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Characterization of BciB: A Ferredoxin-Dependent 8-Vinyl-Protochlorophyllide Reductase from the Green Sulfur Bacterium *Chloroherpeton thalassium*

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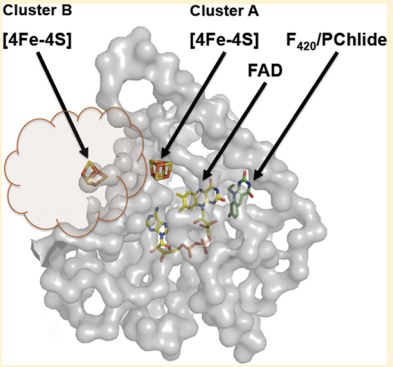
ABSTRACT: Two enzymes, BciA and BciB, are known to reduce the C-8 vinyl group of 8-vinyl protochlorophyllide, producing protochlorophyllide *a*, during the synthesis of chlorophylls and bacteriochlorophylls in chlorophototrophic bacteria. BciA from the green sulfur bacterium *Chlorobaculum tepidum* reduces the C-8 vinyl group using NADPH as the reductant. Cyanobacteria and some other chlorophototrophs have a second, nonhomologous type of 8-vinyl reductase, BciB, but the biochemical properties of this enzyme have not yet been described. In this study, the *bciB* gene of the green sulfur bacterium *Chloroherpeton thalassium* was expressed in *Escherichia coli*, and the recombinant protein was purified and characterized. Recombinant BciB binds a flavin adenine dinucleotide cofactor, and EPR spectroscopy as well as quantitative analyses of bound iron and sulfide suggest that BciB binds two [4Fe−4S] clusters, one of which may not be essential for the activity of the enzyme. Using electrons provided by reduced ferredoxin or dithionite, recombinant BciB was active and reduced the 8-vinyl moiety of the substrate, 8-vinyl protochlorophyllide, producing protochlorophyllide *a*. A structural model for BciB based on a recent structure for the FrhB subunit of F_{420}-reducing [NiFe]-hydrogenase of *Methanothermobacter marburgensis* is proposed. Possible reasons for the occurrence and distribution of BciA and BciB among various chlorophototrophs are discussed.



The conversion of light energy into chemical energy is a process performed by chlorophototrophic bacteria, algae, and plants. (Bacterio)-Chlorophylls [(B)Chls] are the essential cofactors of light-energy conversion in photochemical reaction centers in all types of chlorophototrophs.[1] On the basis of modifications to the tetrapyrrole macrocycle, 14 major types of (B)Chls have been identified to date in chlorophototrophic bacteria; these (B)Chls confer specific advantages for light harvesting in certain ecological niches.[2−4] Most (B)Chls have an ethyl substituent at the C-8 position of the (bacterio)-chlorin macrocycle.[2] A vinyl group occurs less frequently in this position, for example, in [8-vinyl]-Chl *a* and [8-vinyl]-Chl *b* (i.e., divinyl-Chl *a* and divinyl-Chl *b*) in *Prochlorococcus* sp.[5,6] In green sulfur bacteria (GSB), the C-8[2] carbon of the ethyl side chain is usually further methylated by a radical SAM-type methyltransferase, BchQ.[7] This functionally important methylation cannot occur if a vinyl group is present at this position.[7,8]

Suzuki and Bauer first proposed that the product of the *bchJ* gene reduces the C-8 vinyl moiety during BChl *a* biosynthesis in *Rhodobacter capsulatus*.[9] This proposal was made because large amounts of [8-vinyl]-protochlorophyllide *a* (8V-PChlide) (Figure 1) accumulated in a mutant in which the *bchJ* gene had been inactivated, although this mutant still produced a mixture of PChlide and 8V-PChlide.[9] The genome of *Chlorobaculum tepidum* encodes an ortholog of the *R. capsulatus bchJ* gene; this gene was initially proposed to serve the same function on the basis solely of sequence similarity.[10,11] However, functional studies subsequently proved that this is not the case, and another gene was shown to catalyze this reaction.[8] The protein product of open reading frame (ORF) CT1063 has high sequence identity to the plant-type 8V-PChlide reductase encoded by *AT5G18660* in *Arabidopsis thaliana*.[12,13] Further work demonstrated that CT1063, now denoted *bciA*, encodes

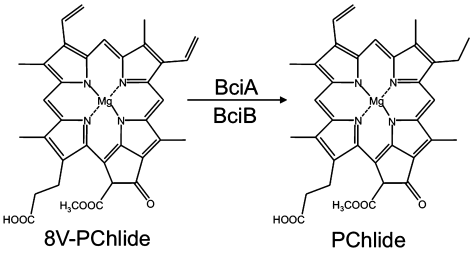


Figure 1. Conversion of 8V-PChlide *a* to PChlide *a* by 8V-PChlide reductase (either BciA or BciB).

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an 8V-PChlide reductase in *Cba. tepidum*, which utilizes NADPH as the reductant and catalyzes the reaction shown in Figure 1.[8]

Although most cyanobacteria synthesize monovinyl Chls, homologues of BciA were only found in a few *Synechococcus* species.[14] This led to the hypothesis that these organisms have a different class of 8V-PChlide reductase that had not yet been discovered. Comparative bioinformatics studies identified ORF slr1923 of *Synechocystis* sp. PCC 6803 as a candidate, and a mutant, in which this gene was inactivated, accumulated the predicted divinyl−Chl *a* product.[14,15] The amino acid sequence of this gene product is homologous to a subunit of archaeal-type $F_{420}H_2$ dehydrogenase (coenzyme F_{420} is 8-hydroxy-5-deazaflavin).[16] However, in vitro activity was not observed when the recombinant slr1923 protein, designated as BciB, was produced in *Escherichia coli*.[14] It was uncertain whether these experiments failed (i) because other proteins were required to form a multiprotein complex, (ii) because of incorrect folding or other problems common to expressing proteins heterologously in *E. coli*, (iii) because of the absence or failed assembly of essential cofactors, or (iv) because of the absence of required electron donors.

