Selective Protein Extraction from *Chlorobium tepidum* Chlorosomes Using Detergents. Evidence That CsmA Forms Multimers and Binds Bacteriochlorophyll *a*†

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ABSTRACT: Chlorosomes of the photosynthetic green sulfur bacterium *Chlorobium tepidum* consist of bacteriochlorophyll (BChl) *c* aggregates that are surrounded by a lipid−protein monolayer envelope that contains ten different proteins. Chlorosomes also contain a small amount of BChl *a*, but the organization and location of this BChl *a* are not yet clearly understood. Chlorosomes were treated with sodium dodecyl sulfate (SDS), Lubrol PX, or Triton X-100, separately or in combination with 1-hexanol, and the extracted components were separated from the residual chlorosomes by ultrafiltration on centrifugal filters. When chlorosomes were treated with low concentrations of SDS, all proteins except CsmA were extracted. However, this treatment did not significantly alter the size and shape of the chlorosomes, did not extract the BChl *a*, and caused only minor changes in the absorption spectrum of the chlorosomes. Cross-linking studies with SDS-treated chlorosomes revealed the presence of multimers of the major chlorosome protein, CsmA, up to homooctamers. Extraction of chlorosomes with SDS and 1-hexanol solubilized all ten chlorosome envelope proteins as well as BChl *a*. Although the size and shape of these extracted chlorosomes did not initially differ significantly from untreated chlorosomes, the extracted chlorosomes gradually disintegrated, and rod-shaped BChl *c* aggregates were sometimes observed. These results strongly suggest that CsmA binds the BChl *a* in *Chlorobium*-type chlorosomes and further indicate that none of the nine other chlorosome envelope proteins are absolutely required for maintaining the shape and integrity of chlorosomes. Quantitative estimates suggest that chlorosomes contain approximately equimolar amounts of CsmA and BChl *a* and that roughly one-third of the surface of the chlorosome is covered by CsmA.

Chlorosomes are unique light-harvesting antenna structures found in the photosynthetic green sulfur bacteria and green filamentous bacteria (*1*−*4*). Recent studies have shown that chlorosomes of *Chlorobium tepidum* contain about 215000 ± 80000 bacteriochlorophyll (BChl)[1] *c* molecules (*5*) that are aggregated in a defined manner to produce rod-shaped structures that have a diameter of 10 nm and lengths of 100−200 nm (*6*). All chlorosomes also contain a very small amount of BChl *a*, typically about 1% (by weight) of the total chlorophyll, that participates in the transfer of excitation energy from the bulk BChl *c* to the photosynthetic reaction centers in the cytoplasmic membrane (*1*, *4*). Although the precise location and organization of this BChl *a* are not known, it is likely to be associated with one or more proteins associated with the chlorosome envelope. Recent studies with chlorosomes of the green filamentous bacterium *Chloroflexus aurantiacus* suggest that BChl *a* is associated with the major chlorosome protein CsmA (*7*). In support of this hypothesis, preparations in which BChl *a* and CsmA copurify have been obtained from the chlorosomes of this bacterium (*7*, *8*).

Chlorosomes of the model green sulfur bacterium *C. tepidum* also contain CsmA and BChl *a*, and they additionally contain nine other proteins uniquely located in chlorosomes (denoted CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, and CsmX) (*1*−*4*, *9*). The genes for all ten proteins have been characterized and overexpressed in *Escherichia coli*, and antisera have been raised to each recombinant protein (*9*−*14*). Immunoprecipitation and protease susceptibility experiments have shown that all ten chlorosome proteins in *C. tepidum* copurify together with BChl *c* and that these proteins are found in the chlorosome envelope (*9*−*14*). Although three of the chlorosome proteins (CsmI, CsmJ, and CsmX) have been shown to be related to adrenodoxin-type [2Fe-2S] ferredoxins (*12*, *14*), and a mutant lacking CsmC has been described in the related organism *Chlorobium vibrioforme* (*15*), no specific functional role(s) has (have) yet been conclusively established for any of these ten proteins. Since it is well established that BChl *c* can form aggregates in vitro with properties very similar to those observed in chlorosomes (*16*−*18*), it is highly unlikely that the aggregation of BChl *c* in chlorosomes is protein-dependent.

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[1] Abbreviations: BChl, bacteriochlorophyll; BPhe, bacteriopheophytin; DTT, 1,4-dithiothreitol; EDC, 1-ethyl-3-(dimethylaminopropyl)-carbodiimide; FMO protein, Fenna−Matthews−Olson protein; HPLC, high-performance liquid chromatography; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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In this study we have examined the effects of the ionic detergent SDS and the nonionic detergents Lubrol PX and Triton X-100 on chlorosome structure and composition. Centrifugal ultrafilters with a molecular mass cutoff of about 100 kDa were used to separate extracted chlorosome proteins, all of which have a molecular mass of less than 30 kDa, and other released small molecules from the larger, residual chlorosome structures with their associated BChl *c* aggregates. Cross-linking studies performed with chlorosomes containing only CsmA are also described. Finally, some quantitative estimates related to the distribution and organization of CsmA and BChl *a* in chlorosomes from *C. tepidum* are presented. From these analyses it is concluded that CsmA binds BChl *a* and that CsmA is present as dimers and higher multimers in the chlorosome envelope.

EXPERIMENTAL PROCEDURES

Chlorosome Isolation. C. tepidum strain ATCC 49652 was grown at 48 °C in 2.4 L bottles in medium CL modified as described (*14*, *19*). Chlorosomes were isolated in the presence of 2 M NaSCN, 1 mM PMSF, and 2 mM DTT as previously described (*14*). The chlorosome fraction was collected from the sucrose gradients, diluted 4-fold with phosphate-buffered saline (10 mM potassium phosphate, pH 7.2, 150 mM NaCl), and centrifuged at 240000*g* for 1.5 h at 4 °C. The resulting chlorosome pellet was resuspended in phosphate-buffered saline containing 1 mM PMSF and 2 mM DTT, and chlorosomes were pelleted again under the same ultracentrifugation conditions. The loose material on top of the firm chlorosome pellet was removed and separately retained; the firm chlorosome pellet was resuspended in a minimal volume of phosphate-buffered saline supplemented with 1 mM PMSF and 2 mM DTT. Protein concentrations were determined as described (*14*). The resuspended chlorosomes were aliquoted into tubes and stored at −80 °C until required.

