protein); lane 3, resuspended chlorosome-containing pellet from the first ultracentrifugation step which is the fraction loaded onto sucrose gradients (2.9 μg of protein); lane 4, chlorosome fraction recovered from the sucrose gradient (3.6 μg of protein); lane 5, resuspended pellet from the sucrose gradient purification of chlorosomes (>12.6 μg of protein); lane 6, light chlorosome fraction (6.4 μg of protein); lane 7, heavy chlorosome fraction (8.3 μg of protein); and lane M, molecular mass standards with masses indicated in kilodaltons on the right.

overnight and pelleted by centrifugation at 13000*g* for 15 min. Proteins from the fraction enriched in reaction centers were precipitated with trichloroacetic acid (5%, w/v) and assayed using a modified Lowry procedure as described (procedure P5656, Sigma, St. Louis, MO). The acetone pellets were solubilized in 50 μL of 0.5 M NaOH by incubation in a boiling water bath for 5 min; the protein content was assayed using a Lowry procedure as described in the same manual with slight modifications. The modified Lowry reagent containing 2.27% (w/v) SDS (0.45 mL; Sigma) was added to 0.05 mL of the dissolved pellet proteins. The sample was mixed, diluted 2-fold with water, and incubated at room temperature for 20 min. Diluted Folin and Ciocalteu's phenol reagent (0.25 mL; Sigma) was added; the samples were incubated for 30 min at room temperature for color development, and the absorbance was measured at 750 nm. The concentration of recombinant proteins was estimated using the dye-binding method of Bradford (*26*). Bovine serum albumin (Pierce, Rockford, IL) was used as the standard in both methods. Amino-terminal amino acid sequence analyses of the membrane-blotted proteins were performed as previously described (*16*, *18*, *19*) at the University of Nebraska and by Dr. J. Zhao at Peking University (Beijing, People's Republic of China) with an Applied Biosystems model 470 protein sequencer.

Protein SDS−PAGE and Immunoblotting Analyses. The protein composition of the cellular fractions of *C. tepidum* was analyzed by polyacrylamide gel electrophoresis in the presence of SDS using the Tris-Tricine buffer system described by Schägger and von Jagow (*27*). The stacking gel was 4% T and 2.6% C, and the resolving gel was 15%

Table 2: Chlorosome Proteins and Three Probable Contaminant Proteins in Chlorosomes of *C. tepidum*

protein	locus[a]	mass (kDa)[b]	localization	comments	ref
CsmA	*CT1942*	8.29	chlorosomes	most abundant chlorosome protein; N-terminal Met removal and C-terminal processing yields mature 6.02-kDa protein	*16*
CsmB	*CT2054*	7.61	chlorosomes	possible N-terminal processing from 8.9-kDa precursor, or removal of initiator Met only to produce mature 7.48-kDa protein	*18*
CsmC	*CT1943*	14.3	chlorosomes	N-terminal Met removed; knockout mutant characterized	*16, 29*
CsmD	*CT2064*	11.1	chlorosomes	N-terminal Met removed	*16, 19*
CsmE	*CT2062*	8.95	chlorosomes	N-terminal Met removal and C-terminal processing yields mature 6.55-kDa protein	*16, 19*
CsmF	*CT1046*	7.74	chlorosomes	N-terminal Met removed	this work
CsmH	*CT1417*	21.8	chlorosomes	N-terminal Met removed; recombinant protein is water-soluble	this work
CsmI	*CT1382*	25.9	chlorosomes	possesses [2Fe-2S] cluster-binding motif	*20*
CsmJ	*CT0651*	23.9	chlorosomes	possesses [2Fe-2S] cluster-binding motif; probable C-terminal processing to 21.8-kDa mature protein	*20*
CsmK	?	18−20	?	provisional chlorosome-associated protein; attempts to sequence this protein have thus far failed, and hence its identity and distribution remain uncertain	this work
CsmX	*CT0652*	24.0	chlorosomes	possesses [2Fe-2S] cluster-binding motif	*20*
AtpF	*CT0019*	19.4	all fractions	*b* subunit of F_0-ATP synthase; previously and provisionally named CsmG; probable contaminant	this work
CT1970	*CT1970*	16.0	all fractions	heat-shock protein; initiator Met removed; previously and provisionally named CsmL; probable contaminant	this work
CT2144	*CT2144*	21.5	membranes, chlorosomes	highly similar to *Wolbachia* sp. outer membrane protein precursor; signal sequence removal produces 18.2-kDa mature protein; previously and provisionally named CsmW; probable contaminant	this work

[a] Gene locus designations from the complete genomic sequence of *C. tepidum* (*40*). [b] Mass of the protein deduced from the gene sequence.

T and 3.3% C. The resulting gels were stained with silver as described previously (*28*), except that incubation time with sodium thiosulfate was prolonged from 1 to 3 min, and the incubation in the developer solution was prolonged from 20 to 30 min. The sample buffer was 0.1 M Tris-HCl (pH 6.8), 24% (v/v) glycerol, 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol, and 0.02% (w/v) Coomassie Blue G-250. When gels were to be silver stained, the aliquots of *C. tepidum* cell fractions were loaded directly without pigment extraction after boiling in dissociation buffer. For immunoblots, the pigments and lipids were extracted, and the proteins were precipitated with cold acetone. Purification of the recombinant proteins was routinely monitored by SDS−PAGE using the system of Laemmli (*23*). For immunodetection, proteins were transferred onto 0.45 μm pore-size "Immobilon-P" PVDF membranes (Millipore) or onto 0.45 μm pore-size nitrocellulose "Protran" membranes (Schleicher and Schuell, Keene, NH) using a semidry transfer cell (Bio-Rad, Richmond, CA). Transferred proteins were visualized by staining the membranes in 2% (v/v) acetic acid containing 0.1% amido black stain (Sigma). The stained membranes were scanned with a La Cie flat-bed scanner prior to development with antisera. The membranes were blocked with nonfat milk (5% w/v), incubated with the specified rabbit antisera directed against recombinant chlorosome proteins of *C. tepidum*, and washed with TBS containing 0.05% (w/v) Tween-20 (U.S. Biochemicals, Cleveland, OH). The antisera for CsmB, CsmC, CsmD, and CsmE were described previously (*18*, *19*). The immunoreactive proteins were detected using goat anti-rabbit-IgG secondary antibodies (Sigma) conjugated with horseradish peroxidase for the enhanced chemiluminesence assay (Amersham Pharmacia Biotech, Piscataway, NJ). After incubation with the secondary antibody, membranes were washed with TBS containing 0.1% Tween-20. Immunoblot membranes were stripped multiple times according to the ECL kit manufacturer instructions (Amersham RPN2106P1/98/10) and redeveloped with different antisera. The precise locations of the immunoreactive bands and molecular mass standards were determined by overlaying the developed film onto the scanned image of the stained membrane. The digitized images of stained protein gels, stained immunoblots, and developed immunoblots were manipulated and assembled into figures using Adobe Photoshop 5.5 software.

