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[2Fe-2S] Proteins in Chlorosomes: CsmI and CsmJ Participate in Light-Dependent Control of Energy Transfer in Chlorosomes of *Chlorobaculum tepidum*

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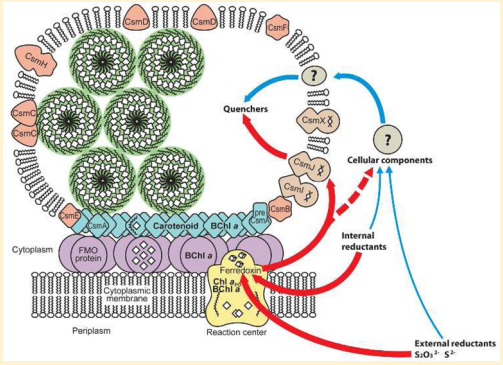
Supporting Information

ABSTRACT: Chlorosomes of *Chlorobaculum tepidum* are formed from stacks of *syn*−*anti* coordinated bacteriochlorophyll *c* dimers, which form a suprastructure comprised of coaxial nanotubes and are surrounded by a glycolipid monolayer envelope containing 10 proteins. Three of these proteins, CsmI, CsmJ, and CsmX, have sequences very similar in their N-terminal domains to those of [2Fe-2S] ferredoxins of the adrenodoxin/putidaredoxin subfamily. The roles of these proteins in chlorosomes were studied in single-, double-, and triple-mutant strains. In each mutant, only the protein(s) corresponding to the mutated gene(s) was missing, and the amounts of other chlorosome proteins did not vary significantly. Electrophoretic analyses and immunoblotting showed that CsmX was much less abundant than CsmI or CsmJ. The growth rates and the pigment and isoprenoid quinone contents of isolated chlorosomes of the mutants were similar to wild-type values. Quenching and recovery of energy transfer in isolated chlorosomes and intact cells were studied by measuring fluorescence emission after exposure to or removal of oxygen. Oxygen-induced activation of the quencher in isolated chlorosomes or in intact cells was largely independent of CsmI and CsmJ. This may be because oxygen can diffuse across the chlorosome envelope easily and directly reacts with the quencher. However, CsmI and CsmJ were required to restore energy transfer fully after isolated chlorosomes were exposed to oxygen. Studies with intact cells suggested that cells contain both light-dependent and light-independent pathways for reducing the quenching species in chlorosomes and that CsmI and CsmJ are components of a light-dependent pathway.



Green sulfur bacteria (GSB) are obligate photolithoautotrophs that normally occur in extremely light-limited and strictly anoxic environments.[1−5] GSB contain remarkable antenna complexes, chlorosomes, which efficiently harvest light at extremely low irradiance levels. Chlorosomes are sac-like structures, which are ∼70−180 nm long and ∼30−60 nm wide and are tightly appressed to the inner surface of the cell membrane. They transfer light energy to the Fenna−Matthews−Olson protein (FMO), which subsequently transfers the energy to homodimeric, type 1 photosynthetic reaction centers in the cytoplasmic membrane.[1−5] Structural cryoelectron microscopy and solid-state nuclear magnetic resonance studies have shown that chlorosomes in wild-type (WT) cells of the model GSB, *Chlorobaculum tepidum*, are assembled from multiple, coaxial nanotubes, which have a lamellar spacing of approximately 2.1−2.3 nm.[6,7] The basic building blocks of these lamellae are stacks of *syn*−*anti* bacteriochlorophyll (BChl) *c* dimers.[8] An alternative suprastructure formed from parallel monomer stacks of all-*syn* and all-*anti* BChls was found in a *bchQR* mutant containing [8-Et,12-Me]BChl *c*.[9] The nanotubular suprastructures probably correspond to "rod elements" that were initially believed to be the main structural features produced by highly aggregated BChl *c*, *d*, or *e*.[10−13]

Proteins are absent from the chlorosome interior, and proteins do not directly participate in organizing the suprastructure of BChl molecules in the chlorosome interior.[1,8,9,13−15] The chlorosome envelope in the model GSB *Cba. tepidum* is an asymmetric, glycolipid-containing monolayer membrane that contains 10 proteins: CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ and CsmX. Comparative sequence analyses have shown that chlorosome proteins belong to four structural motif families, three of which, CsmA/E, CsmB/F, and CsmC/D, share no obvious relationship with other proteins.[16] Complexes of CsmA, BChl *a*, and carotenoids form a paracrystalline baseplate structure that forms the portion of the chlorosome envelope that interacts with the FMO, which in turn directly interacts with the homodimeric type I reaction centers located in the cytoplasmic

Received: October 25, 2012
Revised: January 28, 2013
Published: January 31, 2013

membrane.[5,17,18] A fourth structural motif is found at the N-termini of CsmI, CsmJ, and CsmX. Each of these chlorosome proteins has an N-terminal adrenodoxin-like domain, which ligates one [2Fe-2S] cluster [refs 16 and 19 and the following paper (DOI: 10.1021/bi301455k)].

In *Cba. tepidum*, each chlorosome contains up to 250000 BChl *c* molecules, which harvest light energy for ~25 reaction centers.[1,20,21] The excitation energy absorbed by BChl *c* and carotenoids is transferred to pigments with progressively lower energy levels: BChl *a* associated with CsmA in the baseplate (795 nm), BChl *a* associated with FMO (810 nm), and finally BChls associated with the type 1 reaction centers (820−840 nm).[1,2,4] Energy transfer in chlorosomes and FMO is regulated by redox potential in whole cells and isolated chlorosomes.[22−25] The energy transfer efficiency can decrease from nearly 100 to <10% under oxidizing conditions, and efficient energy transfer can be restored only if reducing reagents are added.[22−26] It is generally believed that the quenching mechanism prevents cells from producing reactive oxygen species, which easily form and can be lethal when GSB are exposed to oxygen.[26,27] Under oxic conditions, quenchers (e.g., chlorobiumquinone and menaquinone) within chlorosomes become oxidized, and these molecules efficiently block energy transfer, which decreases the rate of formation of cytotoxic oxygen species that form when cells are exposed to light and oxygen.[25,27]

CsmI, CsmJ, and CsmX have been proposed to play roles in controlling the transfer of excitation energy from the chlorosomes to the reaction centers.[16,19] In this study, the functions of these three Fe−S proteins were investigated by insertional inactivation of each gene individually, by the construction of all possible double mutants, and by the construction of a triple mutant that lacked all three Fe−S proteins. The kinetics of fluorescence quenching upon exposure to oxygen and the restoration of energy transfer, as monitored by recovery of fluorescence emission upon a return to anoxic conditions, was studied in each of the mutants as well as in the WT. The results suggested that CsmI and CsmJ participate in the reductive inactivation of the quencher when chlorosomes and cells are returned to anoxic conditions. The third Fe−S protein, CsmX, was much less abundant than CsmI and CsmJ, and it made an only minor contribution to fluorescence quenching and recovery in *Cba. tepidum*.