The distribution of *bciA* and *bciB* in GSB was analyzed, and a phylogenetic evaluation of slr1923/*bciB* homologues was performed for all sequenced GSB genomes.[17] Some GSB strains have only *bciA* genes, and for *Cba. tepidum*, the inactivation of *bciA* leads to the synthesis of (B)Chl molecules with an 8-vinyl group.[8] However, some GSB strains have only homologues of *bciB* (slr1923), whereas others have both *bciA* and *bciB*. Liu and Bryant tested four *bciB* homologues, including genes from *Chlorobium limicola* DSM 245 (Clim_1791), *Chlorobium phaeobacteroides* DSM 266 (Cpha266_0188), *Prosthecochloris aestuarii* DSM 271 (Paes_0603), and *Chloroherpeton thalassium* ATCC 35110 (Ctha_1208), for their ability to complement a *bciA* mutation in *Cba. tepidum*.[17] Each of the four genes tested could functionally complement the *bciA* mutation, and the resulting strains produced (B)Chl *c* in amounts similar to wild-type *Cba. tepidum*.[17] These observations strongly suggest that the BciB reductases of GSB do not function as subunits of a multiprotein enzyme complex and that they probably use a reductant that is readily available in *Cba. tepidum*.

BciB exhibits sequence similarity to the FpoF subunit of the 8-hydroxy-5-deazaflavin (F_{420}) dehydrogenase from *Methanosarcina* sp.[18] FpoF catalyzes the reduction of coenzyme F_{420} using reduced ferredoxin as the electron donor and has been shown to contain flavin adenine dinucleotide (FAD) and iron−sulfur (Fe/S) cluster cofactors.[19] Both the sequence similarity and the function in electron transport led to the hypothesis that BciB is also an Fe/S cluster-containing flavoprotein.[14,15] However, as noted above, previous attempts to observe enzymatic activity with recombinant BciB protein failed, possibly because of missing essential cofactors and/or electron donors. In the studies reported here, BciB from *Chloroherpeton thalassium* was selected for further study because this protein functionally complemented a *bciA* mutant of *Cba. tepidum*.[17] The *Chp. thalassium bciB* gene was coexpressed in *E. coli* together with the *isc* operon from *Azotobacter vinelandii*[20] to facilitate Fe/S-cluster insertion into the recombinant protein. Chemical analyses of Fe and sulfide for recombinant BciB as well as electron paramagnetic resonance (EPR) spectroscopy studies suggest that this enzyme binds two Fe/S clusters and an FAD cofactor. In the presence of a suitable reductant, recombinant BciB alone is sufficient to reduce the 8-vinyl group of 8V-PChlide in vitro.

MATERIALS AND METHODS

Cloning of *Chp. thalassium bciB*. The *bciB* gene (Ctha_1208) from *Chp. thalassium* ATCC 35110 was amplified from genomic DNA by polymerase chain reaction (PCR) using the primers 5′-CGCGCGCCATGGGGGCTCAAGCAACCTACTCAT-3′ and 5′-CGCCCGTCGACCTAATGATGATGATGATGATGACCCGAACCCTTTGGAATTCCGTATTCTT-3′ to include a C-terminal hexa-histidine tag on the BciB protein. The resulting PCR product was digested with NcoI and SalI (underlined in the primer sequences) and then ligated into similarly digested pET-28a (Novagen, Madison, WI). The final product was verified by DNA sequencing, which was performed at The Pennsylvania State University Genomics Core Facility (University Park, PA).

Expression of *Chp. thalassium bciB* in *E. coli*. Plasmid pET28-CthaBciB was cotransformed into *E. coli* BL21(DE3) along with the plasmid pDB1282, which contains the genes *iscS*, *iscU*, *iscA*, *hscA*, *hscB*, and *fdx* for Fe/S-cluster assembly from *A. vinelandii* under the control of an arabinose-inducible promoter to produce *E. coli* strain AHS-1.[20] A single colony was used to inoculate 100 mL of Luria−Bertani (LB) media containing 50 μg/mL of kanamycin and 100 μg/mL of ampicillin, and the culture was grown overnight at 37 °C with shaking. A 5 mL portion of the culture was then inoculated into 2.8 L flasks containing 1.7 L of LB media containing 50 μg/mL of kanamycin and 100 μg/mL of ampicillin, and the cultures were incubated at 37 °C with shaking. When the $OD_{600\ nm}$ of the culture reached ∼0.9, 0.04% (w/v) arabinose, 100 μM ferric ammonium citrate, and 50 μg of riboflavin were added to each flask. At an $OD_{600\ nm}$ of ∼1.0, 200 μM IPTG and an additional 100 μM ferric ammonium citrate were added to each flask. The cultures were incubated for an additional 3 h at 37 °C with shaking. Cells were harvested by centrifugation and stored at −80 °C until use. In some experiments, iron-limiting conditions were imposed during expression by including *o*-phenanthroline (100 μM final concentration) in the growth medium during growth of *E. coli* cells.

Purification of *Chp. thalassium* BciB. The purification of *Chp. thalassium* BciB was carried out inside of an anoxic chamber from Coy Laboratory Products, Inc. (Grass Lake, MI) under an atmosphere of N_2 and H_2 (95:5, v/v). Steps requiring centrifugation were performed outside of the anoxic chamber in centrifuge tubes that were sealed before removal from the chamber.

Protein purification was performed by immobilized metal-affinity chromatography using Talon cobalt affinity matrix (Clonetech, Mountain View, CA). In a typical purification, 25 g of frozen AHS-1 cells was resuspended in 80 mL of buffer (50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 20 mM imidazole, 10 μM FAD, and 10 mM 2-mercaptoethanol). Solid egg white lysozyme was added to a final concentration of 1 mg mL^{-1}, and the cells were disrupted by sonication. The cellular debris was removed by centrifugation, and the soluble BciB was loaded onto the cobalt affinity column equilibrated in the same resuspension buffer. The column was washed with resuspension buffer, and BciB was subsequently eluted with buffer containing 250 mM imidazole and 20% (v/v) glycerol. Fractions that were brown in color were pooled and concentrated in an Amicon stirred ultrafiltration cell (Millipore, Billerica, MA) fitted with a YM-10 membrane (10 000 MW cutoff). The protein was

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exchanged into buffer (50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 10 mM dithiothreitol (DTT), and 20% (v/v) glycerol) by anoxic gel filtration using a PD-10 column (GE Healthcare Biosciences, Piscataway, NJ), concentrated, and stored in aliquots in a liquid N_2 dewar until used. In one experiment, the protein was isolated from *E. coli* cells that had been grown under iron-limiting conditions under oxic conditions.

Reconstitution of BciB. To reconstitute the Fe/S cluster(s) of BciB, a 100 μM solution of protein was incubated with 10 mM DTT and 100 μM FAD for 20 min on ice. A 5-fold molar excess of $FeCl_3$ was added, and after a 20 min incubation, an 8-fold molar excess of Na_2S was added slowly over a period of 3 h. The resulting solution was incubated on ice overnight. The reconstituted protein was exchanged into buffer (50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 10 mM DTT, and 20% (v/v) glycerol) by gel filtration using a PD-10 column and stored in aliquots in a liquid N_2 dewar until use.