Detergent Treatment, 1-Hexanol Treatment, and Membrane Separation. Unless otherwise specified, all detergent and 1-hexanol treatments and ultrafiltration experiments were performed in Tris-buffered saline (25 mM Tris-HCl, pH 8.3, 150 mM NaCl). Tris-buffered saline with a saturating amount of 1-hexanol (approximately 0.6% w/v) was prepared as described by Matsuura and Olson (*20*). Chlorosomes were incubated in these buffers containing variable amounts of sodium dodecyl sulfate (SDS) (J. T. Baker, Phillipsburg, NJ), polyoxyethylene-9 lauryl ether (Lubrol PX) (Sigma, St. Louis, MO), or Triton X-100 (Sigma, St. Louis, MO) at ambient temperature in the dark with gentle shaking. After the incubation the chlorosomes were separated from extracted components using centrifugal microconcentrators with a 100000 molecular weight cutoff membrane (Nanosep, Pall Filtron, East Hills, NY, or Microcon, Millipore, Bedford, MA) in a benchtop centrifuge at room temperature. To ensure complete separation, the procedure included two washes with 0.2 mL of buffer. Proteins and other components that pass through the membrane ultrafilter will be referred to as the "filtrate". "Retentate" will refer to the material that did not pass through the filter under the specified conditions. For further characterization, the retentate fraction was washed off the membrane ultrafilter with a small volume of Tris-buffered saline or with buffer containing 2 M NaSCN. For quantitative recovery of BChl, the retentate was extracted three times with 0.1 mL of methanol, and the extracts were pooled.

Cross-Linking of Chlorosome Proteins. Untreated or SDS-treated chlorosomes (16.9 mg of BChl *c* mL^{-1}) from wild-type *C. tepidum* were diluted 2-fold with conjugation buffer [0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid], pH 5.5] and incubated at room temperature for 10 min. One-ninth volume of 50 mM EDC [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride] was added, and the mixture was incubated with gentle shaking at room temperature for variable time ranges (5 min to 3 h). The cross-linking reaction was stopped by addition of one-tenth volume of 1 M ammonium acetate.

For cross-linking of SDS-treated chlorosomes, an aliquot (40 μL) of purified chlorosomes (16.9 mg of BChl *c* mL^{-1}) was diluted in 0.50 mL of buffer A (25 mM Tris-HCl, pH 8.5, 150 mM NaCl), and SDS was added to a final concentration of 0.4% (w/v). Extracted proteins were separated from the chlorosome fraction by using Microcon centrifugal filter devices with YM-100 membranes (Millipore, Beverley, MA) at 13000*g* at room temperature. The retentate fraction containing the chlorosomes was washed twice with 0.2 mL of buffer A to ensure complete removal of the extracted proteins. The chlorosome retentate fraction was washed off the filter membrane with 40 μL of buffer A. SDS-treated chlorosomes were mixed with an equal volume of conjugation buffer [0.1 M MES [2-(*N*-morpholino)ethanesulfonic acid], pH 5.5] and incubated at room temperature for 10 min. EDC was added to a final concentration of 5 mM, and the SDS-treated chlorosomes were incubated with gentle shaking at room temperature for 5 min to 3 h. The cross-linking reaction was stopped by addition of one-tenth volume of 1.0 M ammonium acetate.

SDS−PAGE and Immunoblotting Analyses. After chlorosomes were subjected to the ultrafiltration treatment, the chlorosome proteins were precipitated from both the filtrate and retentate fractions with cold acetone, resuspended in dissociation buffer, and analyzed by polyacrylamide gel electrophoresis in the presence of SDS (SDS−PAGE) in the same relative proportions. Thus, the relative intensities of the protein bands for the retentate and filtrate lanes directly show the distribution of the proteins that remained in the chlorosome fraction in the retentate or that were solubilized and released into the filtrate by the detergent treatment. Proteins were analyzed by SDS−PAGE using the Tris-Tricine buffer system (*21*) and silver stained as described (*22*). The stacking gel was 4% T and 2.6% C, and the resolving gel was 15% or 16% T and 3.3% C unless otherwise stated. For some SDS−PAGE protein analysis of chlorosomes cross-linked with EDC, a resolving gel of 8% T and 3.3% C was used. The conditions for immunoblotting were identical to those described previously (*13*, *14*). Immunoreactions were detected by enhanced chemiluminescence according to the recommendations of the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ).

Absorption Spectroscopy. Absorption spectra were recorded on a Cary 14 spectrophotometer modified for computerized data acquisition by OLIS Inc. (Bogart, GA). The concentration of BChl *c* was determined in acetone or methanol extracts using the specific absorbance coefficients 92.6 or 86 L g^{-1} cm^{-1}, respectively (*23*). Monomeric BChl *c* extracted from the chlorosomes was determined at the Q_y

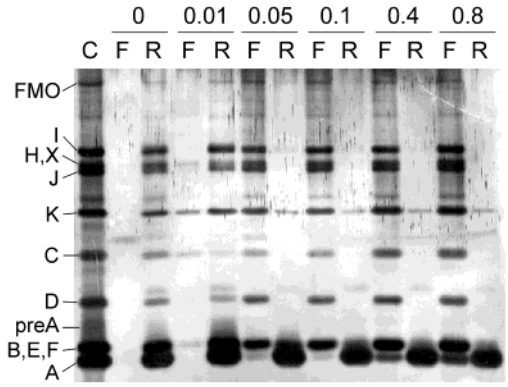

FIGURE 1: SDS−PAGE analysis of the protein composition of SDS-treated chlorosomes separated on centrifugal microconcentrators. Chlorosomes (corresponding to 0.2 mg of BChl *c* mL^{-1}) were incubated for 45 min with various concentrations of SDS (indicated at the top in percent, w/v). Lane C indicates control, untreated chlorosomes, and the lanes marked 0 were subjected to a mock filtration treatment with no SDS (0%) added. F and R indicate filtrate and retentate, respectively. Each lane was loaded with an aliquot of filtrate or retentate prepared from an amount of chlorosomes containing 21 μg of BChl. Chlorosome proteins and the FMO protein are identified on the left.

peak around 669 nm in either undiluted filtrates or filtrates diluted in methanol. The same absorption coefficient was used for monomeric BChl *c* in aqueous filtrates and in methanol extracts.