Immunoagglutination Experiments. The heavy chlorosome fraction was diluted to a final concentration of 1 mg of BChl *c*/mL in phosphate/saline buffer (above) containing 0−30 μL of a specific rabbit antiserum or preimmune serum in a final volume of 0.1 mL. The suspension was incubated for 45 min in the dark at 25 °C. The immunoagglutinated chlorosomes were pelleted in a benchtop centrifuge (13000*g* for 2 min at 4 °C). The efficiency of immunoprecipitation was estimated from the change in absorbance of the Q_Y peak of BChl *c* at 743−748 nm in the supernatants measured after 200-fold dilution in water. The data were normalized relative to those for the BChl *c* measured in the resulting supernatants of the control samples prepared with buffer alone.

RESULTS

Identification and Cloning of Genes Encoding Proteins Identified in Chlorosome Preparations. A summary of the properties of proteins identified in chlorosome preparations from *C. tepidum* is given in Table 2. The N-terminal amino acid sequences of CsmH, CsmI, and CsmF were originally reported by Chung et al. (*16*). The N-terminal amino acid sequence of CsmJ, the cloning of the *csmI* and *csmJ* genes, and the initial identification of the *csmX* gene were recently described by Vassilieva et al. (*20*). The *csmH* gene was cloned using a synthetic, degenerate oligonucleotide, 5′ GAR-GAR-ACC-AAC-ATG-CCN-GCY-GCY-GAR-GC 3′, as the hybridization probe (*17*). Genomic Southern blot hybridization experiments revealed that this probe hybridized to a 1.65-kb *Hinc*II fragment, which was cloned and sequenced. Sequence analysis of this fragment revealed that the *csmH* coding sequence was incomplete, and thus, an overlapping

1.86-kb *Cla*I fragment was cloned and sequenced. The complete nucleotide sequence of this 3.321-kb region was deposited in GenBank under accession number AF060080. Analysis of this sequence revealed that the *csmH* gene encodes a polypeptide of 212 amino acids with a mass of 21 778 Da (Table 2) and a predicted isoelectric point of 4.72. Since the N-terminal methionine is post-translationally removed (*16*), the molecular mass of mature CsmH is predicted to be 21.6 kDa.

Repeated attempts to clone the *csmF* gene using a variety of unique sequence and degenerate oligonucleotide probes failed, primarily due to the fortuitous occurrence of sequences that are very similar to the probe sequences elsewhere in the genome (data not shown). To overcome this problem, a PCR-based approach was employed. A portion of the 5′ coding sequence of *csmF*, whose corresponding amino acid sequence was completely known, was amplified using the degenerate primers 5′ GGY-AAY-ATY-GGY-GTY-TTY-GGY-GA 3′ (forward) and 5′ CC NGC-CAT-RTC NAC-NGC 3′ (reverse). These primers were expected to amplify a 74-bp fragment of *C. tepidum* DNA. Although the use of these primers produced several DNA fragments, a major fragment of 74 bp was amplified as expected. This fragment was purified by gel electrophoresis, cloned in plasmid pUC19, and sequenced. A comparison of the coding sequence predicted from this fragment matched the known amino acid sequence of CsmF and confirmed that the PCR-amplified fragment was derived from the *csmF* gene. Using this sequence information, a nondegenerate 27-nucleotide oligonucleotide, 5′ GGC-GAT-CTC-TTC-ACC-GCT-GTG-GGC-GAT 3′, was synthesized and used as a hybridization probe to clone the *csmF* gene. Hybridization experiments with *C. tepidum* chromosomal DNA revealed that the *csmF* gene was encoded on a 1.7-kb *Eco*RI−*Eco*RV fragment. This fragment was cloned from a size-selected library of electrophoretically fractionated *Eco*RI−*Eco*RV genomic restriction fragments. The 1.666-kb fragment was completely sequenced, and the nucleotide sequence was deposited in GenBank under accession number AF060078. The *csmF* gene encodes a protein of 77 amino acids with a predicted mass of 7742 Da (Table 2) and a predicted isoelectric point of 4.01. Since the N-terminal methionine is removed post-translationally (*16*), the predicted molecular mass for mature CsmF is 7.6-kDa.

Chung et al. (*16*) detected small amounts of a 19.5-kDa protein in chlorosome fractions with the N-terminal sequence MDGEA. Unfortunately, all attempts to acquire additional amino acid sequence data for this protein failed, and no protein containing this sequence has been identified in the genomic sequence of *C. tepidum.* However, further sequencing of proteins from this molecular mass region revealed the N-terminal sequence (E)GXLLNPNPGLIFXTALTFL, which resembles the 10-residue sequence ATXLLNPNXG previously attributed by Chung et al. (*16*) to a minor, unnamed 18-kDa protein, which later was provisionally named CsmG. Moreover, this sequence matched residues 10−29 of a 19.4-kDa protein of 175 amino acids (CT0019) in the *C. tepidum* genome database (*40*). Additional similarity searches of the databases revealed that this protein was in fact subunit *b* (AtpF) of the F_0 subcomplex of the ATP synthase (Table 2).

Chung et al. (*16*) also reported the N-terminal sequence of a minor, 16.5-kDa chlorosome-associated protein (ALT-LYGKDPLTL). This sequence matched residues 2−11 of a 16 kDa (142 amino acids) protein (CT1970) in the *C. tepidum* genomic database (Table 2; after removal of the first methionine, the molecular mass is predicted to be 15.9 kDa). CT1970, which was originally and provisionally designated CsmL, belongs to the HSP-20 family of small heat-shock proteins. A second protein (CT0644), belonging to the same protein family and which is 52% identical to CT1970, was found in the *C. tepidum* genome database. The calculated molecular mass of that protein of 132 amino acids is 15 kDa.