Analytical Methods. Protein concentrations were determined by the Bradford dye-binding procedure with bovine serum albumin as the standard.[21] Quantitative amino acid analysis, conducted at the University of California–Davis Proteomics Core Facility, showed that the Bradford method overestimates BciB protein concentration by a factor of 2.0. Iron and sulfide analyses were performed as previously described.[22,23] The identity of the BciB protein was confirmed by in-gel trypsin digestion followed by mass spectrometry at the Proteomics and Mass Spectrometry Core Facility (University Park, PA).

Electron Paramagnetic Resonance (EPR) Spectroscopy. Samples for EPR spectroscopy were prepared inside an anoxic chamber by treating a 250 μL aliquot of a 70 μM as-isolated (AI) BciB solution with a final concentration of 2 mM sodium dithionite at room temperature for 5 min, placing the sample in EPR tubes, and freezing in liquid N_2. Low-temperature EPR spectroscopy was performed on an X-band Bruker EleXsys E-500 spectrometer (Bruker BioSpin Corporation, Billerica, MA) with an EleXsys super-high-sensitivity probehead resonator equipped with an ESR 900 helium-flow cryostat (Oxford Instruments, Oxford, UK). Typically, 10 scans were averaged for each sample at a microwave power of 2 mW and 10 G modulation amplitude at 100 kHz modulation frequency at temperatures in the range 4 to 70 K.

Flavin Analysis. To determine the type of flavin bound to recombinant BciB, a sample was heated at 100 °C for 7 min and centrifuged at 10 000*g* for 10 min to remove precipitated protein. The resulting supernatant was filtered over a 0.4 μm poly(tetrafluoroethylene) filter (Whatman, Florham Park, NJ) prior to analysis by reversed-phase high-performance liquid chromatography (HPLC) on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) using an analytical Discovery C18 column (4.6 mm × 25 cm) (Supelco, Sigma-Aldrich, St. Louis, MO). The method used was modified from the method previously described.[24] Solvent A was 5 mM ammonium acetate, pH 6, and solvent B was 100% methanol. At the time of injection, the mobile phase was 7% B at a flow rate 0.75 mL/min. After 2 min, the percentage of B was increased linearly to 70% over 11 min and was maintained for 11 min at 70% B. Finally, the percentage of B was reduced to 7% with a linear gradient over 5 min. ChemStation rev. B.02.01 software (Agilent Technologies) was used to analyze the chromatograms and absorption spectra of eluates. The elution times of samples were compared to those for FAD and flavin mononucleotide (FMN) standards (5 nmoles) analyzed under the same conditions.

Activity Assay. The enzymatic activity of BciB was determined by monitoring reduction of the 8-vinyl group of 8V-PChlide. The 8V-PChlide substrate was purified from the spent medium from a culture of a *bchJ* mutant of *Cba. tepidum* as previously described.[8] Activity determinations were performed in a final volume of 300 μL and included 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 14 mM glucose, 1.5 U glucose oxidase, 1 μg catalase, and 10 μM FAD. BciB was added to this assay mixture to a final concentration of 25 μM along with either 2 mM sodium dithionite or 1 mM NADPH, 11 μg spinach ferredoxin, and 0.2 μg spinach ferredoxin–$NADP^+$ oxidoreductase (FNR). The resulting solution was incubated at rt for 5 min. The reaction was initiated by the addition of 8V-PChlide substrate (100 μM in 100% (v/v) acetone), and the solution was incubated for 1 h at rt. Substrate additions were adjusted so that the final concentration of acetone was less than or equal to 5% (v/v). The reaction was quenched by the addition of 300 μL of acetone and centrifuged to remove precipitated protein, and the supernatant was analyzed by HPLC using a method modified from one previously described.[25] Samples were analyzed on an Agilent 1100 series HPLC system (Agilent Technologies) using an analytical ProntoSIL C30 column (4.6 mm × 25 cm) (Bischoff Chromatography, Leonberg, Germany). Solvent A was 30% methanol buffered with 5 mM ammonium acetate, pH 6, and solvent B was 100% methanol. At the time of injection, the mobile phase was 82% B at a flow rate of 0.75 mL/min. After 2 min, the percentage of B was increased linearly to 100% over 44 min and was then maintained for 11 min at 100% B. Finally, the percentage of B was reduced to 82% with a linear gradient over 5 min. Elution of PChlide and 8V-PChlide was detected by monitoring at 440 and 629 nm.

Samples for HPLC with detection by mass spectrometry (LC–MS) were analyzed on an Agilent Technologies 1200 system coupled to an Agilent Technologies 6410 QQQ mass spectrometer with simultaneous UV–vis analysis using an Agilent diode-array detector using the associated MassHunter software package. The assay mixtures were separated on an Agilent Technologies Zorbax Rapid Resolution Extend-C18 column (4.6 mm × 50 mm, 1.8 μm particle size) equilibrated in 60% solvent A (5% (v/v) methanol buffered with 40 mM ammonium acetate, pH 6.6) and 40% solvent B (acetonitrile). Following injection, these initial conditions were maintained for 5 min, the percentage of B was then increased to 95% over a 1 min period, and these conditions were maintained for 1 min. The column was then returned to the starting conditions over a period of 2 min. Detection of the products was performed using electrospray ionization in positive mode (ESI^+) with MS2-scan.

RESULTS

Isolation and Characterization of *Chp. thalassium* BciB. BciB (Ctha_1208) from *Chp. thalassium* was overproduced in the presence of plasmid pDB1282, which encodes genes for Fe/S cluster assembly from *Azotobacter vinelandii*.[20] The genes encoded by the *isc* operon contained in plasmid pDB1282 were induced at an $OD_{600\ nm}$ of 0.9 by addition of arabinose (Figure 2, lanes 2 and 3). Expression of the *Chp. thalassium bciB* gene was subsequently induced at an $OD_{600\ nm}$ of 1.0 by the addition of IPTG (Figure 2, lane 4). Production of the protein, which included a C-terminal hexa-histidine tag, could be observed by the appearance of a polypeptide at 47

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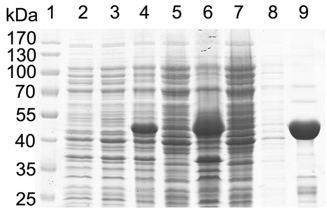