High-Performance Liquid Chromatography Analyses. The chlorosome retentates were diluted into at least 10 volumes of acetone, filtered, and analyzed by high-performance liquid chromatography as previously described (*24*), except that BChl *c* was determined at 635 nm using an absorption coefficient of 20 L g^{-1} cm^{-1}.

Electron Microscopy. Electron microscopy of chlorosomes was performed on a JEOL 1200EXII electron microscope (Peabody, MA). The chlorosomes were adsorbed on Formvar-coated, copper grids and negatively stained with 2% (w/v) uranyl acetate.

RESULTS

Effects of SDS Treatment. Figure 1 shows the results obtained when *C. tepidum* chlorosomes were incubated for 45 min in increasing concentrations of SDS in Tris-buffered saline and subjected to centrifugal ultrafiltration, and the resulting filtrates and retentates were analyzed by SDS−PAGE. Treatment of chlorosomes with very low concentrations of SDS (0.01% w/v) led to the release of some CsmC, CsmB, CsmH, and CsmK (Figure 1, lane 0.01, F). It should be noted that not all chlorosome preparations contain CsmK and that this protein has not conclusively been shown to be a component of chlorosomes (see ref *14*). Incubation of chlorosomes with 0.05% (w/v) SDS released nearly all of the chlorosome proteins except CsmA and a small proportion of the CsmB from the envelope (Figure 1, lanes 0.05). Increasing the SDS concentration to 0.1% (w/v) resulted in nearly complete extraction of all chlorosome proteins except CsmA (Figure 1, lanes 0.1). The identities of the proteins released (CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, CsmK, CsmX) were established by comparing the electrophoretic profiles to controls and by immunoblotting experiments (data not shown; see ref *14*). When the SDS concentration was increased to 0.4% or 0.8% (w/v), some time-dependent solubilization of CsmA was observed, although only a portion (~20%) was solubilized under the time and highest SDS concentration conditions employed in this experiment (Figure 1, lanes 0.4 and 0.8).

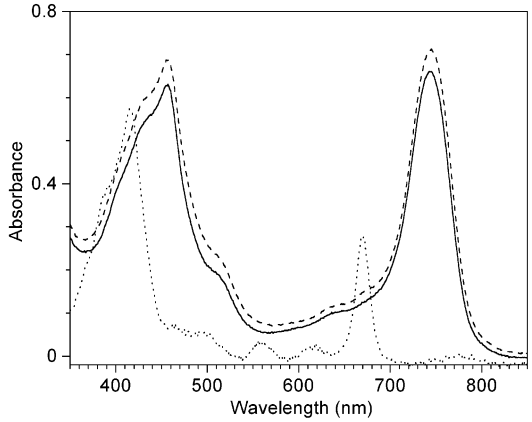

FIGURE 2: Absorption spectra of SDS-treated chlorosomes. Chlorosomes (corresponding to 0.2 mg of BChl *c* mL^{-1}) were incubated for 45 min with no SDS (solid line, 20-fold diluted) or 0.4% (w/v) SDS (dashed line, 20-fold diluted). The chlorosomes incubated with SDS were separated on a centrifugal microconcentrator, and the filtrate was recorded (dotted line, undiluted).

Figure 2 shows absorption spectra of control chlorosomes, chlorosomes treated with 0.4% (w/v) SDS, and the undiluted filtrate obtained from this sample by centrifugal ultrafiltration. The absorption spectrum of the chlorosomes was hardly changed by this treatment. After incubation with SDS and ultrafiltration, the chlorosomes formed a surface layer on the membrane filter that was very difficult to resuspend into a well-dispersed suspension, possibly due to changes in the surface hydrophobicity of the chlorosomes after most of the envelope proteins and lipids had been extracted. Incubation with SDS also caused the release of very small amounts of bacteriopheophytin (BPhe) *c* and BChl *a* that were recovered in the filtrate (Figure 2, dotted line). If the incubation time with SDS was less than about 1 h, the BPhe *c* accounted for no more than 1−2% of the total BChl *c* in the sample. The stability of the chlorosomes during the SDS treatment was also tested by incubation with 0.5% (w/v) SDS for 18 h at room temperature in the dark by recording the absorption spectrum before and after incubation. The Q_y absorbance peak decreased less than 9%, most of which was due to solubilization and pheophytinization of BChl *c* (data not shown).

Figure 3 shows electron micrographs of control chlorosomes (Figure 3A) and chlorosomes that had been incubated in 0.1% (w/v) SDS for either 2 h (Figure 3B) or 50 h (Figure 3E). The overall appearance of the control and SDS-treated chlorosomes is very similar. Chlorosomes treated with SDS for 2 h (Figure 3B; dimensions, 164 ± 30 long and 56 ± 9 nm wide, 63 samples) were about 10% longer but had the same width as the control chlorosomes (Figure 3A; dimensions, 149 ± 30 nm long and 54 ± 9 nm wide, 106 samples), although after longer exposure to SDS the length shortened to some extent (Figure 3E; dimensions, 135 ± 29 nm and 60 ± 9 nm wide, 102 samples). When the SDS-treated chlorosomes were subjected to ultrafiltration, washed, and resuspended in Tris−saline buffer in the absence of SDS but with 0.4 M NaSCN, clumps of rod-shaped aggregates