A third minor protein with a molecular mass of approximately 20 kDa and the N-terminal sequence AESPYV-SLS was detected in the chlorosome fraction. This sequence matches residues 20−28 of a 21.5-kDa protein (192 amino acids) encoded in the *C. tepidum* genome (CT2144). Similarity searches of the databases revealed that CT2144, which was originally and provisionally designated CsmW, was very similar to outer surface proteins of the intracellular parasitic bacteria of the genus *Wolbachia* (*30*). Further analysis of this protein sequence indicated that a signal-sequence cleavage site is predicted between residues 19 and 20 (*31*). If cleaved at this position, CT2144 would have a predicted molecular mass of 18.2 kDa and would have precisely the N-terminus that was determined experimentally.

One potential chlorosome protein has not yet been characterized in detail. This protein, which is prominent in the light chlorosome fraction but is variably present in "heavy" chlorosome preparations (compare Figure 5B, lanes 1 and 2), was initially thought to be AtpF (*32*) on the basis of its apparent molecular mass (see above). However, the protein detected by the antiserum to $AtpF_{trunc}$ (see below) has a greater electrophoretic mobility than this unidentified protein. It seems likely that the N-terminus of the protein in question may be blocked, since numerous attempts to sequence this protein have failed. Thus, it is possible that chlorosomes may contain one additional minor protein, nominally denoted CsmK (see Figure 5B below), which is typically seen on SDS−PAGE analyses as a somewhat diffuse protein with a mass of 18−20 kDa.

Chlorosome Purification and Fraction Analysis. Aliquots taken from the major fractions obtained during a standard chlorosome purification (as described in Experimental Procedures) were analyzed by SDS−PAGE (Figure 2) and by immunoblotting (Figure 3). To facilitate comparison, the subcellular fractions are numbered identically in Figures 2 and 3. It should be noted that the total amount of protein loaded from the sucrose gradient pellet fraction (Figures 2 and 3, lane 5) exceeds that of other fractions because this fraction was extremely difficult to resuspend. The isolation of chlorosomes was routinely monitored by following the enrichment of BChl *c* and the smallest and most abundant chlorosome polypeptide, CsmA (6.0−6.15 kDa; see Table 1). CsmA could not be detected by silver staining in the whole-cell extract (Figure 2, lane 1) at the total protein load employed in this experiment, but it could be detected by immunoblotting (Figure 3, lane 1). In contrast, significant amounts of AtpF and CT1970 were detected by immunoblotting in the whole-cell extract (Figure 3A, lane 1) and in all other subcellular fractions. The antiserum to CsmC cross-reacted with two polypeptides. One of these had an apparent

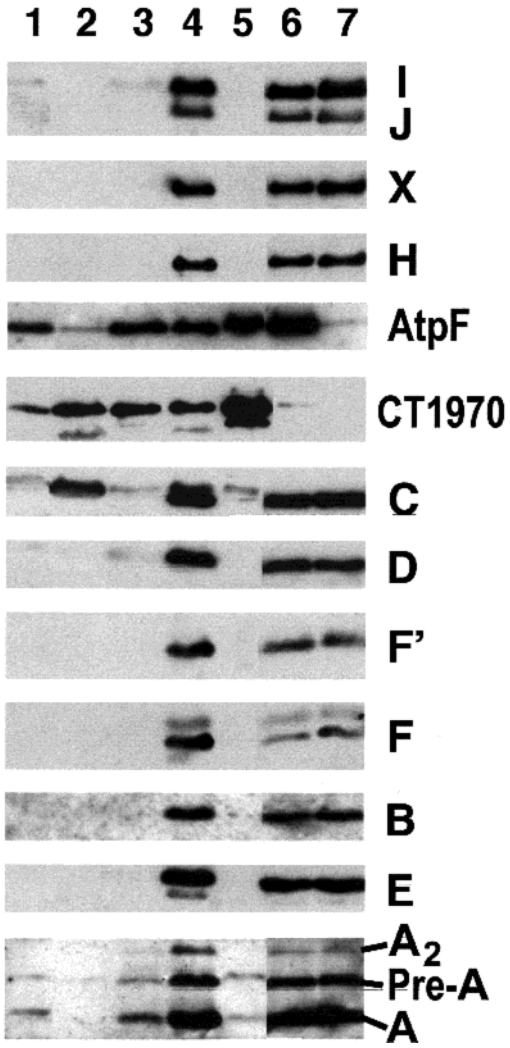

FIGURE 3: Immunoblot detection of chlorosome and chlorosome-associated proteins in various cellular fractions produced during chlorosome purification (see the legend of Figure 2 and Experimental Procedures). Lane numbers and corresponding samples are identical to those in Figure 2. The letters at the right indicate the chlorosome proteins as detected by specific antisera (see Table 1). Note that the anti-CsmF antiserum cross-reacted with two protein species on a freshly prepared membrane (panel F) but only reacted with the more slowly migrating species after the membrane had been stripped (panel F′). Note that the anti-CsmA antiserum recognized mature CsmA, precursor CsmA (Pre-A), and CsmA dimer (A_2). Protein loads were as follows: lane 1, 20 μg; lane 2, 40 μg; lane 3, 20 μg; lane 4, 46 μg; lane 5, >126 μg; lane 6, 20 μg; and lane 7, 20 μg. For immunodetection of CsmA, CsmF, and CT1970, proteins were transferred onto a nitrocellulose membrane. In all other cases, the proteins were transferred onto a PVDF membrane. Antisera dilutions and the number of times the filter had been stripped and reprobed: anti-CsmA, 1:3000 (three strips); anti-CsmB, 1:300 (one strip); anti-CsmC, 1:1000 (fresh membrane); anti-CsmD, 1:800 (fresh membrane); anti-CsmE, 1:300 (fresh membrane); anti-CsmF, 1:3000 on fresh membrane (panel F) and 1:400 on a twice-stripped membrane (panel F′); anti-AtpF, 1:5000 (one strip); anti-CsmH, 1:2000 (one strip); anti-CsmI, 1:10000 (fresh membrane); anti-CsmJ, 1:5000 (fresh membrane); anti-CT1970, 1:10000 (two strips); and anti-CsmX, 1:1000 (two strips).