MATERIALS AND METHODS

Strains and Growth Conditions. The WT2321 strain of *Cba. tepidum* [formerly *Chlorobium tepidum* (see ref 28)], which is a plating strain derived from *Cba. tepidum* strain ATCC 49652,[29] was used in these studies.[30] The solid and liquid growth media for *Cba. tepidum* were previously described.[31] Manipulations and the growth of small volumes of cells were performed in an anoxic chamber at ~40 °C (Coy Laboratory Products, Grass Lake, MI). Large cultures were grown in tightly sealed 2.4 L bottles in an external incubator at 45−48 °C.[31] Growth rates were determined at 47 °C by measuring the optical density at 600 nm as previously described.[15,27,32,33] Standard molecular biological procedures were performed in *Escherichia coli* strain DH5α as previously described.[15,34]

Insertional Inactivation of the *csmI*, *csmJ*, and *csmX* Genes. Three different antibiotic resistance markers were used in the construction of *csmI*, *csmJ*, and *csmX* mutants: *aacC1*, which confers gentamicin resistance, from plasmid pMS255; *aadA*, which confers resistance to streptomycin and spectinomycin, from plasmid pHP45Ω; and a dual resistance *cat-ermC* cassette, which confers resistance to both chloramphenicol (used in *E. coli*) and erythromycin (used in *Cba. tepidum*), from plasmid pRL409.[35] Resistance markers were separately inserted into *csmI*, *csmJ*, and *csmX* gene fragments by exploiting available protein expression constructs pET3d::*csmI*, pET3d::*csmJ*, and pET32a::*csmX*, respectively[16,19] (see Table S1 of the Supporting Information). Restriction maps of the resistance markers and gene inactivation constructs are shown in Figure S1 of the Supporting Information.

The plasmids for insertional inactivation of the *csmI*, *csmJ*, and *csmX* genes were linearized with AhdI, and natural transformation was performed as described previously.[31] The *csmI*, *csmJ*, and *csmX* single mutants were first constructed by transformation of pET3d::*csmI*::*erm-cat* (Em^R), pET3d::*csmJ*::*aacCI* (Gm^R), and pET32a::*csmX*::*aadA* (Sm^R, Sp^R) into WT *Cba. tepdium* cells (see ref 15). Plasmid pET3d::*csmJ*::*aacCI* was subsequently used to transform the *csmI* and *csmX* mutants, which produced the *csmI csmJ* and *csmJ csmX* double mutants, respectively. Plasmid pET3d::*csmI*::*erm-cat* was also used to transform the *csmX* mutant to produce a *csmI csmX* double mutant. The *csmI csmJ csmX* triple mutant was constructed by transforming this *csmI csmX* mutant strain with pET3d::*csmJ*::*aacCI*. The complete segregation of alleles and validation of mutants was performed by analytical polymerase chain reaction (PCR) analyses with primers specific for *csmI*, *csmJ*, and *csmX*[15,16,19] (data not shown). Strains were also validated by immunoblotting with rabbit polyclonal antibodies specific for CsmI, CsmJ, and CsmX (see Results).

Protein Electrophoresis and Immunoblotting Analyses. The protein composition of chlorosomes was analyzed by polyacrylamide gel electrophoresis (PAGE) on Tris-Tricine gels containing sodium dodecyl sulfate (SDS) as previously described.[17] Silver staining was performed as described previously.[16,36] For immunoblotting, chlorosome proteins corresponding to 100−200 μg of BChl *c* were precipitated with cold acetone to extract the lipids and pigments, and the protein pellet was resuspended in sample loading buffer and loaded onto the gel after the mixture had been boiled for 1 min. Proteins were transferred onto nitrocellulose membranes (0.45 μm; Schleicher and Schuell, Keene, NH) by using a semidry transfer cell (Bio-Rad, Richmond, CA). Rabbit polyclonal antibodies to each Fe−S protein were generated by using recombinant proteins purified from inclusion bodies produced in *E. coli* as the immunizing antigen.[16,19] Conditions for immunoblotting and protein detection by enhanced chemiluminescence were previously described.[16,17]

Chlorosome Isolation. Chlorosomes were isolated on linear 7 to 47% (w/v) sucrose gradients essentially as described previously.[16] Freshly harvested cells were disrupted by three passages through a chilled (4 °C) French pressure cell operated at 138 MPa, after lysozyme treatment (3 mg/mL, room temperature for ~20 min); chlorosomes were pelleted by ultracentrifugation at 220000*g* for 2 h at 4 °C. The resuspended chlorosomes were applied to 7 to 47% (w/v) continuous sucrose gradients and subjected to ultracentrifugation at 220000*g* for 18 h at 4 °C. The chlorosome fraction was collected, diluted 4-fold with phosphate-buffered saline (pH 7.2), and concentrated by centrifugation at 240000*g* for 1.5 h. The resulting pellet was resuspended in the same buffer, and the resuspended chlorosomes were pelleted again under the same conditions. The loosely pelleted chlorosome fraction was collected separately from the firm pellet, and the firmly pelleted

chlorosomes were resuspended in a minimal volume of phosphate-buffered saline containing 1 mM phenylmethanesulfonyl fluoride and 2 mM dithiothreitol. Purified chlorosomes were aliquoted and stored at −80 °C for future use. The BChl *c* concentration was determined by measuring the absorbance of methanol extracts using the specific absorption coefficient of 86 L g^{-1} cm^{-1}.[37]

Fluorescence Quenching and Recovery in Chlorosomes. Fluorescence quenching and recovery of cells and chlorosomes were measured with an SLM-Aminco 8100 Series 2 spectrofluorometer (Spectronic Instruments, Inc., Rochester, NY) at 22−25 °C. Chlorosomes were excited with 460 nm light generated by an ELXE-500 Xenon lamp, and the fluorescence emission was recorded at 772 nm with a time resolution of 0.5 s. For quenching experiments, chlorosomes were diluted to a BChl *c* concentration of 20 μg/mL in oxygen-free buffer [10 mM KH_2PO_4 (pH 7.0), saturated with nitrogen by sparging] that contained 1 mM dithionite and were incubated for 1 h to allow full fluorescence recovery. Anoxic chlorosomes were then mixed 1:1 (v/v) with oxic buffer [10 mM KH_2PO_4 (pH 7.0), saturated with air by sparging] by using a model SFA-20 rapid kinetics stopped-flow accessory (Hi-Tech Limited, Salisbury, U.K.) with a silica observation cell. For the restoration of fluorescence, chlorosomes were diluted to a BChl *c* concentration of 200 μg/mL with oxic buffer for 1 h. The oxic chlorosome solution was then mixed 1:1 (v/v) with anoxic buffer to produce a final dithionite concentration of 12.5, 25, or 50 mM by using the rapid mixing device described above.