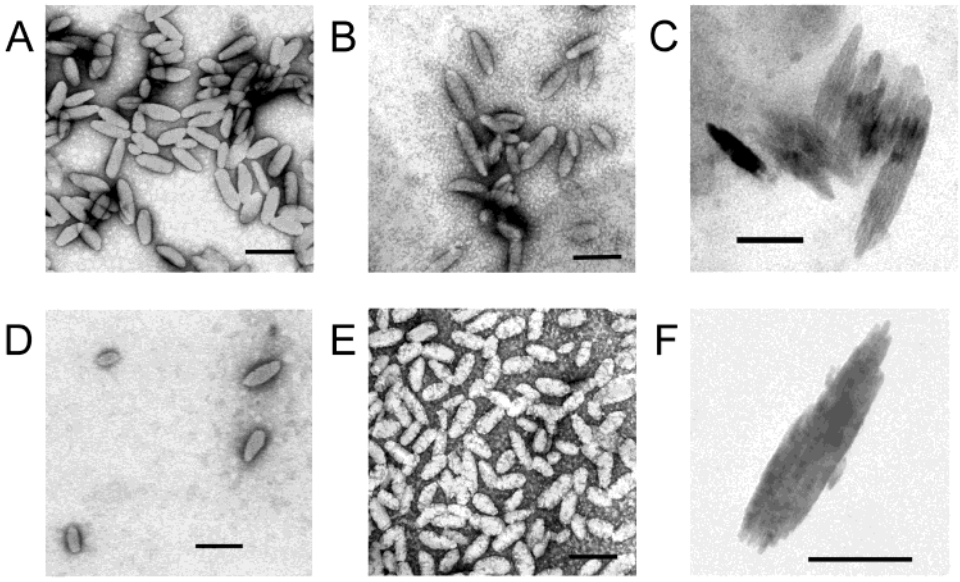

FIGURE 3: Electron micrographs of untreated chlorosomes (0.176 mg of BChl *c* mL^{-1}) (panel A), chlorosomes incubated with 0.1% (w/v) SDS (panels B and E), the resuspended retentate fractions of chlorosomes incubated with 0.1% (w/v) SDS (panels C and F), and chlorosomes incubated with 0.1% (w/v) SDS and 0.6% (w/v) 1-hexanol (panel D). The samples were incubated at room temperature for 2 h (panels A and B), 24 h (panel C), 45 min (panel D), or 50 h (panels E and F). For the samples shown in panels C and F, 0.4 M NaSCN was added to the treated chlorosomes immediately prior to the ultrafiltration to decrease their tendency to aggregate. The resulting retentates were resuspended in the detergent-free buffer containing 0.4 M NaSCN. Chlorosomes were negatively stained with 2% (w/v) uranyl acetate. The bars in panels A, B, D, and E represent 200 nm, and the bars in panels C and F represent 50 nm.

with average dimensions approximately one-half of those of intact chlorosomes were observed (Figure 3C,F). Since about 95% of the BChl *c* was retained in the retentate fraction after these treatments, the observed structures must represent the BChl *c* aggregates which have been extruded from chlorosomes, whose envelopes have been destroyed by the SDS treatment and resuspension procedure. The aggregates observed after 2, 24, and 50 h incubations were very similar in size and appearance. In the best of the electron micrographs, these structures appeared to be bundles of rods with a diameter of about 8−10 nm with faint striations perpendicular to their long axis.

HPLC analysis of the retentate fraction was performed to assess the distribution of BChl *a* after solubilization and release of the chlorosome proteins by SDS. The HPLC analyses showed that the weight-to-weight ratio of BChl *a* to BChl *c* in untreated chlorosomes was about 0.014 and that about 90% of this BChl *a* was still present in the retentate after quantitative extraction of all proteins except CsmA by incubation with either 0.1% or 0.4% (w/w) SDS for 45 min. The content of carotenoids and isoprenoid quinones also did not change significantly (data not shown), in agreement with previous observations (*25*).

Effects of Lubrol and Triton X-100 Treatments. Figure 4 shows the results obtained when *C. tepidum* chlorosomes were incubated for 45 min in increasing concentrations of Lubrol PX in Tris-buffered saline and subjected to centrifugal ultrafiltration, and the resulting filtrates and retentates were analyzed by SDS−PAGE. At the lowest concentration tested, 0.01% (w/v), Lubrol PX extracted most of the CsmC and CsmH as well as substantial amounts of CsmB, CsmD, and CsmK. However, in contrast to the results obtained with SDS, no CsmA was solubilized even at the highest concentrations tested (0.8% w/v). Substantial amounts of CsmJ and CsmI remained associated with the chlorosomes at this concentration as well. Similar results were obtained with Triton X-100

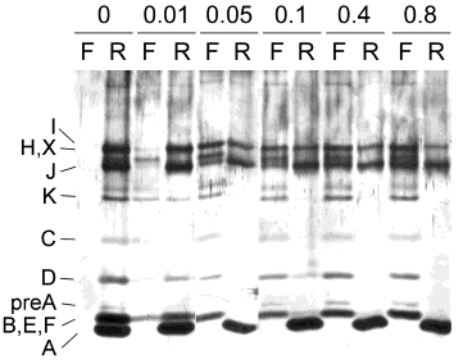

FIGURE 4: SDS−PAGE analysis of the protein composition of Lubrol PX-treated chlorosomes separated on centrifugal microconcentrators. Chlorosomes (corresponding to 0.2 mg of BChl *c* mL^{-1}) were incubated for 45 min with various concentrations of Lubrol PX (indicated at the top in percent, w/v). The lanes marked 0 were subjected to a mock filtration treatment with no Lubrol PX (0%) added. F and R indicate filtrate and retentate, respectively. Each lane was loaded with an aliquot of filtrate or retentate prepared from an amount of chlorosomes containing 21 μg of BChl *c*. Chlorosome proteins are identified on the left.