Figure 2. SDS-PAGE analysis monitoring the expression and purification of *Chp. thalassium* BciB. Lane 1, molecular mass markers; lane 2, preinduction whole-cell lysate of *E. coli* strain AHS-1; lane 3, whole-cell lysate of *E. coli* strain AHS-1 after arabinose induction of *isc* genes on plasmid pDB1282; lane 4, whole-cell lysate of *E. coli* strain AHS-1 after IPTG induction; lane 5, crude supernatant from strain AHS-1; lane 6, insoluble pellet fraction from strain AHS-1; lane 7, flow-through fraction from loading of the affinity column; lane 8, affinity column wash fraction; lane 9, affinity-purified BciB after desalting.

kDa, corresponding to the expected molecular mass of BciB. Following cell lysis and centrifugation to remove unbroken cells and large debris, the majority of BciB remained in the pellet (Figure 2, lane 6). However, some soluble protein was found in the crude extract (Figure 2, lane 5). BciB was purified in the absence of oxygen by affinity chromatography on the basis of the hexa-histidine tag, and the resulting protein solution was desalted by gel-exclusion chromatography (Figure 2, lane 9). Purified BciB was identified by tryptic peptide mass fingerprinting; 47 unique tryptic peptides covering 86% of the BciB protein sequence were identified (data not shown). Cross-linking experiments with the zero-length cross-linker, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, did not yield BciB dimers; this result suggested that the native protein is not oligomeric (data not shown).

The yellow-brown color of the purified BciB solution along with its UV−vis spectrum (Figure 3) suggested the presence of

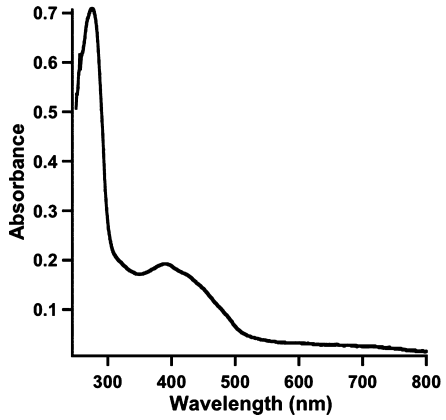

Figure 3. UV−vis absorption spectrum of AI-BciB.

flavin and Fe/S cluster cofactors. The UV−vis spectrum had a broad absorption peak around 400 nm, typical of Fe/S clusters, with additional features at 389 and 426 nm that are commonly observed in flavin-containing proteins. Quantitative iron and sulfide analysis detected 6.45 ± 0.10 irons and 4.67 ± 0.54 sulfides per protein; these values confirmed the presence of Fe/S cluster(s). Chemical reconstitution of the Fe/S cluster(s) resulted in an absorbance increase at 400 nm and a corresponding increase in the iron content (11.3 ± 0.68) and sulfide content (5.71 ± 0.49) per protein. However, because this procedure often leads to adventitious binding of iron to proteins, the iron-content values of reconstituted samples cannot reliably be used to determine the number of Fe/S clusters present. Although excess iron can often be removed from proteins by gel-exclusion chromatography on a Sephacryl S200 column,[26] for the purposes of the studies presented here, the as-isolated enzyme (designated AI-BciB) was used without further reconstitution treatment.

EPR Spectroscopy. EPR spectroscopy was used to investigate the number and type(s) of Fe/S clusters bound to the BciB protein[27]. The EPR spectrum of AI-BciB reduced with sodium dithionite was obtained at different temperatures and a microwave power of 2 mW (Figure 4). At temperatures above

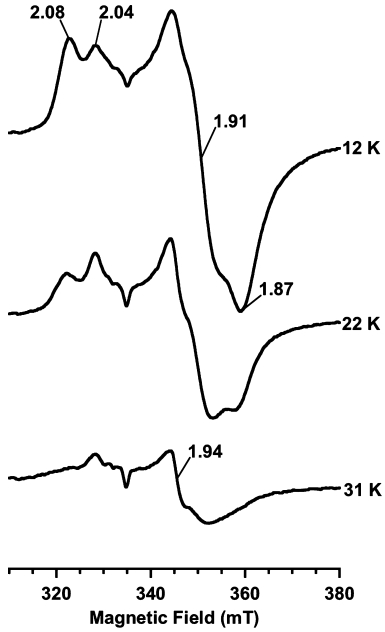

Figure 4. EPR spectra of AI-BciB. Samples were reduced with 2 mM sodium dithionite, and the spectra were recorded as described in the Materials and Methods at the temperatures indicated to the right. Approximate *g* values are indicated in the figure.

40 K, few features could be seen above the noise. At 31 K, a spectrum with rhombic symmetry appeared with *g* values of 2.04, 1.94, and 1.91 that may represent a single Fe/S cluster (termed Cluster A). As the temperature was lowered to 22 K, the amplitude of Cluster A appeared to increase, and additional features appeared at 322, 350, and 358 mT, which indicated the presence of a second Fe/S cluster (termed Cluster B). At 12 K, the amplitude of Cluster B appeared to increase, but at temperatures less than 10 K the amplitude of all of the resonances diminished because of microwave power saturation (data not shown). Clusters A and B appear to represent spin systems that are not sufficiently close to interact magnetically. Additionally, given the iron and labile sulfide values, a mixture of proteins with differentially loaded Clusters A and B may be present, which would make the interpretation of the spectra especially problematic. If the two spin systems are not interacting, then Cluster B could be assigned *g* values of 2.08, 1.91, and 1.87. The temperature dependence of the resonances suggest that Clusters A and B are both [4Fe−4S] clusters.

When BciB was isolated from *E. coli* cells that had been grown with phenanthroline under Fe-limiting and oxic conditions, the enzyme was still brown in color and bound

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3.17 ± 0.21 irons and 4.71 ± 0.28 sulfides per protein. The EPR spectrum of the dithionite-reduced cluster at 12 K (Figure 5, dashed line) displayed features with *g* values similar to those

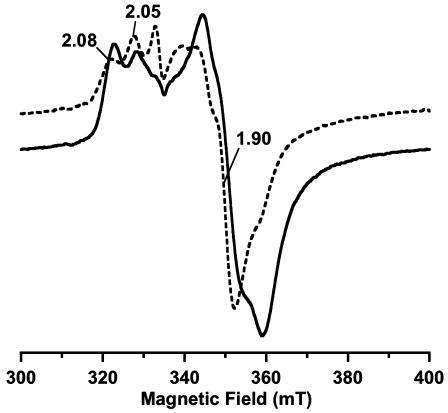

Figure 5. EPR spectra of AI-BciB purified under anoxic (solid line) and oxic (dashed line) conditions. The protein was reduced with 2 mM sodium dithionite. Spectrometer conditions: 2 mW microwave power at 12 K. Approximate *g* values are indicated for the sample that had been exposed to oxygen (dashed line). Approximate *g* values for AI-BciB purified under anoxic conditions are shown in Figure 4.

observed at higher temperatures, consistent with a greater amount of Cluster A relative to Cluster B, whereas the prominent features of the anoxic sample have *g* values similar to those described above for a mixture of Clusters A and B (Figure 5, solid line). These data collectively suggest that the protein isolated under oxic conditions was depleted for one of the [4Fe−4S] clusters, namely, Cluster B, that was observed when the protein was isolated in the absence of oxygen.