Fluorescence Recovery in *Cba. tepidum* Cells. *Cba. tepidum* cells in the late exponential phase were pelleted at 13000*g* for 30 s and resuspended in oxic potassium phosphate buffer (pH 7.0). The cells were exposed to air for 72 h (3 d) in the dark prior to centrifugation and resuspension in anoxic potassium phosphate buffer containing 2 mM $S_2O_3^{2-}$, 2 mM S^{2-}, or no addition. Fluorescence recovery was performed in darkness, at low irradiance (0.2 μmol of photons m^{-2} s^{-1}) and at room light (~50 μmol of photons m^{-2} s^{-1}) for the specified time periods.

RESULTS

Construction and Verification of Mutants Lacking CsmI, CsmJ, and CsmX. Strains carrying single null mutations in the *csmI*, *csmJ*, and *csmX* genes were constructed and partly characterized in a previous study.[15] Those studies, as well as others,[33] suggested that some chlorosome envelope proteins might be functionally redundant. Thus, mutants lacking all combinations of two of these Fe−S proteins as well as a mutant lacking all three Fe−S proteins were also constructed in hopes of revealing functions for these Fe−S proteins of the chlorosome envelope. Analytical PCR experiments showed that the WT and mutant alleles of *csmI*, *csmJ*, and *csmX* were segregated completely in all of the mutant strains (data not shown).

To verify that the corresponding proteins were absent as anticipated in each of the strains, chlorosomes were isolated from each mutant strain, and the protein compositions of their envelope proteins were assessed by SDS−PAGE and immunoblotting (Figure 1). Figure 1A shows that the abundances of other chlorosome proteins (CsmA−CsmF and CsmH) were similar in the chlorosomes of the WT and mutants lacking various combinations of the Fe−S proteins. These results showed that the expression and incorporation of other proteins into chlorosome envelopes were not affected by

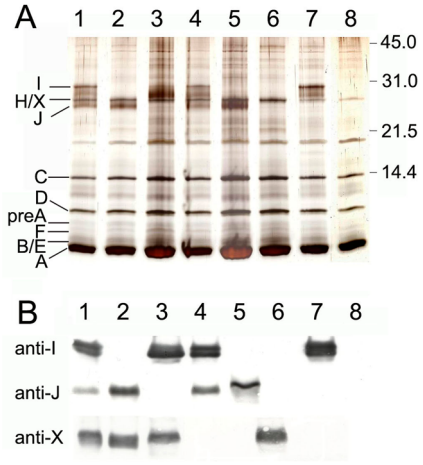


Figure 1. SDS−PAGE analysis of the protein composition of chlorosomes isolated from WT and mutants *csmI*, *csmJ*, *csmX*, *csmI csmX*, *csmI csmJ*, *csmJ csmX*, and *csmI csmJ csmX*. (A) SDS−PAGE analysis of chlorosome proteins detected by silver staining. The proteins are identified at the left, and the positions of selected marker proteins in kilodaltons are indicated at the right. (B) Immunoblots probed with rabbit polyclonal antibodies to CsmI, CsmJ, and CsmX. Chlorosomes were isolated from *Cba. tepidum* WT (lane 1) and mutants *csmI* (lane 2), *csmJ* (lane 3), *csmX* (lane 4), *csmI csmX* (lane 5), *csmI csmJ* (lane 6), *csmJ csmX* (lane 7), and *csmI csmJ csmX* (lane 8). Note that the cross-reacting polypeptides shown are derived from three separate immunoblots that were made with similar but nonidentical gels. The strips corresponding to the three cross-reacting proteins were excised and repositioned for emphasis and clarity.

the absence of CsmI, CsmJ, and/or CsmX. Using polyclonal antibodies against CsmI, CsmJ, and CsmX, immunoblotting experiments (Figure 1B) confirmed that each of the expected combinations of Fe−S protein(s) was completely missing in the single and double mutants, and that all three Fe−S proteins were absent from the chlorosome envelope of the *csmI csmJ csmX* triple mutant. The results clearly demonstrated that none of the three Fe−S proteins was essential for the viability of *Cba. tepidum* when cells were grown under standard laboratory conditions. The results further showed that no compensatory increase in the amount(s) of the remaining Fe−S protein(s) occurred in mutants lacking one or two of these proteins. On the basis of silver staining band intensities of SDS−PAGE gels (Figure 1A), and consistent with previous observations (e.g., see Figure 3 of ref 15 and discussion in ref 17), we concluded that CsmI and CsmJ occurred in an ~1:1 molar ratio in WT chlorosomes.

Pigment and Quinone Contents in Mutants Lacking CsmI, CsmJ, and CsmX. The pigment and quinone contents of isolated chlorosomes are listed in Table S2 of the Supporting Information. The BChl *a* and carotenoid contents of the mutant chlorosomes were similar to those of WT chlorosomes. These data suggest that the Fe−S proteins on the chlorosome envelope probably do not play a role in pigment uptake or in the assembly of the BChl *c* suprastructure in chlorosomes. Although the total quinone contents of the chlorosomes of the mutant strains were similar to those of WT, the chlorosomes of the *csmI csmJ* and *csmI csmJ csmX* mutants contained less total chlorobiumquinones (~65% of the average of the other strains) and correspondingly more menaquinone-7 (~180% of the average of the other strains). The *csmI* single mutant also had a lower content of chlorobiumquinones and somewhat higher content of menaquinone-7, but this trend was not observed in

dx.doi.org/10.1021/bi301454g | *Biochemistry* 2013, 52, 1321−1330

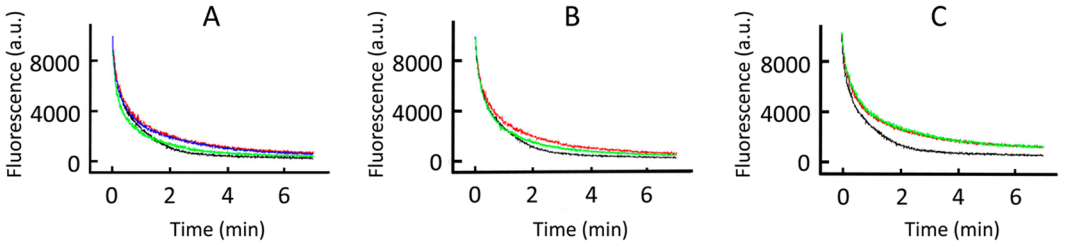


Figure 2. Quenching of fluorescence emission in isolated chlorosomes. The final chlorosome BChl *c* concentration corresponds to 10 μg/mL. Fluorescence emission was recorded at the emission maximum (772 nm) with a time resolution of 0.5 s. (A) Chlorosomes from the WT (black), *csmI* mutant (red), *csmJ* mutant (green), and *csmX* mutant (blue). (B) Chlorosomes from the WT (black), *csmI csmX* mutant (red), and *csmJ csmX* mutant (green). (C) Chlorosomes from the WT (black), *csmI csmJ* mutant (red), and *csmI csmJ* csmX mutant (green).

the chlorosomes of the *csmI csmX* mutant. Because of this result, it is not certain that these differences in quinone content are directly correlated with the absence of CsmI.