(data not shown). CsmA was not solubilized even when chlorosomes were incubated in 2% (w/v) Triton X-100 for 45 min. Electron microscopic observations showed that the size and shape of chlorosomes treated with Lubrol PX did not significantly differ from untreated chlorosomes (data not shown).

Effects of 1-Hexanol and Detergents. Treatment of chlorosomes with 1-hexanol and other alcohols has been shown to cause spectroscopic changes in the chlorosomes that have been interpreted to indicate that the BChl *c* aggregates become disaggregated (*20*, *26*, *27*). The subsequent dilution of this alcohol reverses these absorption changes and apparently causes BChl *c* aggregates to form again inside chlorosomes (*20*), although the original structural appearance

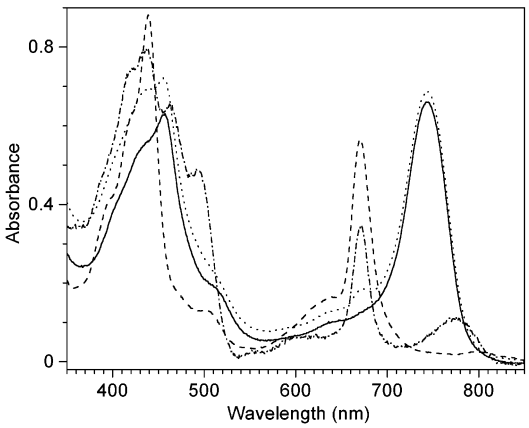

FIGURE 5: Absorption spectra of chlorosomes treated with 1-hexanol or 1-hexanol plus 0.4% (w/v) SDS. Chlorosomes (corresponding to 0.2 mg of BChl *c* mL^{-1}) were incubated for 45 min with no additives (solid line, 20-fold diluted), 0.6% (w/v) 1-hexanol (dashed line, 20-fold diluted), or 0.6% (w/v) 1-hexanol and 0.4% (w/v) SDS (dotted line, 20-fold diluted). (Samples were diluted in the same buffer used for incubation.) The chlorosomes incubated with 1-hexanol and SDS were separated on a centrifugal microconcentrator, and the spectrum of the filtrate was recorded (dashed−dotted line, undiluted). The filtrate from chlorosomes treated with 1-hexanol alone did not exhibit any detectable absorption in the visible range.

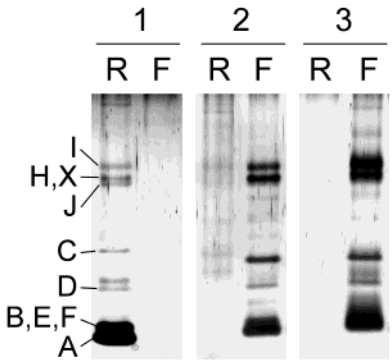

FIGURE 6: SDS−PAGE analysis of the filtrate and retentate fractions obtained by ultrafiltration from chlorosomes treated with 1-hexanol in the absence or presence of SDS or Lubrol PX. Chlorosomes (corresponding to 0.05−0.08 mg of BChl *c* mL^{-1}) were incubated for 45 min with 0.6% (w/v) 1-hexanol (lanes 1), 0.6% (w/v) 1-hexanol and 0.1% (w/v) SDS (lanes 2), or 0.6% (w/v) 1-hexanol and 0.8% (w/v) Lubrol PX (lanes 3). F and R indicate filtrate and retentate, respectively. Each lane was loaded with an aliquot of filtrate or retentate prepared from an amount of chlorosomes containing 26 μg (lanes 1) or 40 μg (lanes 2 and 3) of BChl *c*. Chlorosome proteins are identified on the left.

of the chlorosomes is not restored (*26*, *27*). As shown in Figure 5, when chlorosomes were treated with 0.6% (w/v) 1-hexanol, the absorption maximum was blue shifted from 745 to 671 nm. This shift is indicative of the disaggregation of the BChl *c* aggregates in the chlorosome. However, even after incubation for 45 min this 1-hexanol-induced disaggregation was not accompanied by the release of any proteins or pigments from the chlorosomes (Figure 6, lanes 1F and 1R).

The addition of 0.6% (w/v) 1-hexanol in combination with 0.1% or 0.4% (w/v) SDS produced very different results. The absorption spectrum was only slightly changed from the control sample by the appearance of a small absorption peak around 670 nm due to production of a small amount of monomerized BChl or BPhe *c* (Figure 5). The reason for BChl *c* not being monomerized by 1-hexanol under these conditions is possibly that the addition of detergent decreases the effective concentration of 1-hexanol in the soluble phase. Upon filtration, about 95% of the BChl *c* was found in the retentate and the remainder in the filtrate in the form of monomeric BChl (or BPhe) *c* (peak at 670 nm; Figure 5, dashed−dotted line). The filtrate also contained a component with an absorption maximum around 770 nm, suggesting that BChl *a* had been released from the chlorosomes as well. This 770 nm component was not aggregated BChl *c* because addition of an equal volume of acetone:1-hexanol (25:1 v/v) (which completely monomerizes BChl *c* in control chlorosomes; data not shown) did not eliminate this peak. The amount of this 770 nm absorbing species in the filtrate roughly corresponds to the amount of BChl *a* present in the chlorosome (i.e., roughly 1/100 of the BChl *c* absorption). Although most of the carotenoids were retained in the retentate, the filtrate contained a small amount of carotenoids which probably originates from the chlorosome envelope. All chlorosome proteins, including CsmA, were found in the filtrate fraction, and no proteins were detected in association with the BChl *c* aggregates in the retentate fraction (Figure 6, lanes 2F and 2R). An examination of chlorosomes which had been treated with both 1-hexanol and SDS after negative staining in the electron microscope showed that the chlorosomes from which all proteins had been released were similar in size and appearance to those treated for extended periods of time with SDS alone (Figure 3D).