mass of 14.3 kDa and was only observed in fractions highly enriched in BChl *c* (Figure 3, lanes 4, 6, and 7). The other had an apparent mass of 14.5 kDa, seemed to have a higher affinity for PVDF membranes than for nitrocellulose membranes, and is considered to represent a nonspecific cross-reaction to the antiserum. Under these conditions of comparison, the smallest detectable protein of the soluble fraction (Figure 2, lane 2) had a molecular mass of ~12 kDa. The abundant protein band with an apparent mass of ~14.5 kDa was the lysozyme that had been added prior to cell disruption, and this protein can be seen in other fractions as well (Figure 2, lanes 1−5). On the silver-stained gel shown in Figure 2, CsmA is faintly visible in the fraction enriched in chlorosomes, which were pelleted from the soluble extract at the first ultracentrifugation step (Figure 2, lane 3). This fraction was further resolved by sucrose gradient centrifugation yielding two major fractions: the chlorosome fraction in the upper part of the gradient (Figure 2, lane 4) and a viscous, brownish pellet on the bottom of the gradient (Figure 2, lane 5). The pellet fraction probably includes fragments of the cell wall and the cytoplasmic membranes (*33*). The abundant protein migrating more slowly than CsmA in both pellet fractions (Figure 2, lanes 3 and 5) is similar in size to some small chlorosome polypeptides (Figure 2, lanes 6 and 7),

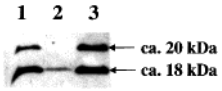

FIGURE 4: Immunodetection of the chlorosome-associated protein CT2144 in various subcellular fractions of *C. tepidum*: lane 1, whole-cell extract (30 μg of protein); lane 2, heavy chlorosomes (50 μg of protein); and lane 3, pellet from the sucrose gradient (80 μg of protein). A PVDF membrane was used for protein blotting, and the antiserum dilution was 1:1000.

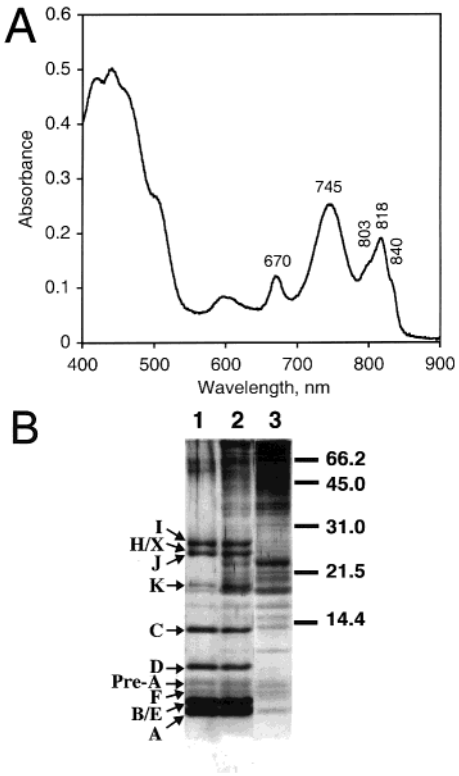

FIGURE 5: Analysis of the sucrose gradient fraction enriched in reaction centers. (A) Absorption spectrum of the fraction derived from the cytoplasmic membrane and enriched in reaction centers. (B) SDS−PAGE analysis: lanes 1 and 2, fractions of heavy and light chlorosomes, respectively (3 μg of protein per lane); and lane 3, fraction enriched in reaction centers (3 μg of protein). Positions of the molecular mass markers are indicated with bars. The letters on the left identify the corresponding chlorosome proteins (see Table 1).

but unlike them, it produces a greenish color when stained with silver. This protein did not cross-react with antibodies to any chlorosome protein.

In the final routine purification step, proteins nonspecifically copurifying with chlorosomes on the sucrose gradient were removed by pelleting the chlorosomes twice from a phosphate/saline buffer. The less rapidly sedimenting particles, or "light" chlorosomes (Figure 2, lane 6), formed a loose pellet on the top of the more tightly packed heavy chlorosomes (Figure 2, lane 7). The loose chlorosome fraction normally represented only ~10% of the total BChl *c* recovered. The protein:BChl ratios (w/w) in the isolated chlorosomes of two independent preparations, measured after overnight protein precipitation with 96−98% (v/v) acetone at −20 °C, were 0.232 ± 0.033 ($n = 5$) and 0.46 ± 0.05 ($n = 3$) in the heavy and light fractions, respectively. These values are in reasonable agreement with the value of 0.45−0.55 previously reported by Chung et al. (*16*) for the total chlorosome fraction.

In an alternative final purification step, chlorosomes isolated from sucrose gradients were subjected to an additional fractionation by flotation on analytical 5 to 40% (w/v) sucrose gradients. The sucrose gradients were prepared either with the isolation buffer containing 2 M NaSCN or with phosphate/saline buffer for examination of whether the ionic strength of the buffer might affect the protein association with chlorosomes. The chlorosomes were layered on the bottom of the gradients, and they floated up and banded between 12 and 20% (w/v) sucrose and between 25 and 28% (w/v) sucrose in the gradients prepared with and without NaSCN, respectively. SDS−PAGE analysis showed that the protein composition of the recovered chlorosomes was identical to that of heavy chlorosomes (data not shown, but see lane 7 of Figure 2 and lane 1 of Figure 5B). Consistent with previously published reports (*16*, *24*), the chaotropic agent NaSCN effectively released chlorosomes from the membranes and allowed a very high degree of purification by sucrose gradient centrifugation.