Flavin Analysis. The UV−vis spectrum of AI-BciB displays features suggesting the presence of a flavin (Figure 3). To identify which flavin is present, the protein was purified without addition of any exogenous FAD or FMN to the buffers, and the purified protein was heated to release the flavin and to denature and precipitate the protein. The supernatant was analyzed by HPLC, and elution times of the observed peaks were compared to those of authentic FAD and FMN standards (Figure 6). The FAD standard eluted at approximately 12.1 min, whereas the FMN standard eluted at about 13.3 min. The major flavin peak derived from BciB eluted at 12.1 min, which established that FAD is the flavin cofactor associated with the enzyme.

Activity Assay. BciB was incubated with 8V-PChlide in the absence of oxygen to observe reduction of the 8-vinyl group, forming PChlide. In contrast to *Cba. tepidum* BciA, which only requires NADPH to perform this reaction, BciB required an additional electron donor, ferredoxin, together with NADPH and ferredoxin−$NADP^+$ oxidoreductase (FNR). The product of the BciB reaction, PChlide, was separated from the substrate, 8V-PChlide, by HPLC. Only the 8V-PChlide substrate peak, which eluted at 23 min, was observed in the absence of either ferredoxin or sodium dithionite or in the no-enzyme control, which contained all assay components except BciB (Figure 7A). For the complete reaction, the PChlide reaction product eluted at approximately 17 min and displayed a blue shift of ∼5 nm in the 440 nm Soret peak compared to that of the 8V-PChlide substrate (eluting at 23 min) (Figure 7B). The properties of the product, PChlide, were similar to those observed in studies performed with *Cba. tepidum* BciA,[8] for which a blue shift of the Soret peak (∼5 nm) was also observed. This shift

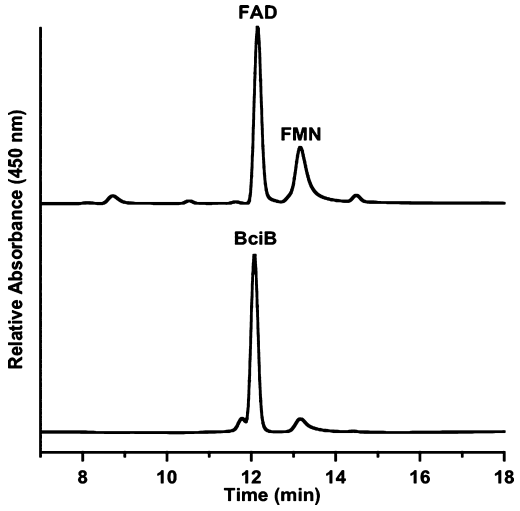

Figure 6. Identification of the flavin cofactor associated with BciB. HPLC elution profiles monitored at 450 nm of FAD and FMN standards (5 nmole each; top trace) and BciB extract (bottom). Under the conditions employed, FAD eluted at ∼12.1 min, and FMN eluted at ∼13.3 min.

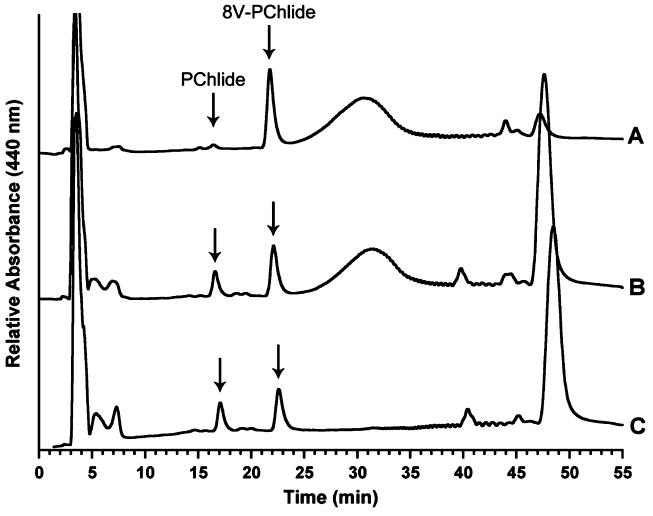

Figure 7. HPLC elution profiles of reactions of 8V-PChlide. Profile A shows the elution profile for the reaction mixture to which no BciB was added. Profile B shows the elution profile obtained with BciB using ferredoxin/FNR/NADPH as the reductant. Profile C shows the elution profile obtained with BciB using sodium dithionite as the reductant. The substrate, 8-vinyl-PChlide (8V-PChlide), eluted at 23 min and the product, PChlide, eluted at 17 min under the conditions employed.

demonstrated clearly that BciB also has C-8 vinyl reductase activity in vitro. No activity was observed when NADPH was replaced with NADH (data not shown). BciB was also functional when the enzyme was reduced with the chemical reductant, sodium dithionite, instead of ferredoxin/FNR/NADPH (Figure 7C). 8V-PChlide reductase activity was observed for both the AI and reconstituted preparations of BciB, and activity was also still observed when the protein was isolated under oxic conditions (data not shown). The latter result suggested that one of the [4Fe−4S] clusters (Cluster B) was not essential for the reductase activity of the enzyme. We were unable to observe time-dependent product formation by BciB in the presence of excess substrate. Enzyme activity was

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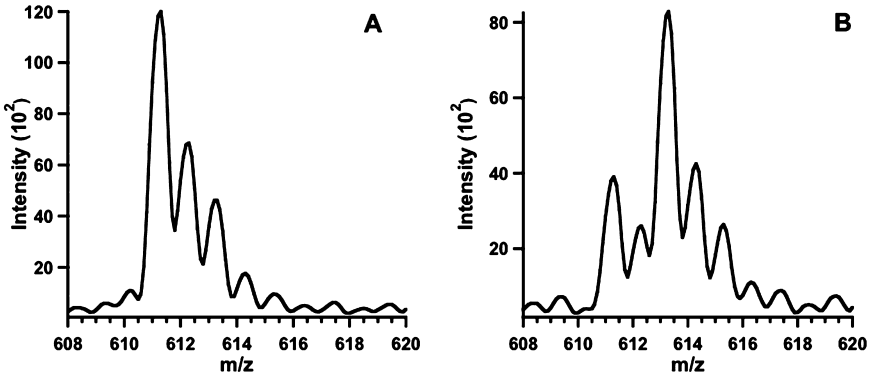

Figure 8. MS analysis of 8V-PChlide assay mixtures. Panel A shows the Q-Tof mass analysis of the reaction mixture when BciB was not added. Panel B shows the mass analysis of the reaction mixture in presence of BciB. The *m*/*z* values correspond to the +1 charge state. See the text for additional details.