HPLC analysis of the retentate fraction was performed to determine the distribution of BChl *a* after treatment of chlorosomes with both 0.6% (w/v) 1-hexanol and SDS [0.1% or 0.4% (w/v)] for 45 min. These analyses showed that less than 10% of the BChl *a* remained in association with the chlorosomes after the release of CsmA from the chlorosome envelope. Together with the presence of the 770 nm absorption band in the filtrate fraction (Figure 5), these results provide strong evidence that the BChl *a* has been released from the chlorosomes along with CsmA by the combined action of 1-hexanol and SDS.

When chlorosomes were treated with 0.6% (w/v) 1-hexanol and 0.8% (w/v) Lubrol PX, different results were obtained. Unlike the results obtained with 1-hexanol and SDS, the absorption maximum shifted from 745 to 672 nm; this shift indicated that the BChl *c* aggregates had been disrupted (data not shown). When these chlorosomes were subjected to ultrafiltration, all ten proteins of the chlorosome envelope and 79% of the BChl *c* were found in the filtrate fraction (Figure 6, lanes 3F and 3R).

Cross-Linking Studies. Figure 7 shows the results obtained when chlorosomes are subjected to cross-linking with the zero-length cross-linker EDC for increasing periods of time. After a treatment of only 5 min, dramatic changes had already occurred. The most readily apparent changes are the substantial decreases in the amounts of non-cross-linked pre-CsmA, CsmC, CsmD, CsmF, and CsmH. The readily apparent cross-linking products that appear at the shortest cross-linking time are a CsmA dimer and a product with an apparent mass of about 35 kDa whose composition is presently unknown. As the cross-linking time increased, there was a steady decrease in the amount of CsmA and a corresponding increase in the amount of CsmA dimers, trimers, tetramers, and larger species. CsmB was also cross-

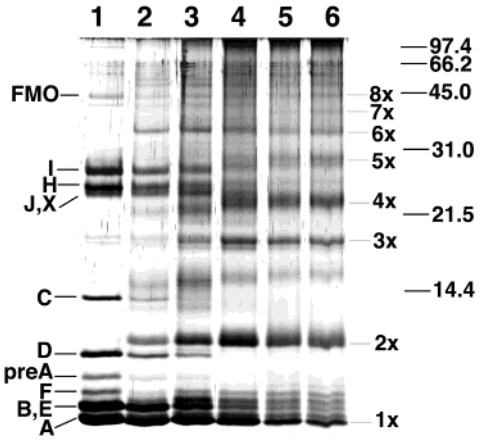

FIGURE 7: SDS−PAGE analysis of the protein composition of chlorosomes cross-linked with EDC for various periods of incubation. Chlorosomes (16.9 mg of BChl *c* mL^{-1}) that had been resuspended in 10 mM potassium phosphate buffer, pH 7.2, and 150 mM NaCl were diluted 2-fold with millimolar MES buffer, pH 5.5. Aliquots equal to 15 μg of BChl *c* were loaded for each lane. Lane 1: control, wild-type chlorosomes. Lanes 2−6: wild-type chlorosomes incubated at room temperature in the presence of 5 mM EDC for 5 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), and 3 h (lane 6).

linked to form larger products, possibly including the products with an apparent mass of about 18 and 35 kDa. Moreover, there was a steady decrease over time in the amounts of CsmI and CsmJ. The increasing apparent complexity of the lowest molecular mass species may arise due to modification of surface residues of these proteins, which can cause changes in their charge and mass. Alternatively, the removal of some CsmA and CsmB through cross-linking to form higher molecular mass species may unmask otherwise unresolved heterogeneity in these components. Attempts to identify the cross-linked products formed are ongoing and will be the subject of future work.

To simplify the interpretation of the cross-linking experiments, chlorosomes which had been treated with 0.4% (w/v) SDS to extract all proteins except CsmA were subjected to cross-linking and immunoblotting analysis with antibodies to CsmA. As shown in Figure 8A, as the cross-linking time increased, these studies showed that CsmA was cross-linked as dimers, trimers, tetramers, and other intermediate species up to octamers. Immunoblotting analysis with anti-CsmA antibodies confirmed that all of these species arise from CsmA (Figure 8B). Since these same species were observed in cross-linking experiments with control chlorosomes (see Figure 7), the results indicate that CsmA maintains its quaternary structure after all other chlorosome proteins are released from the chlorosome envelope by the SDS treatment.

DISCUSSION

The chlorosome envelope is a highly unusual and asymmetric membrane that is typically described as a lipid monolayer membrane (*6*, *28*). It is known that the galactose moieties of the galactosyl diacylglycerol headgroups are exposed on the outer surface of the chlorosome (*11*, *29*). Present structural models for chlorosomes depict the farnesyl tails of the BChl *c* rod aggregates as being directed outward toward the chlorosome surface (*30*, *31*). These farnesyl tails would be available to form hydrophobic interactions with the fatty acyl moieties of the envelope galactolipids.

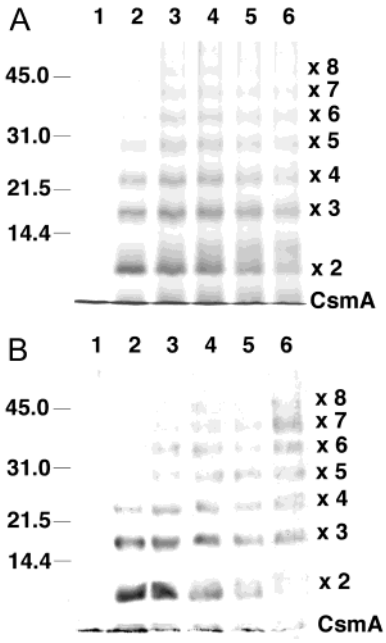

FIGURE 8: (A) SDS−PAGE analysis of the protein composition of chlorosomes that had previously been incubated with 0.4% (w/v) SDS and then subjected to ultrafiltration. The extracted chlorosomes were resuspended in 20 mM Tris-HCl buffer, pH 8.0, and were then diluted 2-fold into 100 mM MES buffer, pH 5.5. EDC was added to a final concentration of 5 mM, and the chlorosomes were then incubated at room temperature for various times. Aliquots equal to 15 μg of BChl *c* (for silver staining) or 60 μg of BChl *c* (for immunoblotting) were mixed with dissociation buffer, heated, and analyzed by SDS−PAGE (8% T, 3.3% C; see Experimental Procedures). The resulting gel was silver stained (panel A), or the cross-linked proteins were transferred electrophoretically to a nitrocellulose filter and probed with rabbit polyclonal antibodies to CsmA (*13*) as described in Experimental Procedures (panel B). Lane 1: control, wild-type chlorosomes. Lanes 2−6: chlorosomes incubated at room temperature with 5 mM EDC for 5 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), or 3 h (lane 6). The positions of CsmA and various cross-linked species (dimers, trimers, up to octamers) are indicated at the right. The positions of molecular mass markers in kilodaltons are indicated at the left.