Figure 2 clearly shows that, under these conditions of comparison, the characteristic small polypeptides of the chlorosomes, CsmA and CsmB, are only visible in the fractions enriched in chlorosomes (lanes 4, 6, and 7). The antiserum to CsmA cross-reacted strongly with proteins of ~6.15, ~8.2, and ~12 kDa (Figure 3). The 8.2 kDa polypeptide corresponds in mass to the unprocessed, precursor form of CsmA and is readily detected in both the light and heavy chlorosome fractions (Figure 3, lanes 6 and 7). However, since the precursor form of CsmA (8.3 kDa; *16*) was overproduced as an antigen for the production of the antiserum, the antibody titer may be higher toward this form of CsmA than to the processed form. The immunoreactive species of approximately 12 kDa corresponds to a CsmA dimer. The nature of this dimer is not clear, but it was previously observed by amino-terminal sequence analyses (*16*) and in immunoblotting experiments performed with a different anti-CsmA antiserum (*19*). This CsmA dimer surprisingly did not dissociate when chlorosome proteins were boiled in the presence of SDS and 2-mercaptoethanol. The pellet collected from the bottom of the sucrose gradient (Figure 3, lane 5) reacted very weakly with the CsmA antiserum, but did not cross-react with any other antiserum to an established chlorosome protein (see Table 2 and Figure 3), even though the amount of protein tested for this fraction far exceeded that for other fractions (see Figure 2).

The antiserum to CsmF reacts with two protein species on freshly prepared immunoblots (Figure 3, panel F). One of these proteins has the same electrophoretic mobility as CsmB and CsmE. The other cross-reactive species has an electrophoretic mobility that is slightly faster than that of the 8.2-kDa CsmA precursor as would be expected for the 7.6-kDa CsmF (Table 2). The species of lower mobility was more readily detected when nitrocellulose membranes were used than when PVDF membranes were used. Moreover, after a membrane had been stripped (see Experimental Procedures), only the species of apparently greater molecular mass was detected (Figure 3, panel F′). This may have been due to the removal of the faster migrating protein from the membrane. These observations suggest that the CsmF antiserum cross-reacts with the more abundant protein CsmB, which is 29% identical and 63% similar to CsmF (see below). Preliminary results obtained with whole-cell extracts of mutant strains lacking either CsmB or CsmF confirm that the immunoreactive species of faster electrophoretic mobility is due to a cross-reaction of the anti-CsmF antiserum with CsmB (data not shown).

As shown in Figure 3, proteins CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, and CsmX copurify in a constant proportion together with the BChl *c*, and none of these proteins were enriched in any other subcellular fraction that was analyzed. However, AtpF and CT1970 apparently copurified with chlorosomes as contaminants and were found in significant amounts in all subcellular fractions. At present, it is not known why some AtpF copurifies with the chlorosome fraction. However, the amount of this protein in the light chlorosome fraction varied significantly in different preparations. It should be noted that no other ATP synthase subunit was identified in the chlorosome fraction.

As mentioned above, the *C. tepidum* genome encodes two similar proteins of the Hsp-20 family of small heat-shock proteins, CT1970 and CT0644. The anti-CT1970 antiserum detected both of these proteins (Figure 3, panel CT1970), although it is likely that the cross-reaction to the immunizing antigen is much stronger. Although only trace amounts of CT1970 were observed in the chlorosome fraction (Figure 3, lanes 6 and 7), it is possible that the CT1970 protein found in association with the chlorosomes might have a specific, chlorosome-related function, since the related protein CT0644 was not detected in this fraction. Alternatively, this result could merely be an artifact caused by a higher but nonspecific affinity of CT1970 for the chlorosomes. Similarly, CT2144 was more abundant in the whole-cell extract and in the cell wall/cell membrane fractions than in chlorosomes (Figure 4). Two immunoreactive species were detected with apparent molecular masses of ~18 and ~20 kDa. On the basis of these apparent molecular masses and the N-terminal amino acid sequencing data (above), it follows that a small amount of the processed form of CT2144, the signal sequence of which has been cleaved, is present in the chlorosome fraction (Figure 4B, lane 2).

A minor, light green-colored fraction was collected from below the chlorosome fraction in the lower portion of the sucrose gradient, and this fraction was analyzed separately (Figure 5). The absorption spectrum of this fraction (Figure

5A) clearly revealed the presence of a reaction center-associated BChl *a* peak at 818 nm with shoulders representing P840 and 803 nm antenna BChl *a*. All of these features indicate that this fraction is derived from the cytoplasmic membrane. The presence of an absorption band at 745 nm (aggregated BChl *c*) suggests that the fraction might contain a small amount of chlorosomes as well. The absorption band at 670 nm is probably a combination of chlorophyll *a* from the reaction center and monomeric and/or pheophytinized BChl *c*. A comparison of the polypeptide patterns of purified heavy chlorosomes (Figure 5, lane 1), light chlorosomes (Figure 5, lane 2), and this membrane-derived, reaction center-containing fraction (Figure 5, lane 3) shows that some of the contaminating proteins observed in the light chlorosome fraction are probably derived from contaminating cytoplasmic membranes. However, immunoblot analyses of the membrane fraction showed that chlorosome proteins were not enriched in this fraction, although as expected for the proton-translocating ATP synthase, AtpF was abundant (data not shown).

Immunoprecipitation of Chlorosomes by Antisera against Chlorosome Proteins. It has previously been reported that antibodies against CsmA, CsmB, CsmC, CsmD, and CsmE can immunoprecipitate chlorosomes with different efficiencies (*18*, *19*). Figure 6A shows immunoprecipitation profiles for the anti-CsmH antiserum and for antisera against the iron−sulfur proteins CsmI, CsmJ, and CsmX. Figure 6B shows the immunoprecipitation profile for a higher-titer antiserum developed against recombinant CsmA (see Experimental Procedures). After prolonged storage at −80 °C and freezing and thawing, some preparations of chlorosomes contained aggregated chlorosomes that were easily pelleted by low-speed centrifugation. The reason for this chlorosome aggregation is not understood, but it might indicate that the chlorosome envelope layer was damaged by freezing and thawing. If the lipid/protein monolayer were to be partially disrupted, the underlying BChl *c* aggregates might become exposed, and this could promote clumping. Therefore, in the experiments performed with the different chlorosome preparations, the potent anti-CsmH antiserum (see below) was always used as a positive control to evaluate different preparations.