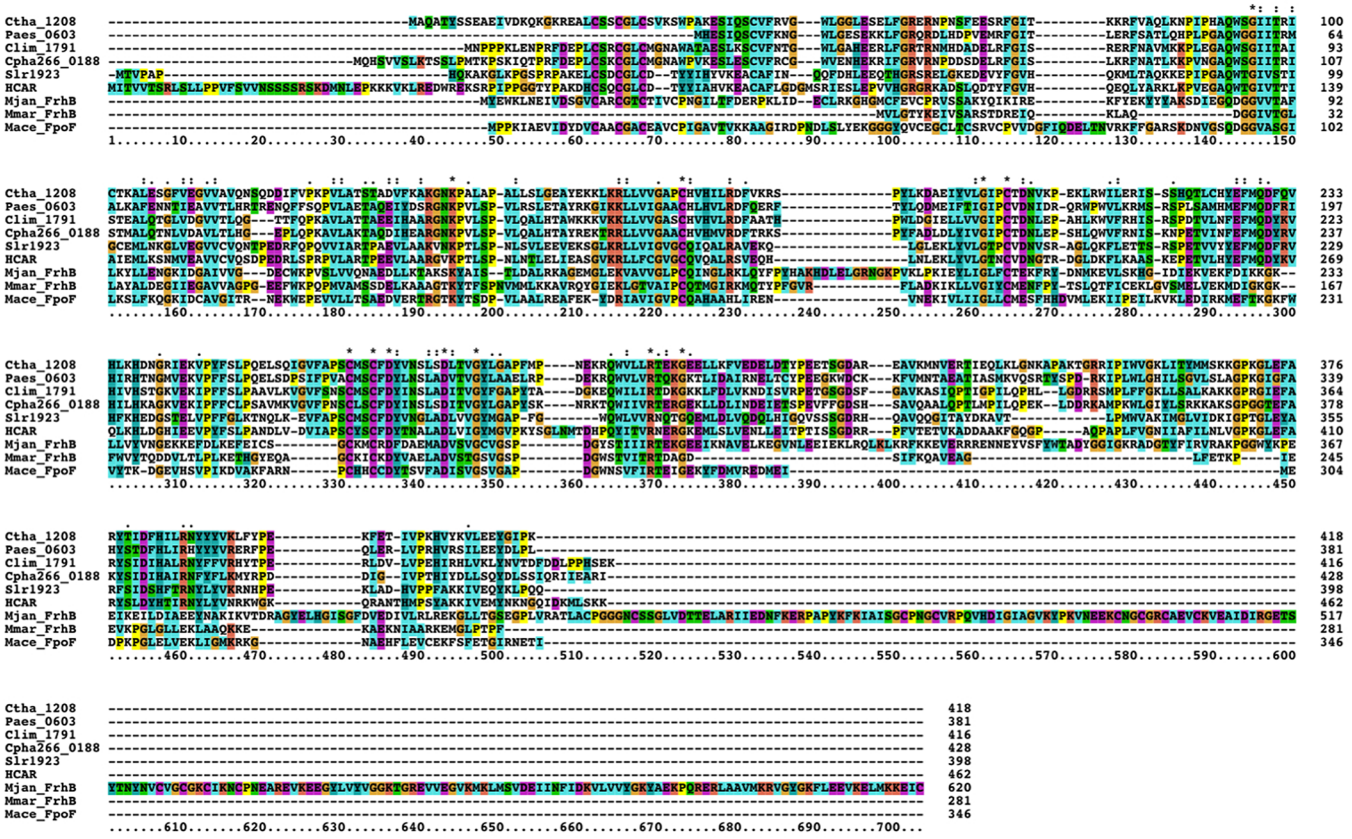

Figure 9. Sequence alignment of BciB proteins from GSB and cyanobacteria and F_{420} dehydrogenase sequences from archaea. BciB from *Chp. thalassium* (Ctha_1208), BciB from *Ptc. aestuarii* (Paes_0603), BciB from *Chl. limicola* (Clim_1791), BciB from *Chl. phaeobacteroides* DSM 266 (Cpha266_0188), BciB from *Synechocystis* sp. PCC 6803 (Slr1923), HCAR from *Arabidopsis thaliana*, FrhB from *Methanocaldococcus jannaschii*, FrhB from *Methanothermobacter marburgensis*, FpoF from *Methanosarcina acetivorans*.

only observed when the reaction was initiated by the addition of substrate. This might have been due to aggregation of the 8V-PChlide substrate under the aqueous assay conditions employed. No product formation was observed if the substrate was first incubated in the complete aqueous assay mixture and the reaction was then started by the addition of BciB. In general, more product was observed in assays in which more enzyme was added, which might indicate that the product was not released from the enzyme or inhibited the enzyme strongly.

Reduction of the 8-vinyl group by BciB was also confirmed by liquid chromatography−mass spectrometery (LC−MS). Assays were performed with the ferredoxin/FNR/NADPH reduction system in the presence and absence of BciB. The assay mixtures were analyzed by LC−MS, monitoring at 440 nm by UV detection and also by mass spectrometry. 8V-PChlide has a mass of 610.2 g/mol, and a peak exhibiting a mass-to-charge ratio (*m*/*z*) of 611.2 (corresponding to the +1 charge state) was observed as the major peak in the mass spectrum of the control reaction lacking BciB (Figure 8A). The presence of the two minor peaks at *m*/*z* of 612.2 and 613.2 are due to the stable isotopes of the magnesium atom of the substrate. In the presence of BciB in the complete reaction

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mixture, the major peak observed shifted to an m/z of 613.2 with minor peaks at 614.2 and 615.2, as expected for the reduction of the 8-vinyl group (Figure 8B).