Growth of the Mutants Lacking CsmI, CsmJ, and CsmX. The growth rates of the WT and all mutant strains were tested under three irradiance levels: 8, 30, and 150 μmol of photons m^{-2} s^{-1} (Table S3 of the Supporting Information). The growth rates of the *csmJ* and *csmJ csmX* mutant strains were ∼15% slower than that of WT at 47 °C, but all other mutants except *csmI csmJ* had growth rates that differed by less than 10% from those of the WT. Unexpectedly, the initial isolate of the *csmI csmJ* mutant did not grow at high irradiance, and it grew at a rate only approximately half of that of the WT at low irradiance and 47 °C. Further studies showed that unlike other strains, this *csmI csmJ* mutant strain was sensitive to both light and temperature (data not shown). The WT grows optimally at 47−48 °C, but the optimal growth temperature for the *csmI csmJ* mutant strain was 39−40 °C. High irradiance levels (>60 photons m^{-2} s^{-1} at 45 °C or >250 photons m^{-2} s^{-1} at 40 °C) greatly decreased the growth rate of this particular *csmI csmJ* mutant strain. Therefore, two other *csmI csmJ* mutant strains were studied. Although they were not as severely affected as the initial mutant, each grew more slowly than the WT at high irradiance (growth rates were 50−60% of that of the WT) (Table S3 of the Supporting Information). Surprisingly, the growth behavior of the *csmI csmJ csmX* triple mutant was similar to that of WT. Therefore, we concluded that the phenotype of the *csmI csmJ* mutant was not specifically correlated with the loss of either CsmI or CsmJ, that the growth defect was probably due to a secondary mutation(s), and that the Fe−S proteins did not affect the fitness of cells grown under standard anoxic conditions.

Cell Viability after Aerobic Exposure. Table S4 of the Supporting Information shows a comparison of the viability of WT cells and *csmI csmJ csmX* triple-mutant cells after incubation for 3 or 6 days under dark anoxic conditions or under two oxic conditions: in darkness or in ambient room light (∼50 μmol of photons m^{-2} s^{-1}). No loss of viability was observed when WT cells were incubated under dark anoxic conditions for up to 6 days, but only 69% of the *csmI csmJ csmX* cells survived after dark anoxia for 6 days. After 3 days under oxic conditions in the dark, 58% of the WT cells and 49% of the mutant cells were still viable, and these values further decreased to 28 and 13%, respectively, after 6 days. After being exposed to air under room light for 3 days, only 31% of the WT cells and 16% of the mutant cells were viable. However, neither the WT nor the *csmI csmJ csmX* triple-mutant cells were viable after being exposed to air under room light for 6 days. The data in Table S4 of the Supporting Information show that the *csmI csmJ csmX* triple mutant is somewhat more sensitive to oxygen than the WT, especially when cells were also illuminated. These data are consistent with the hypothesis that the Fe−S proteins in the chlorosome envelope play a role in protecting GSB cells from the toxic effects of oxygen exposure.

Fluorescence Quenching in Chlorosomes after Their Exposure to Oxygen. As described above, mutants lacking the Fe−S proteins associated with the chlorosome envelope had growth rates similar to that of the WT and had chlorosomes with normal pigment contents and otherwise normal envelope protein contents as well. The *csmI csmJ csmX* mutant was slightly more sensitive to oxygen exposure than the WT strain (other strains were not studied). EPR studies and redox titrations [see the following paper (DOI: 10.1021/bi301455k)] suggested that the [2Fe-2S] clusters had midpoint potentials that might allow these Fe−S proteins to participate in oxidation and/or reduction of chlorobiumquinone(s) and menaquinone, which are abundant in the chlorosomes of *Cba. tepidum* (Table S2 of the Supporting Information) and are thought to quench energy transfer in chlorosomes when oxidized.[38,39] Thus, we next tested whether energy transfer quenching and recovery were affected in these mutants.

The fluorescence emission amplitude of chlorosomes from *Cba. tepidum* is much lower for chlorosomes that have been incubated under oxic conditions than for chlorosomes that have been completely reduced with dithionite.[22,25,40] Figure 2 shows the quenching of fluorescence emission that occurred when isolated chlorosomes that had been stored under anoxic conditions were mixed 1:1 (v/v) with oxygen-saturated buffer. Figure 2A shows the decay in fluorescence emission during the first ∼8 min for chlorosomes from the WT as well as the three single mutants: *csmI*, *csmJ*, and *csmX*. Panels B and C of Figure 2 show the fluorescence quenching that was observed for chlorosomes isolated from the double and triple mutants, *csmI csmX*, *csmJ csmX*, *csmI csmJ*, and *csmI csmJ csmX*. The fluorescence emission amplitude decreased rapidly for chlorosomes isolated from the WT and all of the mutants, and this indicated that energy transfer was rapidly quenched in all of the chlorosomes. However, the magnitude of the decrease, as assessed by the ratio of the maximal and minimal fluorescence emission amplitudes, differed for the chlorosomes from the various strains. After ∼8 min, the ratio for WT chlorosomes was consistently greater than 40 (Figure 2, black lines), while this ratio was only ∼20 for all single and double mutants except *csmI csmJ*. For chlorosomes from the *csmI csmJ* and *csmI csmJ csmX* mutants, this ratio was ∼10 (Figure 2C).

With the exception of chlorosomes from the *csmJ* mutant (Figure 2A), which exhibited a faster initial phase of quenching than WT chlorosomes, the rate of quenching of the