Blood plasma may prevent nonspecific aggregation and could even increase the solubility of the chlorosomes in the suspension, possibly by nonspecific interactions with the chlorosome surface (see curves for the preimmune sera in panels A and B of Figure 6). In contrast, addition of as little as 2 μL of anti-CsmH antiserum caused substantial chlorosome immunoprecipitation, and when 10 μL of the antiserum was added, nearly complete immunoprecipitation of the chlorosomes was observed (Figure 6A). Thus, CsmH appears to be highly exposed at the chlorosome envelope surface and is readily available for reaction with antibodies. This result is consistent with studies showing that CsmH is very easily extracted from the chlorosome envelope by detergents (*32*), and this protein is also highly susceptible to digestion by added proteases (data not shown). Similar results were previously obtained for CsmC and CsmD by Chung and Bryant (*19*). CsmC and CsmD were highly sensitive to protease treatment of chlorosomes, and antisera to these proteins very effectively immunoprecipitated intact chlorosomes. These observations are also consistent with the

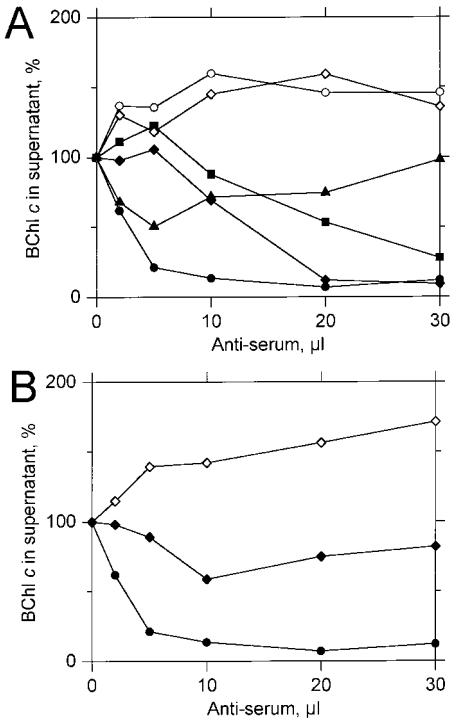

FIGURE 6: Chlorosome immunoprecipitation curves. The percentage recovery of BChl *c* in the supernatant of each sample is plotted as a function of the amount of antiserum added to the sample (see Experimental Procedures for details). (A) Precipitation of chlorosomes by antisera against chlorosomal [2Fe-2S] proteins compared with immunoprecipitation by the anti-CsmH antiserum. Filled circles, squares, diamonds, and triangles represent anti-CsmH, anti-CsmI, anti-CsmJ, and anti-CsmX antisera, respectively. Empty circles and diamonds represent preimmune sera for CsmH and CsmJ, respectively. (B) Immunoprecipitation with anti-CsmA antiserum compared with immunoprecipitation by anti-CsmH antiserum. Filled diamonds and circles represent anti-CsmA and anti-CsmH antisera, respectively. Empty diamonds represent the preimmune serum for CsmA. Note that the addition of preimmune serum appeared to solubilize some nonspecific chlorosome aggregates, which would otherwise be removed by centrifugation. This leads to an apparently enhanced recovery of BChl *c* in the supernatant fraction. The results shown are from a single experiment, but the results were repeated several times with similar overall results each time.

observed sequence similarities among these proteins (see the Discussion below).

Antisera against the iron−sulfur proteins CsmI, CsmJ, and especially CsmX were less effective than the anti-CsmH serum but still caused immunoprecipitation of chlorosomes (Figure 6A). The slight increase in chlorosome solubility observed after addition of small amounts of these antisera could reflect the nonspecific solubilization of the partially aggregated chlorosomes as noted above. However, as increasing amounts of antisera were added, the extent of immunoprecipitation clearly increased. The anti-CsmX antiserum had a somewhat different immunoprecipitation pattern (Figure 6A). The immunoprecipitation began at low concentrations of antiserum, but as more antiserum was added, the degree of precipitation actually decreased. The reason for this behavior is not clear, but it is possible that only a small part of the CsmX protein is exposed at the chlorosome envelope.

When compared to the preimmune serum, it is obvious that the anti-CsmA antiserum was able to immunoprecipitate

chlorosomes (Figure 6B), but this antiserum was much less effective than the anti-CsmH antiserum. Addition of more than 10 μL of anti-CsmA serum did not cause a further increase in the extent of precipitation of chlorosomes. The immunoprecipitation data for anti-AtpF, anti-CT1970, and anti-CT2144 antisera were very similar to those obtained with the corresponding preimmune sera (data not shown) and indicate these antisera do not agglutinate chlorosomes.

DISCUSSION

This work extends data obtained previously concerning the protein composition of the chlorosome envelope (*16*) by establishing the subcellular distribution of these proteins and their copurification with one another together with BChl *c*. The results obtained in these studies indicate that chlorosomes of *C. tepidum* contain 10 proteins (11 if the CsmK protein is a bona fide chlorosome protein; see above). Three proteins (AtpF, CT1970, and CT2144; see Table 2) were identified as low-abundance components in the light chlorosome fraction, but it is not clear why only CT1970, but not the related CT0644 protein, and only the processed form of CT2144 were found in this chlorosome fraction. It is our assumption that these probably represent copurifying contaminants in the less purified chlorosome preparations, although the possibility of a function in chlorosome biogenesis cannot be rigorously excluded.

Analysis of the immunoprecipitation profiles (Figure 6) indicated that chlorosome proteins CsmA, CsmH, CsmI, CsmJ, and CsmX are exposed on the chlorosome envelope surface to differing extents. The difference in the immunoprecipitation profiles in Figure 6A may also reflect differing amounts and topographies for the three Fe−S proteins on the chlorosome surface. The amounts of CsmI and CsmJ in chlorosomes are probably greater than the amount of CsmX, since CsmX was not detected by protein sequencing while CsmI and CsmJ were both identified and sequenced (*16*, *20*). The surface-exposed regions of these three [2Fe-2S] proteins could be relatively small when compared with their overall sizes. Consistent with this notion, thermolysin treatment of chlorosomes produced two large fragments of CsmJ, but this protein was not digested further (data not shown). Finally, substantial differences in the cross-reactivities of antibodies to these three presumably native proteins of the chlorosome envelope could arise from the fact that the antisera were raised against SDS-denatured polypeptides.