The highly asymmetric structure of the chlorosome envelope membrane is reminiscent of the asymmetry found in the outer membranes of Gram-negative bacteria (*32*, *33*). The lipids of the two leaflets of the outer membrane are distinctly different. The lipopolysaccharide units forming the outer leaflet are chemically distinct from the phospholipids of the inner leaflet and of the cytoplasmic membrane. The proteins of the outer membrane are structurally distinct from the proteins localized in the cytoplasmic membrane and usually form β-barrels of differing diameters. No outer membrane proteins with transmembrane α-helices have yet been reported (*34*, *35*). Present evidence indicates that all chlorosome proteins are associated with the chlorosome envelope layer (*10*−*14*). One role of the chlorosome proteins could be to stabilize the unusual membrane structure of the chlorosome in the cytoplasm. Hydropathy and secondary structure prediction analyses of chlorosome proteins suggest that the chlorosome proteins lack transmembrane α-helices and are likely to form predominantly β-sheet secondary structures. The absence of signal peptides and transmembrane α-helices in chlorosome proteins could ensure the proper targeting of chlorosome proteins to the envelope.

It is shown here that the proteins of the chlorosome envelope are easily extracted from the envelope membrane at detergent concentrations that are equal to or greater than the critical micelle concentration (*36*). Both the anionic detergent SDS and the nonionic detergents Lubrol PX and Triton X-100 were extremely effective in extracting proteins other than CsmA from the chlorosome envelope. However, the removal of most, or even all, chlorosome proteins from the envelope has little or no effect on the absorption properties of the BChl *c* aggregates of the chlorosomes (Figures 2 and 5). Moreover, the size and gross morphology of the chlorosomes lacking most or even all proteins was similar to that of control chlorosomes (Figure 3).

During the 1980s and the first half of the 1990s, the roles of proteins in chlorosomes, and for a while even the occurrence of proteins in chlorosomes, were hotly debated and controversial issues. At that time, all well-studied photosynthetic light-harvesting systems were composed of pigment−protein complexes that could form larger, organized structures. It was natural to assume that chlorosomes would present a similar structural organization. Although it is now clear that pigment−pigment interactions are the predominant structural interactions in chlorosomes, this idea was not immediately embraced by some workers in the field.

Several factors conspired to obscure the protein composition of chlorosomes as well as the locations of these proteins in the structure. First, two types of organisms, the green gliding bacterium *Cfx. aurantiacus* and various green sulfur bacteria of the genus *Chlorobium*, have been widely used in studies of chlorosomes. The chlorosomes from these organisms have often been treated as though they are identical, although it is clear that significant differences exist (see refs *1*, *3*, and *4*). Second, various workers have used many different procedures for the isolation of chlorosomes over the past 20 years. In particular, many investigators have included detergents in their isolation buffers at concentrations ranging from about 0.1% (w/v) up to 5% (w/v) (*37*−*44*). From the results presented here, it is clear that the inclusion of detergents at high concentrations would cause most proteins to be extracted from the chlorosome envelope and that this extraction would increase with the time of exposure to the detergent. On the basis of results in some of the papers cited above and those of Miller et al. (*45*), this is also clearly true for *Cfx. aurantiacus*. Finally, all chlorosome proteins have molecular masses of less than 27 kDa; some of these proteins do not stain reliably with Coomassie blue, and some proteins even diffuse out of polyacrylamide gels during staining procedures if they are not previously fixed by formaldehyde cross-linking.

The introduction of sodium thiocyanate by Gerola and Olson (*46*) as a chaotropic agent to release chlorosomes from the FMO and cytoplasmic membranes of *Chlorobium limicola* f. *thiosulfatophilum* was a significant advance in obtaining reproducible preparations of chlorosomes from green sulfur bacteria. Using this isolation method, Chung et al. (*9*) showed by N-terminal protein sequence analyses that the chlorosomes of *C. tepidum* contained ten proteins. Although some mass differences have been observed for specific chlorosome proteins, the same ten proteins have been shown to be present in chlorosomes isolated from the BChl *d*-containing strain *C. vibrioforme* strain 8327D and the BChl *e*-containing strain *Chlorobium phaeobacteroides* strain 1549 (*3*). These results differ dramatically from those of Stolz et al. (*41*), who observed that chlorosomes from a *C. phaeobacteroides* strain isolated in the presence of 2% (w/v) miranol contained only two proteins. One was probably CsmA, while the other was possibly a contaminant, since no proteins of this mass have been observed in more recent studies (*3*, *12*−*14*). These results, as well as those obtained with *Cfx. aurantiacus* chlorosomes treated with SDS or LDS (*39*−*45*), demonstrate how completely different results and conclusions can be obtained depending on the isolation and treatment conditions employed.