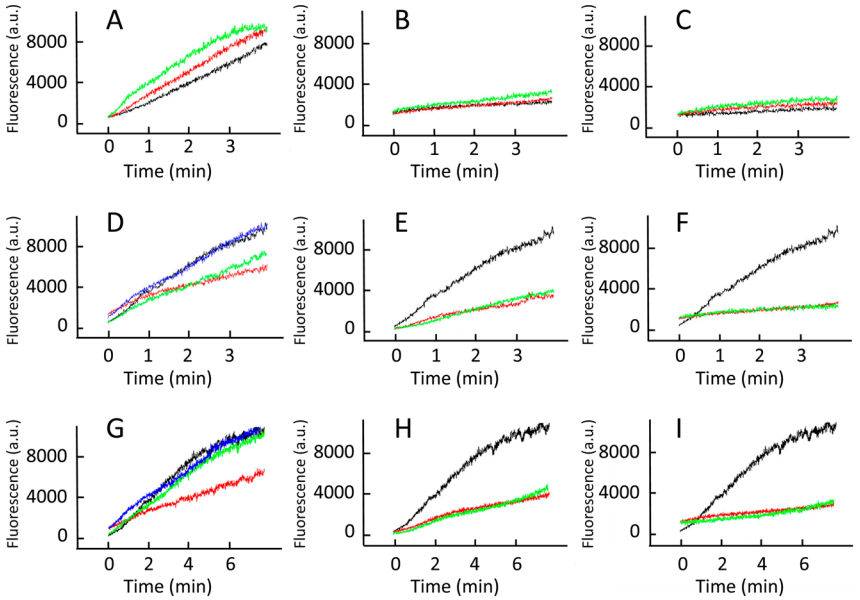


Figure 3. Recovery of fluorescence emission in isolated chlorosomes. The final chlorosome solution corresponded to 100 μg/mL BChl *c*. Fluorescence emission was recorded at the emission maximum (772 nm) with a time resolution of 0.5 s. The units for fluoresence emission amplitude are arbitrary units (a.u.). (A–C) Fluorescence recovery for chlorosomes from the WT (A), *csmI csmJ* mutant (B), and *csmI csmJ csmX* mutant (C) with 12.5 (black), 25 (red), and 50 mM dithionite (green). (D–F) Recovery of chlorosome fluorescence emission in the presence of 25 mM dithionite. (D) Chlorosomes from the WT (black), *csmI* mutant (red), *csmJ* mutant (green), and *csmX* mutant (blue). (E) Chlorosomes from the WT (black), *csmI csmX* mutant (red), and *csmJ csmX* mutant (green). (F) Chlorosomes from the WT (black), *csmI csmJ* mutant (red), and *csmI csmJ csmX* mutant (green). (G–I) Recovery of chlorosome fluorescence emission in the presence of 12.5 mM dithionite. (G) Chlorosomes from the WT (black), *csmI* mutant (red), *csmJ* mutant (green), and *csmX* mutant (blue). (H) Chlorosomes from the WT (black), *csmI csmX* mutant (red), and *csmJ csmX* mutant (green). (I) Chlorosomes from the WT (black), *csmI csmJ* mutant (red), and *csmI csmJ csmX* mutant (green). Note that the time range on the *x*-axis for panels G–I is twice that for panels A–F.

fluorescence emission was fastest for WT chlorosomes and was slower for some mutant strains than others. It took ~130 s for the fluorescence emission amplitude of WT chlorosomes to decrease to an amplitude that was 10% of the maximum. However, it took up to twice as long (~3–4 min) for the fluorescence emission amplitude of chlorosomes of the single and double mutants (except *csmI csmJ*) to decrease by this amount (Figure 2A,B). It took ≥6 min to quench the fluorescence of chlorosomes from the *csmI csmJ* and *csmI csmJ csmX* mutants by 90% (Figure 2C). When the fluorescence emission amplitude of WT chlorosomes had decreased to ~10%, the emission from chlorosomes of the *csmI csmJ* and *csmI csmJ csmX* mutants was still more than 2-fold higher (~23% of the maximum). Conversely, when the fluorescence emission of the chlorosomes from the *csmI csmJ* and *csmI csmJ csmX* mutants decreased to 10%, emission from WT chlorosomes was as low as 2.5% of the initial value. Because the *csmI csmJ* and *csmI csmJ csmX* mutants had the most obvious quenching phenotype, and because their chlorosomes lack both CsmI and CsmJ, we concluded that CsmI and CsmJ participate in the mechanism by which fluorescence quenching is activated under oxidizing conditions. Furthermore, we concluded that these proteins at least accelerate the rapid quenching of energy transfer that occurs immediately upon exposure of chlorosomes to oxygen. Although it is possible that CsmX may also function in the quenching mechanism (Figure 2A), any role of CsmX was clearly much less important than that of CsmI and CsmJ.

Recovery of Fluoresence Emission after the Addition of Dithionite to Oxidized Chlorosomes. Figure 3 shows the recovery of fluorescence emission of isolated chlorosomes when chlorosomes that had been equilibrated with oxygen-saturated buffer were mixed 1:1 (v/v) with nitrogen-saturated, anoxic buffer to produce a final sodium dithionite concentration of 12.5, 25, or 50 mM. At the lowest of these dithionite concentrations, the restoration of fluorescence was approximately linear during the initial 3–5 min after mixing. Figure 3 (panels A–C) shows that the recovery of fluorescence emission was faster at higher sodium dithionite concentrations. The recovery of fluorescence emission of WT chlorosomes had faster rates (28, 38, and 42 s^{-1}; the fluorescence amplitude values were arbitrary units) as the dithionite concentration increased (12.5, 25, and 50 mM, respectively), but the response to dithionite concentration was clearly not linear over this range. The recovery of fluorescence emission of chlorosomes of the *csmI csmJ* mutant was much slower overall but nevertheless also increased slowly as the dithionite concentration increased (3.4, 6.7, and 7.9 s^{-1}) from 12.5 to 50 mM dithionite, respectively. The recovery rates for fluoresence emission from chlorosomes of the *csmI csmJ csmX* mutant were very similar (2.4, 4.9, and 7.3 s^{-1}) to those for the *csmI csmJ* double mutant. These results clearly establish that the CsmI and CsmJ proteins participate in reduction of the quenching species in chlorosomes when oxidized chlorosomes are incubated with sodium dithionite. They further show that CsmX plays an only minor role in the recovery of fluorescence emission under these conditions.

Figure 3 shows the recovery of fluorescence emission for chlorosomes from WT and all mutants in the presence of 25 and 12.5 mM dithionite (panels D–F and G–I, respectively). The rate of fluorescence emission recovery with 12.5 mM dithionite was ~30–50% slower than that with 25 mM dithionite, but given a sufficiently longer time (e.g., 8 min instead of 4 min), the recovery of fluorescence emission with

dx.doi.org/10.1021/bi301454g | *Biochemistry* 2013, 52, 1321–1330

Table 1. Fluorescence Emission Recovery Rates of Chlorosomes for Various Mutants at Two Dithionite Concentrations

strain	recovery rate (s^{-1}) at 12.5 mM dithionite	percentage of WT rate	recovery rate (s^{-1}) at 25 mM dithionite	percentage of WT rate
WT	28 ± 2	100	39 ± 3	100
csmI	12 ± 2	43	20 ± 3	51
csmJ	20 ± 2	71	27 ± 2	69
csmX	27 ± 3	96	37 ± 3	95
csmI csmX	9 ± 2	32	13 ± 2	33
csmJ csmX	10 ± 1	36	15 ± 2	38
csmI csmJ	3 ± 1	11	6 ± 2	15
csmI csmJ csmX	3 ± 1	11	5 ± 2	13