The inability of antisera to AtpF, CT1970, and CT2144 to immunoprecipitate chlorosomes was anticipated, since these three proteins are here shown to be copurifying contaminants in the light chlorosome fraction. However, the failure of the anti-CsmF antiserum to cause precipitation was not expected, since this protein clearly copurifies with the chlorosomes (see above). Moreover, CsmF is obviously related in sequence to an abundant chlorosome protein CsmB (see Figure 7), the antiserum of which does cause immunoprecipitation of chlorosomes (*18*). Immunoprecipitation typically requires multiple epitopes that must occur in suitable geometries, and these criteria might not be met for CsmF. Alternatively, the linear epitopes that are recognized by the antibodies after complete denaturation by SDS may not be recognizable in the folded, native protein. At present, there are no data that indicate that CsmF is associated with the chlorosome envelope in a manner different from those of the other chlorosome proteins. CsmF can be extracted from chlorosomes by very low concentrations of SDS (data not shown), and therefore, CsmF is probably localized in the chlorosome envelope as shown for all other chlorosome proteins.

Sequence Comparisons of the Chlorosome Proteins. All chlorosome proteins apparently share very low levels of overall sequence similarity. Pairwise sequence alignments show the existence of limited motifs with sequence similarity, but these motifs may or may not be conserved in other proteins of the chlorosome envelope. In this respect, chlorosome proteins appear to exhibit a "mosaic" pattern of sequence similarity. Nevertheless, it is possible to distinguish four protein motif families with more closely related members. Figure 7 shows sequence alignments for three families of chlorosome proteins: CsmB/CsmF (and CsmO from *Ch. aurantiacus*; see below), CsmC/CsmD, and CsmA/CsmE. A sequence alignment of the fourth family, including the Fe−S proteins CsmI, CsmJ, and CsmX, was recently reported (*3*). As shown in Figure 7, the CsmB and CsmF proteins are 29% identical and 63% similar in sequence. Moreover, these two proteins are substantial similar in sequence to the N-terminal domain of CsmH. The CsmC and CsmD proteins are also clearly related in sequence and are 26% identical and 45% similar. Interestingly, the C-terminus of CsmH is related in sequence to CsmC and CsmD. These structural relationships provide an explanation for the copurification of these proteins with chlorosomes as well as the similarities in biochemical behaviors of these proteins as described above. A summary scheme for the structural relationships among chlorosome proteins of *C. tepidum* is shown in Figure 8.

The N-terminal domains of CsmI, CsmJ, and CsmX have previously been shown to be closely related in sequence to adrenodoxin-type ferredoxins and to bind [2Fe-2S] clusters (*20*; see Figure 8). Since most ferredoxins are water-soluble proteins, a relevant question is how these proteins interact with the chlorosome envelope. Figure 7 suggests how this may occur. The C-terminal domains of CsmI, CsmJ, and CsmX have been subjected to ClustalW alignment with the precursor forms of CsmA and CsmE. Although the overall level of sequence identity is low, the C-terminal domains of CsmI, CsmJ, and CsmX clearly appear to be distantly related to CsmA and CsmE. The multiple alignment further suggests that the C-terminal domains of CsmI, CsmJ, and CsmX contain an insertion of 28−33 amino acids between two conserved sequence motifs. The sequence alignment also suggests that the C-terminus of CsmJ may be endoproteolytically processed in a manner similar to that of CsmA and CsmE, since a sequence (L-P-G-S↓T-C) that closely resembles the processing site (L-K/R-G-S↓S-P) for CsmA and CsmE (*16*, *19*) is found in this protein. Consistent with this suggestion, CsmJ was found to have a greater electrophoretic mobility than CsmH by immunoblotting (see Table 2 and Figure 5). The CsmI and CsmX proteins probably lack this processing site, since these proteins appear to have retained little similarity in sequence to CsmA and CsmE in the vicinity of this site.

Chlorosomes from the green filamentous bacterium *Ch. aurantiacus* have been reported to contain only three or four polypeptides (*34*, *35*), and thus, these chlorosomes appear to be simpler in composition than those of green sulfur bacteria. One of the four proteins found in chlorosomes of