DISCUSSION

Previous studies have shown that, during the biosynthesis of (B)Chls in chlorophototrophic bacteria, reduction of the C-8 vinyl group is catalyzed by at least two types of 8V-PChlide reductases. (However, see below about the existence of additional, uncharacterized 8V-PChlide reductases.) The BciA-type 8V-PChlide reductase of *Arabidopsis thaliana* was the first to be identified,[12,13] and soon thereafter it was demonstrated that the GSB, *Cba. tepidum*, has a very similar enzyme, the product of ORF CT1063, that catalyzes 8-vinyl reduction during the synthesis of (B)Chls a_P and c_F and Chl a_{PD}.[8] Recombinant BciA used NADPH as the reductant coenzyme and did not contain Fe/S clusters or other cofactors. However, not all GSB have homologues of BciA, and, similarly, only a few cyanobacteria and other chlorophototrophs have homologues of BciA.[4,17,28] Subsequent studies in *Synechocystis* sp. PCC 6803 showed that the product of ORF slr1923, now designated BciB, is also an 8V-PChlide reductase.[14,15] BciB has sequence similarity with F_{420} dehydrogenases (Figure 9), but initial attempts to study the properties of recombinant BciB failed. It was not clear whether this failure was due to missing polypeptides (i.e., if the complex was hetero-oligomeric), to cofactor synthesis, assembly, or insertion, to or other factors that can interfere with the heterologous expression of proteins in *E. coli*.

We report here that recombinant BciB from the GSB *Chp. thalassium* contains three redox cofactors: two [4Fe−4S] clusters and FAD. Although we were unable to identify conditions to observe multiple turnovers of the enzyme, this enzyme nevertheless specifically reduced the 8-vinyl group of 8V-PChlide using reduced ferredoxin or sodium dithionite as electron donors. Previous complementation studies with a *bciA* deletion mutant of *Cba. tepidum* had already showed that *Chp. thalassium* BciB is active in vivo.[17] Thus, it is not clear whether the failure to observe multiple turnovers in vitro was because of the limited solubility of the substrate or because BciB does not release PChlide *a* in the absence of a specific product-binding protein (e.g., BchJ; see ref 29). When BciB was isolated under oxic conditions, the Fe and sulfide content of the resulting enzyme preparation were lower, and the EPR signal attributed to one of the [4Fe−4S] clusters (Cluster B) was diminished, but the protein retained enzymatic activity. These results suggested that one of the [4Fe−4S] clusters is not required for enzymatic activity (see below). A definitive determination of the number, type, and mode of interaction of the Fe/S cluster(s) will require additional multifrequency EPR and Mössbauer experiments as well as holoprotein preparations more completely loaded with Fe/S clusters. Attempts to express the BciB protein under conditions required to label the protein with ^{57}Fe for Mössbauer experiments were unsuccessful because of protein expression and solubility issues.

The amino acid sequence of BciB is homologous to archaeal F_{420} dehydrogenase, an enzyme that also binds FAD and two Fe/S clusters and reduces F_{420}[19] (Figure 9). Another homologous enzyme is 7-hydroxymethyl Chl *a* reductase (HCAR), which converts 7-hydroxymethyl Chl *a* to Chl *a*.[30] HCAR is involved in the interconversion of Chl *a* and Chl *b*, known as the Chl cycle, in land plants and is important for the processes of greening and senescence.[31] HCAR was recently produced in and purified from *E. coli*, and the resulting enzyme reduced the 7-hydroxymethyl group of 7-hydroxymethyl Chl *a* to a methyl group.[30] Despite the low iron content of the protein (0.85 Fe atoms per HCAR protein), the authors of that study concluded that the enzyme contains FAD and Fe/S cluster cofactors.

A recent cryo-electron microscopy study of the F_{420}-reducing [NiFe]-hydrogenase from *Methanothermobacter marburgensis* provides the first structural model of a group-3 hydrogenase.[32] This hydrogenase contains a subunit, FrhB, which is an Fe/S flavoprotein that displays sequence homology to F_{420} dehydrogenases (FpoF) and BciB (Figure 9). In the model, FrhB binds FAD, like HCAR, F_{420} dehydrogenase, and, as shown here, BciB. FrhB also binds one [4Fe−4S] cluster that is ligated by four conserved cysteine residues, and all BciB proteins also have these four conserved cysteines (residues 172, 199, 264, and 267; Figures 9 and 10), which suggests that they

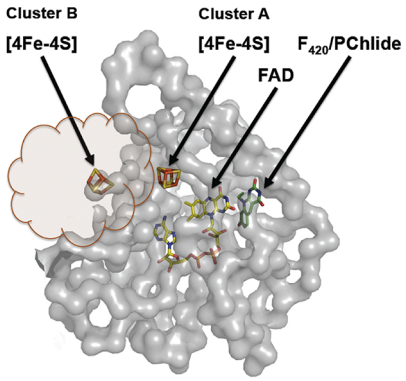

Figure 10. Model for *Chp. thalassium* BciB on the basis of the structural model for FrhB proposed by Mills et al.[32] The FrhB model was produced from the deposited coordinates (~4 Å resolution from cryo-electron microscopy) for the F_{420}-reducing [NiFe]-hydrogenase of *Methanothermobacter marburgensis* in PDF file 3ZFS. The arrow labeled FAD points to the isoalloxazine ring of the FAD cofactor. The F_{420} substrate (shown in green) in FrhB binds in close proximity to the FAD cofactor as shown by the labeled arrow. The substrate, 8-vinyl-PChlide (PChlide), for BciB is proposed to bind in a similar location as indicated. The [4Fe−4S] cluster, which is buried in the protein and is adjacent to FAD, is the conserved cluster found in all members of the F_{420} dehydrogenase superfamily (provisionally assigned here as Cluster A). The second [4Fe−4S] cluster (provisionally assigned here as Cluster B) is positioned at a similar position as the proximal [4Fe−4S] cluster in FrhG and is shown within a diffuse cloud that represents the N-terminal ferredoxin domain of some BciB proteins. See the Discussion for additional details.

are probably the ligands to the [4Fe−4S] cluster. FrhB from *M. marburgensis* contains only these four conserved cysteines and does not contain any additional Fe/S cluster-binding motifs. However, FrhB from *Methanocaldococcus jannaschii* has an N-terminal ferredoxin domain with a Cys−X_2−Cys−X_2−Cys−X_3−Cys motif that coordinates a second [4Fe−4S] cluster.[33] Interestingly, in the *M. marburgensis* [NiFe]-hydrogenase, this ferredoxin domain and its associated [4Fe−4S] cluster are instead associated with the FrhG subunit.