12.5 mM dithionite was nearly identical to that which occurred at 25 mM dithionite (note that the time range for panels G–I is twice that for panels A–F of Figure 3). Table 1 compiles the recovery rates for fluorescence emission of chlorosomes for all of the mutants as percentages of the rates for WT chlorosomes. The *csmI csmJ* and *csmI csmJ csmX* mutants showed the largest effects and had recovery rates that were only ~10% of that of the WT. The *csmI csmX* and *csmJ csmX* double mutants had recovery rates that were ~35% of that of the WT, while the recovery rates of the *csmI* and *csmJ* single mutants were even higher (~45 and ~70%, respectively). The fluorescence emission recovery rate for chlorosomes from the *csmX* mutant was nearly the same as that for WT chlorosomes (~95–96%). When either CsmI or CsmJ was present in the chlorosome envelopes, the recovery rate was 30–70% of the recovery rate for WT chlorosomes, and the presence of CsmX under these conditions seemed to enhance the recovery rate partially. Conversely, when both CsmI and CsmJ were absent, the recovery rate was only ~10% of the rate for WT chlorosomes. These data clearly establish that CsmI and CsmJ play important roles in the fluorescence emission recovery process in vitro. Considering the relatively low level of CsmX (~5% compared to the amount of CsmI and CsmJ combined) in the chlorosome envelope, and the important roles of CsmI and CsmJ in both fluorescence quenching and recovery, we concluded that CsmI and CsmJ are probably directly involved in transferring electrons to and from the quenchers [i.e., chlorobiumquinone(s) and menaquinone] of energy transfer in chlorosomes. CsmX may also play a minor or specialized role in these processes but was clearly less important overall.

Recovery of Fluorescence Emission after Their Exposure of Cells to Oxygen. Fluorescence emission from chlorosomes and other components of the photosynthetic apparatus also decreases when intact cells are exposed to oxic conditions. In preliminary experiments, numerous background studies were performed to study how fluorescence emission (quenching of energy transfer) was restored after exposure to oxygen for varying periods of time. In particular, irradiance conditions and the effect of external reductants (i.e., sulfide or thiosulfate) were tested. Cellular fluorescence emission rapidly decreased from a maximal relative amplitude of ~130 (at OD_{600} = 0.1) to a minimal value of ~6–8 when *Cba. tepidum* cells were resuspended in oxic buffer in the dark for only 15 min (data not shown). This quenching of fluorescence emission was similar for WT and all mutant strains. Surprisingly, however, in initial experiments, the recovery of fluorescence emission occurred at similar rates for cells of the WT or *csmI csmJ csmX* triple mutant under dark conditions, even in the absence of external reductants, after a 1 h exposure to air (data not shown). We reasoned that this might be due to the availability of reductant(s) within cells (e.g., polysulfides, reduced ferredoxins, reduced pyridine nucleotides, or storage polysaccharides), and that these reductants could provide electrons to reduce the quencher within the chlorosome once the oxygen stress was removed. Thus, the exposure time to oxygen was prolonged in hopes of consuming the pool of cellular reductant(s) and thereby modifying the recovery behavior of cells.

Figure 4 shows the recovery of fluorescence emission that occurred with or without light and/or reductant for cells of the

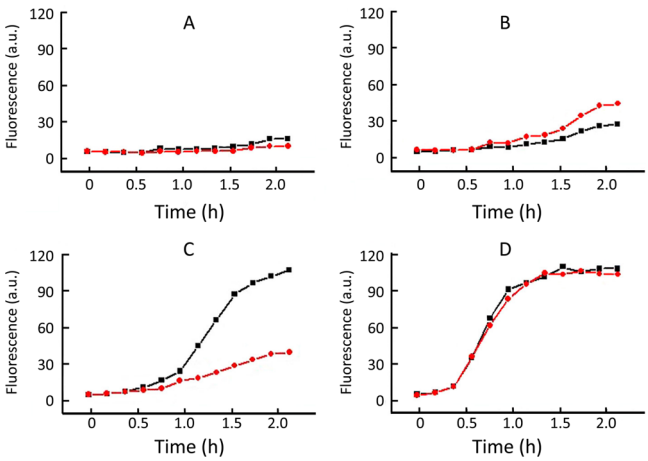


Figure 4. Recovery of fluorescence emission from chlorosomes in whole cells of *Cba. tepidum* WT (black) and the *csmI csmJ csmX* mutant (red). The cells were exposed to air for 3 days (72 h) in the dark. Fluorescence emission was recorded at the fluorescence emission maximum (772 nm) with a time resolution of 1 s. (A) Fluorescence recovery in the dark with no external reductant. (B) Fluorescence recovery in the dark with 2.0 mM $S_2O_3^{2-}$. (C) Fluorescence recovery with 0.2 μE m^{-2} s^{-1} light without external reductant. (D) Fluorescence recovery with 0.2 μE m^{-2} s^{-1} light and 2.0 mM $S_2O_3^{2-}$. This experiment was repeated several times, and the data shown are typical results.

WT and *csmI csmJ csmX* mutant that had been exposed to air for 72 h (3 days) in the dark. As shown in Table S4 of the Supporting Information, the viability of the two strains was similar under these conditions (58 and 49% viability, respectively). As shown in Figure 4A, upon transfer to anoxic conditions in the absence of light and an external reductant, the fluorescence emission of cells of the WT and the triple mutant increased very slowly over a period of ~2 h to final values that were only ~13 and 9% of the maximal fluoresence emission amplitude, respectively. Figure 4B shows the recovery that occurred in the dark when 2 mM thiosulfate was added to cells when they were transferred to anoxic conditions. Surprisingly,

dx.doi.org/10.1021/bi301454g | *Biochemistry* 2013, 52, 1321–1330

the triple mutant recovered to a somewhat greater extent than WT cells under these conditions but recovered only ~35% of the maximal fluorescence emission after 2 h. Very similar results were obtained when 2 mM S^{2-} was added and cells were transferred to anoxic conditions in the dark (data not shown). Figure 4C shows the fluorescence recovery that occurred in cells when they were transferred to anoxic conditions in the absence of an external reductant under very low irradiance (0.2 μmol of photons m^{-2} s^{-1}). After a lag period of ~30 min, fluorescence emission rapidly recovered for WT cells, but the *csmI csmJ csmX* mutant cells recovered to only approximately the same extent as cells incubated in the dark with an external electron source. After 2 h, the WT cells had recovered ~82% of the maximal fluorescence emission but the triple mutant had recovered only ~30% of the maximal fluorescence emission. In the absence of added reductants but at a higher light intensity (~50 μmol of photons m^{-2} s^{-1}), the WT and *csmI csmJ csmX* triple-mutant cells recovered faster than at very low irradiance (data not shown). Figure 4D shows the recovery of fluorescence emission that occurred when both 2 mM thiosulfate and low irradiance (0.2 μmol of photons m^{-2} s^{-1}) were provided. The fastest recovery occurred under this condition, and both the extent and the rates of recovery were nearly identical for cells of the WT and *csmI csmJ csmX* triple mutant. After a lag period of ~30 min, 84% of the WT fluorescence emission and 82% of fluorescence emission of the triple mutant were restored during the 2 h observation period.