The present study shows that BChl *a* is not extracted from chlorosomes of *C. tepidum* when all proteins except CsmA are extracted by SDS treatment. However, BChl *a* is extracted from the chlorosome when all proteins, including CsmA, are released by incubation with SDS and 1-hexanol. Preparations from chlorosomes of *Cfx. aurantiacus* that are highly enriched in CsmA and BChl *a* have been reported (*7*, *8*). These correlations between BChl *a* and CsmA extraction imply that, if BChl *a* is protein-bound, then it must be bound to CsmA. Mutational studies are consistent with this conclusion. Nine mutant strains, each lacking a single chlorosome protein (CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, or CsmX), have been constructed, and the chlorosomes from all of these mutants retain normal levels of BChl *a* (*15*; N.-U. Frigaard, H. Li, and D. A. Bryant, manuscript in preparation). Attempts to inactivate the *csmA* gene have thus far failed (*15*); this suggests that CsmA may play an essential role in light harvesting. These mutational studies strongly imply that the BChl *a* in the chlorosomes of *C. tepidum* is bound to CsmA. The present study and previous reports suggest that the CsmA−BChl *a* complex may also contain bound carotenoids. Figure 5 shows that carotenoids are extracted along with BChl *a* from chlorosomes by the SDS and 1-hexanol treatment. The CsmA−BChl *a*-enriched preparations made from the chlorosomes of *Cfx. aurantiacus* contain appreciable amounts of carotenoids (*7*, *8*). Finally, kinetic spectroscopy suggests a close association between carotenoid and BChl *a* in chlorosomes isolated from both *Cfx. aurantiacus* and *Chlorobium* species (*47*, *48*).

The results presented in this paper suggest that it might be possible to isolate a CsmA−BChl *a* complex from the chlorosome envelopes of *C. tepidum* by first extracting all proteins except CsmA from SDS-treated chlorosomes and then solubilizing CsmA and BChl *a* in a subsequent incubation step by using 1-hexanol and SDS. Some improvements, such as the use of a milder detergent, will probably be necessary. SDS might denature CsmA and cause the liberation of BChl *a* from CsmA. The 795 nm absorption peak due to bound BChl *a* in BChl *c*-free chlorosomes shifts to 770 nm and decreases in amplitude when such chlorosomes are incubated with SDS (N.-U. Frigaard and D. A. Bryant, unpublished data). It is not known whether these changes are due to degradation of the BChl *a* or to detergent-induced changes in the environment of the BChl *a*. Similar effects of detergents on protein-bound BChl *a* have been observed in the BChl *a*-containing light-harvesting complex LH2 from purple bacteria (*49*). In this case, reconstitution of the native BChl *a* absorption is possible by careful removal of SDS from the preparation.

Quantitative estimates of the BChl *a*:CsmA ratio support the hypothesis that CsmA binds BChl *a* in a defined, stoichiometric manner. Although protein:BChl *c* ratios of 0.4−0.5 (w/w) have been reported for chlorosomes isolated from *Chlorobium* species (*9*), the most highly purified chlorosome preparations have protein to BChl *c* ratios as low as 0.23 (w/w) (*14*). As estimated from stained SDS−PAGE gels, CsmA comprises roughly one-third to one-half of the total protein of chlorosomes. Thus, the experimentally observed content of CsmA per BChl *c* in chlorosomes is about 0.08−0.12 (w/w). The experimentally determined BChl *a*:BChl *c* ratio in chlorosomes from *C. tepidum* is about 0.014 (w/w) (see Results). If BChl *a* and CsmA are present in equimolar amounts, a BChl *a*:BChl *c* ratio of 0.014 (w/w) corresponds to a CsmA content of about 0.09 (w/w) per BChl *c*, which is within the observed range. (This value would indicate that CsmA accounts for about 40% of the total protein of the chlorosome.) These calculations strongly suggest that CsmA binds one BChl *a* per monomer. If CsmA bound only one BChl *a* per dimer, CsmA would then be estimated to be 80% of the total protein. Correspondingly, if CsmA bound two BChl *a* molecules per monomer, CsmA should only account for 20% of the total protein. Both of these values are sufficiently outside the observed range that they are unlikely to be correct. Gerola and Olson (*46*) found that the BChl *a*−protein complex associated with chlorosomes of *C. limicola* f. *thiosulfatophilum* has a large, conservative circular dichroism spectrum that indicates a strong, excitonic interaction between two or more BChl *a* molecules. Although the data reported here suggest that each CsmA monomer binds a single BChl *a* molecule, the demonstration that CsmA forms dimers, tetramers, and octamers could still account for the circular dichroism properties of the complex.

If an average *C. tepidum* chlorosome is prolate to cylindrical in shape, with dimensions of about 150 nm long by 50 nm wide, its volume would be about 250000 nm^3, and it would have a surface area of about 25000 nm^2. If roughly 60% of the volume is BChl *c* (*4*) and the ratio of CsmA to BChl *c* is 0.1 (v/v), the volume of CsmA per chlorosome would be roughly 15000 nm^3. Thus, if CsmA forms a layer of about 2−3 nm in thickness, which corresponds to the measured thickness of the chlorosome envelope (*6*, *28*), CsmA could cover 20−35% of the chlorosome surface. Although this calculation provides only a rough estimate, it nevertheless suggests that CsmA could at least form a dense layer in the contact region where the chlorosome is connected to the FMO protein and the cytoplasmic membrane. In freeze−fracture images of chlorosomes of *Cfx. aurantiacus*, a regular, striated structure can be observed in the chlorosome envelope in the vicinity of the membrane attachment site but not on the cytoplasmic surface of the chlorosome envelope (*6*, *50*). It seems plausible that this striated layer could represent a paracrystalline array of CsmA.

In conclusion, the experimental evidence and calculated estimates from the compositional data for chlorosomes strongly suggests that CsmA forms an equimolar complex with BChl *a* in chlorosomes of *C. tepidum*. This complex, which likely contains one or more carotenoid molecules (*8*), can form dimers, tetramers, and at least octamers. In *C. tepidum* and other green sulfur bacteria, CsmA probably forms a baseplate BChl *a* complex that transfers light energy from the BChl *c*-containing rod aggregates of the chlorosomes to the FMO trimers that make close contact with the underlying reaction centers. By using an appropriate combination of detergent and 1-hexanol, it should be possible to purify this protein from either carotenosomes (*24*) or chlorosomes that have been previously stripped of all other proteins by the detergent treatments described here. Attempts to purify such a complex are in progress.

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