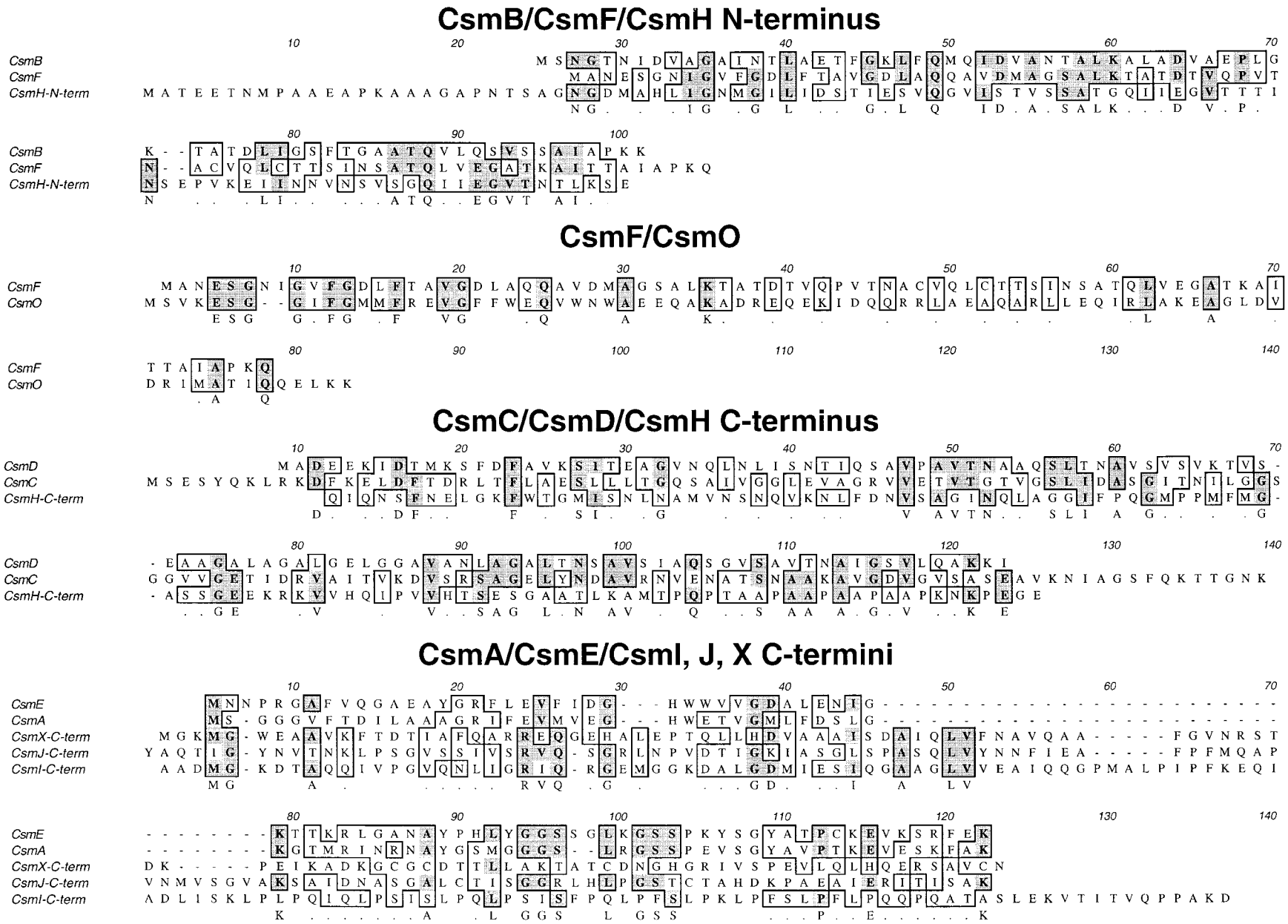

FIGURE 7: Multiple sequence alignments showing sequence similarities for three families of chlorosome proteins. A multiple alignment showing the sequence similarities of the N-terminal domains of chlorosome proteins CsmI, CsmJ, and CsmX can be found in Vassilieva et al. (*3*). Hyphens indicate insertions/deletions included to maximize the sequence similarities. All alignments were performed with the ClustalW module of MacVector version 7.

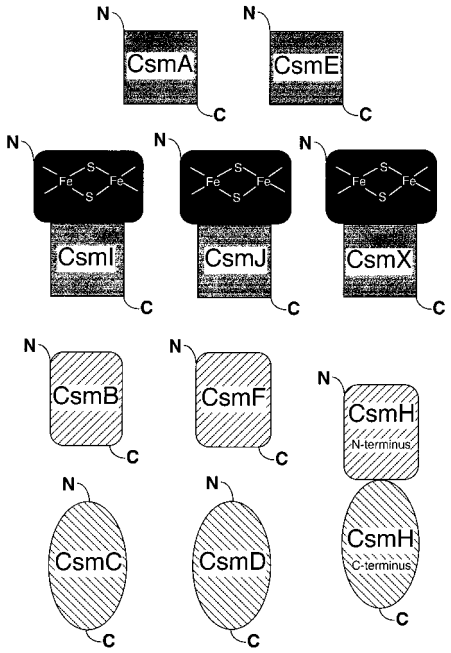

FIGURE 8: Scheme showing the four sequence and structure domains of chlorosome proteins found in *C. tepidum*. For details, see the text.

Ch. aurantiacus is an obvious homologue of CsmA, and this protein is even C-terminally processed in a manner similar to that of CsmA in *C. tepidum* (*16*, *36*). *Ch. aurantiacus* chlorosomes also contain proteins with apparent masses of 11 and 18 kDa, which are the products of the *csmM* and *csmN* genes, respectively (*37*). These two proteins are distantly related in sequence to the CsmC/CsmD proteins of *C. tepidum* (data not shown). Lehmann et al. (*35*) reported an N-terminal amino acid sequence (SVKESGGIFGM-MFREVGFFWEQVXNWAEE) for a protein found in *Ch. aurantiacus* chlorosomes. A database search against randomly sequenced *Ch. aurantiacus* genomic DNA (produced by the Department of Energy's Joint Genomic Institute, http://www.jgi.doe.gov) revealed the presence of a corresponding gene in the contig 1051 that encodes the 9.5 kDa polypeptide, here designated CsmO, that is clearly related in sequence to the CsmB/CsmF family of sequences as shown in Figure 7. The same database was searched against CsmJ, and an ORF encoding a similar protein was found on contig 926; this 22 kDa protein has been designated CsmY (alignment not shown). CsmY is related to both CsmM and CsmH, and moreover, CsmY has recently been detected in chlorosomes from *Ch. aurantiacus* (*4*). Thus, each protein (CsmA, CsmO, CsmM, CsmN, and CsmY) found in the chlorosomes of *C. aurantiacus* is clearly related in sequence to a protein found in the chlorosome envelopes of the green sulfur bacteria.

Figure 8 shows a schematic representation of the sequence relationships and domain structures for the 10 chlorosome proteins of *C. tepidum*. This figure shows that, although 10 proteins are found in the chlorosome envelope of *C. tepidum*, these proteins represent only four structural motifs. Most of the observed diversity has arisen by gene duplication and divergence and/or by gene fusion events to produce new proteins from only four structural motifs. It also appears that the structural complexity of the proteins of chlorosomes of green sulfur and green filamentous bacteria are rather similar, since members of the same four motif families are encoded in the *Ch. aurantiacus* genome. Recent phylogenetic analysis of the Mg−tetrapyrrole biosynthesis genes showed that the *Chloroflexaceae* and *Chlorobiaceae* might be more closely related than some analyses based upon other markers (e.g., 16S rRNA) had suggested (*38*). Given the metabolic and biochemical diversity of these two groups, it is plausible to propose that lateral gene transfer events, or even a reaction center exchange and displacement, may have occurred in one of these two lineages. Some minor variations are even found for certain chlorosome proteins of the two very closely related species, *C. tepidum* and *Chlorobium vibrioforme* 8327 (*39*). Differences between members of the same family may help to explain the relatively low, fragmented level of sequence similarity observed for homologous proteins of *Chloroflexaceae* and *Chlorobiaceae*. The differences found in the chlorosomes of the two groups (see refs *1* and *2* for reviews) have possibly arisen through evolutionary adaptations to accommodate protein−protein contacts between the chlorosome envelope and the different types of reaction centers (type I vs type II) and primary antennae (FMO protein vs the B808−B866 complex) found in the two groups. Elements of sequence similarity for chlorosome proteins indicate that they may all have a related structure. Since the recombinant CsmH protein is produced in *E. coli* in a water-soluble form, future structural studies by either multidimensional NMR analysis or X-ray crystallography will focus on this protein.

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