DISCUSSION

GSB occur naturally in anoxic aquatic environments that are rich in reduced sulfur compounds and in which light is also available.[41−44] Although GSB can occur in densely layered microbial mats or at substantial depths in stratified lakes, GSB cells may nevertheless be challenged by exposure to oxygen under certain conditions. For example, exposure to oxic conditions can be caused by vertical mixing of the oxic and anoxic water layers in stratified lakes. The presence of many genes for oxygen protection in the genomes of *Cba. tepidum* and other GSB is strong evidence that these organisms are occasionally exposed to oxygen.[27] Even if only low levels of oxygen are transiently present, the strongly reducing ferredoxins and other Fe−S proteins in GSB would rapidly react with oxygen to form highly toxic, reactive oxygen species. Furthermore, previous studies have shown that *Cba. tepidum* is most sensitive to oxygen when light is also present.[27] To prevent oxidative stress from occurring, energy transfer in both the chlorosomes and the FMO is regulated by redox mechanisms.[22−26,38] The rapid and strong quenching of energy transfer in these antenna components of the photosynthetic apparatus is an effective protection mechanism for suppressing the activity of photochemical reaction centers, which blocks the reduction of ferredoxins and thereby minimizes the formation of reactive oxygen species. The results presented here strongly suggest that chlorosome proteins CsmI and CsmJ, and to a much lesser extent CsmX, all of which contain motifs similar to those of [2Fe-2S] adrenodoxin-type ferredoxins, play important roles in the redox regulation of energy transfer in chlorosomes.

Vassilieva et al.[16] suggested that the Fe−S proteins found in the chlorosome envelope comprised an adrenodoxin domain together with a CsmA/E-like domain that could target these proteins to and anchor them in the chlorosome envelope. Cross-linking studies provided strong evidence of the formation of CsmI and CsmJ homodimers, heterodimers, and heterotetramers [$(CsmI)_2(CsmJ)_2$] and further showed that these proteins interacted with CsmB in the envelope.[17] The redox potentials of CsmI and CsmJ in WT chlorosomes were determined to be −348 and 92 mV, respectively (DOI: 10.1021/bi301455k). These potentials shifted to −205 and 8 mV, respectively, in chlorosomes of mutants with only CsmI and CsmJ, respectively (DOI: 10.1021/bi301455k). The midpoint potential of CsmJ lies outside the range observed for typical vertebrate ferredoxins, which usually have midpoint potentials between −235 and −273 mV.[45] The [2Fe-2S] clusters in these chlorosome proteins are stable in the presence of oxygen, which is consistent with their proposed role in coping with oxygen stress. The EPR properties of chlorosomes prepared under oxic conditions were identical to those of chlorosomes isolated and maintained under anoxic conditions.[19] Moreover, the clusters in recombinant CsmI and CsmJ survived treatments with oxygen, 2 M sodium thiocyanate, or 6 M urea. This behavior differs from that for vertebrate-type adrenodoxin, putidaredoxin, and spinach ferredoxin, all of which are irreversibly denatured by oxygen in urea or guanidinium hydrochloride solutions.[19,45]

Quinones have been shown to quench excited states of chlorophylls by accepting an electron from an excited chlorophyll molecule and by facilitating extremely rapid charge recombination to the ground state.[46,47] Three isoprenoid quinone species are found in the chlorosomes of *Cba. tepidum*: chlorobiumquinone (1′-oxo-menaquinone-7), 1′-hydroxymenaquinone-7, and menaquinone-7.[38,39] The quinones in chlorosomes constitute more than half of the total cellular quinones and occur at a ratio of ~1:10 relative to BChl *c*.[1,38] Fluorescence emission and energy transfer exhibited a strong dependency on redox state when quinones were added to artificial, chlorosome-like BChl *c* aggregates in an aqueous solution, and chlorobiumquinone was most effective in this respect.[38] Chlorosomes from the green filamentous bacterium *Chloroflexus aurantiacus* contain menaquinone-10, but energy transfer processes in these chlorosomes are less dependent upon the redox state than processes in other chlorosomes.[25,40,47,48] It has been suggested that chlorobiumquinone plays a significant, but not exclusive, role in quenching the fluorescence emission and inhibiting the energy transfer in chlorosomes in *Cba. tepidum* under oxic conditions.[38,47] The suggestion that the 1′-oxo group neighboring the dicarbonyl group of the quinone is essential to the quenching mechanism of chlorobiumquinone seems unlikely to be correct.[47] The chlorosomes of "*Candidatus* Chloracidobacterium thermophilum" contain only menaquinone-7, but the fluorescence emission from the chlorosomes of this organism is quenched to an even greater extent than that in *Cba. tepidum* under oxic conditions.[40] The midpoint potential of fluorescence quenching in GSB chlorosomes is approximately −100 mV,[22,26] which just falls into the range between the redox potentials of CsmI and CsmJ, which is consistent with the possibility that CsmI and CsmJ serve as the carriers of electrons to and from the quinones in the chlorosome interior across the chlorosome envelope (ref 19 and see following paper, DOI: 10.1021/bi301455k).

Fluorescence emission quenching and recovery experiments described here with isolated chlorosomes clearly established roles for CsmI and CsmJ in both of these processes. Although fluorescence emission was rapidly quenched even in chlorosomes lacking CsmI and CsmJ, the extent and kinetics of this quenching were altered when these Fe−S proteins were

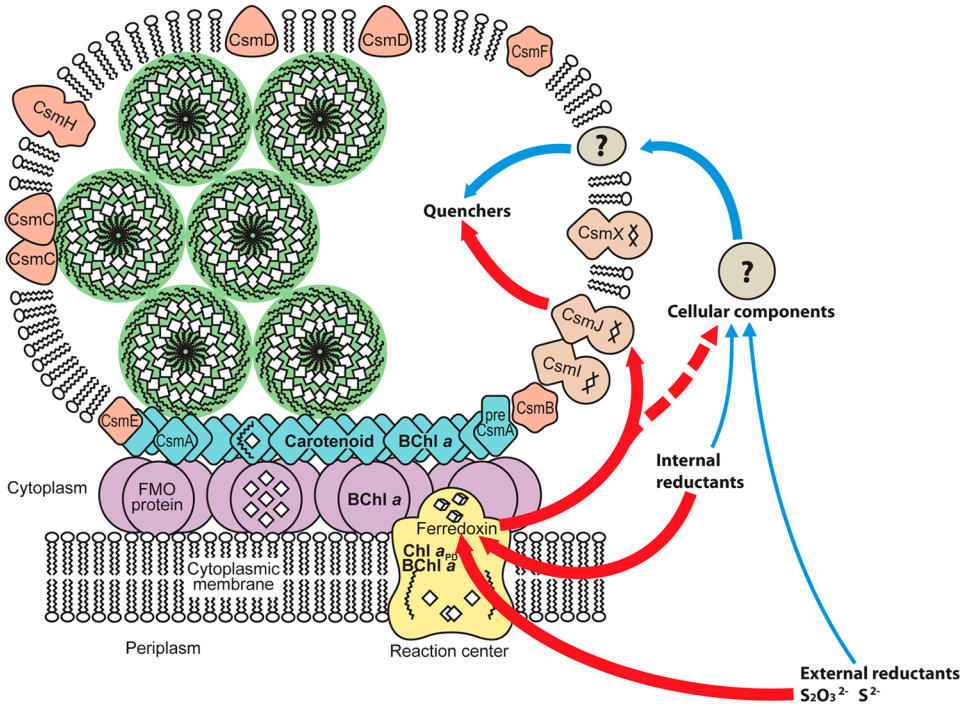


Figure 5. Model of electron transfer pathways functioning during the recovery of energy transfer in cells of *Cba. tepidum*. The blue arrows show electron transport pathways that lead to reduction of the quencher of energy transfer in chlorosomes in the dark and that are independent of CsmI, CsmJ, and CsmX. The cellular component(s) responsible for this pathway is unknown and may not be present in isolated chlorosomes. The red arrows show the light-dependent electron transport pathways that can also reduce the quencher of energy transfer in chlorosomes. Reduction of the quencher requires CsmI and CsmJ, is apparently more rapid than the dark pathway, leads to reduction of a larger fraction of the oxidized quencher, in vivo, and is responsible for essentially all of the reduction of the quencher that occurs in isolated chlorosomes. External reductants can be sulfide, thiosulfate, sulfur, and polysulfides. Internal reductants might be storage polysaccharides or other reduced organic compounds.

missing. When sodium dithionite was added as the reductant to chlorosomes under oxic conditions, even stronger evidence of the participation of CsmI and CsmJ in recovery from the quenched state was obtained by studying the increase in fluorescence emission. The differences in the magnitudes of the observed effects in the two situations were probably due to the absence of the natural electron donor and acceptor partners for the Fe−S proteins in these reactions in vitro. During the recovery of fluorescence emission in isolated chlorosomes, reduction of the oxidized quinone quenchers must occur. Electrons are probably transferred from the reductant (i.e., sodium dithionite) directly to CsmI, CsmJ, and CsmX, and then to the quencher. However, upon transfer of chlorosomes from anoxic to oxic conditions, oxygen might directly oxidize the quencher, which could greatly reduce the dependency of this reaction on CsmI and CsmJ. This might account for the apparent rapid initial quenching and slower subsequent quenching response exhibited by chlorosomes exposed to oxygen in vitro (see Figure 2).

Attempts to compare quenching and recovery in WT and mutant cells was only partly successful. Quenching by oxygen was activated in intact *Cba. tepidum* cells within minutes in both WT and mutants. With respect to recovery, *Cba. tepidum* cells seem to have large pools of reductants that can be used to reduce the oxidized quencher in chlorosomes and reactivate energy transfer as monitored by fluorescence emission. The availability of these reductants made it difficult to demonstrate a specific role for CsmI and CsmJ in reducing the quenchers in chlorosomes. To overcome this problem in part, cells were incubated under oxic conditions in the dark for 3 days in hopes of depleting these reductant pools. After dark oxic incubation, the recovery of fluorescence emission in the absence of light was very slow in both WT and mutant cells. Cells only partly recovered after 2 h even when sulfide or thiosulfate was added to the medium. However, the recovery of fluorescence emission was much more complete if dim light was provided, even if no external electron source was provided. The *csmI csmJ csmX* triple-mutant strain also recovered to some extent under these conditions, but the WT strain was clearly superior and recovered more rapidly and to a much greater extent. The very long lag periods (~30 min) observed in the recovery experiments with intact cells (Figure 4) suggested that cells initially reduce or repair cellular proteins before the quencher in chlorosomes can be reduced. The clear difference between WT and *csmI csmJ csmX* mutant cells observed in Figure 4C suggested that CsmI and CsmJ participate in a light-dependent electron transport pathway that connects internal reductants to reduction of the quenching species in chlorosomes. However, the in vivo recovery experiments also clearly demonstrated that at least one additional pathway for the reduction of the quenchers in chlorosomes exists in cells. This pathway connects both internal and external reductants to reduction of quenchers in chlorosomes and is greatly enhanced by light. This latter pathway does not exist (or has not yet been demonstrated) in isolated chlorosomes.

Although the components of this alternative electron transport pathway are not known, comparisons of the genes and proteins of chlorosomes suggest at least one possibility. The chlorosome envelopes of "*Candidatus* Chloracidobacterium thermophilum" contain a type II NADH dehydrogenase that probably participates in reducing oxidized quinones in the chlorosomes of this organism.[40] The envelopes of these

dx.doi.org/10.1021/bi301454g | *Biochemistry* 2013, 52, 1321−1330

chlorosomes additionally contain an Fe−S protein related to those described here.[40] A similar type II NADH dehydrogenase is encoded in an operon that includes several proteins of the chlorosome envelope, including CsmM and CsmN, in *Cfx. aurantiacus*.[3] *Cba. tepidum* also has a type II NADH dehydrogenase (CT0369), but there is currently no evidence that this protein exists in chlorosome envelopes. Loss of such a protein during chlorosome isolation might partly explain the results observed here. Because of the possible need to use different electron donors as well as the importance of restoring energy transfer in chlorosomes, it would not be surprising that cells would have redundant mechanisms for reactivating energy transfer after an exposure to oxygen.

Figure 5 presents a model that summarizes the main observations and conclusions reported in this study. CsmI and CsmJ contain adrenodoxin-like domains at their N-termini that harbor [2Fe-2S] clusters. These proteins form a heterotetramer (a dimer of dimers)[17] and participate in a light-dependent pathway that connects internal reductants to the reduction of the oxidized quencher in chlorosomes for reactivation of energy transfer (Figure 5). CsmX may perform a similar function; however, but this protein is much less abundant than CsmI and CsmJ, and its specific function is not yet clear. CsmI and CsmJ may also participate in reactions that oxidize the quencher in chlorosomes. An alternative electron transfer pathway that is still operative in a *csmI csmJ csmX* mutant can still reduce the quencher in the dark or in light and seems to require the participation of the type 1 reaction centers of *Cba. tepidum* (Figure 5). Electrons for these processes can be derived from either external sources, internal sources, or both. CsmI and CsmJ seem to be most important in connecting the internal reductant pool to quencher reduction in the light.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional experimental details and supplemental tables and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by Grant DE-FG02-94ER20137 from the U.S. Department of Energy to D.A.B.

Notes

The authors declare no competing financial interests.

■ ABBREVIATIONS

BChl, bacteriochlorophyll; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; FMO, Fenna−Matthews−Olson protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; WT, wild type or wild-type.

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