Because of the sequence homology with the F-subunit (FpoF) of the 8-hydroxy-5-deazaflavin (F_{420}) dehydrogenase from *Methanosarcina* sp., it was originally proposed that the cysteines involved in ligating the Fe/S cluster of the BciB proteins are those that occur in the Cys−X_2−Cys−X_2−Cys motif at the N-terminal region of the polypeptide.[19,30] An N-

dx.doi.org/10.1021/bi401172b | *Biochemistry* 2013, 52, 8442−8451

terminal Cys motif also occurs in several BciB proteins, including *Chp. thalassium* BciB characterized in this study, but it is not present in all BciB proteins of GSB (Figure 9). One BciB paralog lacking this domain and cysteine motif, Paes_0603, however, has been shown to have 8V-PChlide reductase activity in vivo.[17] Therefore, it is clear that the Fe/S cluster associated with this N-terminal domain is not absolutely required for reduction of 8V-PChlide. Repeated attempts to isolate active, recombinant *Ptc. aestuarii* BciB (Paes_0603) protein by approaches that were successful for *Chp. thalassium* BciB failed to produce a soluble, active enzyme. Future studies with *Ptc. aestuarii* BciB isolated from *Cba. tepidum* should provide more definitive information about the numbers and types of Fe/S clusters bound to 8V-PChlide reductases.

Chp. thalassium BciB contains the N-terminal cysteine motif, Cys−X_2−Cys−X_2−Cys−X_n−Cys, in which the number of amino acids between the third and fourth cysteines is variable in different organisms and is 13 residues in *Chp. thalassium* BciB (Figure 9). This motif differs from the canonical Cys−X_2−Cys−X_2−Cys−X_3−Cys motif that ligates the ferredoxin-type [4Fe−4S] cluster of the F_{420} dehydrogenases. Four cysteines, corresponding to residues 172, 199, 264, and 267 in *Chp. thalassium* BciB, are conserved in BciB and other homologous proteins (HCAR, FpoF, and FrhB), and these four cysteines are thus the most likely candidates to ligate the conserved [4Fe−4S] cluster (provisionally assigned here as Cluster A) that is required for activity. We propose that the N-terminal cysteine motif in BciB binds the second [4Fe−4S] cluster (provisionally assigned here as Cluster B) that does not appear to be essential for activity and that was depleted when BciB was isolated under oxic conditions from iron-limited cells.

Using the structural model of FrhB proposed by Mills et al.[32] as a template, we propose a model for the structure of *Chp. thalassium* BciB in Figure 10. The conserved [4Fe−4S] cluster (Cluster A) would be buried in the interior of the protein in close proximity to the FAD cofactor. The more exposed N-terminal domain that ligates the second [4Fe−4S] cluster (Cluster B) probably fills a natural cavity that is normally occupied by the FrhG subunit of the group-3 hydrogenase of *M. marburgensis*.[32] The FrhG subunit of this hydrogenase is also an Fe/S protein that binds two [4Fe−4S] clusters.[32] The model is consistent with the observations that this cluster is not required for enzymatic activity and that it is not present in all 8V-PChlide reductases. The binding site for 8V-PChlide would presumably occur near the binding site for F_{420} in the group-3 hydrogenase (Figure 10).

The distribution of BciA and BciB across chlorophototrophic bacteria suggests that these genes are possibly subject to horizontal transfer. As noted above, some GSB have BciA, some have one or more BciB paralogues, and some have both types of 8V-PChlide reductase.[17] For example, *Chlorobium clathratiforme* DSM 5477 has one copy of *bciA* and 2 *bciB* paralogues, but *Chlorobium phaeobacteroides* DSM 266 lacks *bciA* but has 3 *bciB* paralogues. The presence of multiple 8V-PChlide reductases in these organisms may reflect the importance of this reaction in producing specifically modified side chains of (B)Chls and could also be related to the very slow release of PChlide *a* from BciB observed in vitro. The C-8^2 carbon of (B)Chl *c* in GSB can be methylated up to three times by BchQ,[7] a modification that cannot occur if the 8-vinyl group is not reduced.[8] The latter study showed that the presence of a vinyl side chain at this position modified (B)Chl *c* aggregation in chlorosomes, which resulted in a decreased molar adsorptivity for the (B)Chl aggregates in chlorosomes.[8]

Database searches show that, among cyanobacteria, only a few marine *Synechococcus* spp., *Acaryochloris marina*, and a few *Oscillatoriales* strains have BciA homologues. Most cyanobacteria have homologues of BciB, and most have only a single copy of this gene that is highly similar to ORF slr1923 of *Synechocystis* sp. PCC 6803,[14,15] and a small number of cyanobacterial strains have both, as found in some GSB strains. Alternatively, species of *Prochlorococcus* synthesize 3-,8-divinyl derivatives of Chl *a* and Chl *b*, and there is presently no evidence for any 8-vinyl reductase in these organisms.[9,14] Among the chlorophototrophic members of the Chloroflexi, *Chloroflexus* spp. and *Oscillochloris trichoides* lack BciA and have BciB, but *Roseiflexus* spp. do not have homologues of BciA or BciB.[28] Nevertheless, *Roseiflexus* spp. produce (B)Chl *a* with an ethyl side chain at C-8 and thus *Roseiflexus* spp. must have a third type of 8V-PChlide reductase.[28] The recently discovered chlorophototrophic acidobacterium, "*Candidatus* Chloracidobacterium thermophilum", only has BciB. BciA and BciB are missing in heliobacteria, which produce (B)Chl *g*, and in members of the Proteobacteria that synthesize (B)Chl *b*. Although a recent study has suggested that (B)Chl *g* is synthesized from 8-vinyl-chlorophyllide *a*,[34] it remains possible that hydration of the 8-vinyl side chain precedes the formation of the ethylidene side chain found at C-8 in (B)Chls *g* and *b*.[4] Like GSB, several BChl-synthesizing Proteobacteria contain BciA, but many others, especially purple sulfur bacteria, contain apparent orthologues of BciB. A recent study in the purple nonsulfur bacterium *Rhodobacter sphaeroides* showed that this organism has an active, BciA-type 8V-PChlide reductase. However, a *bciA* deletion mutant was still able to synthesize (B)Chl *a* with a C-8 ethyl side chain.[25] Because *R. sphaeroides* lacks genes homologous to *bciB*, this study suggests that a third or fourth type 8V-PChlide reductase, depending upon whether the alternative enzyme is related to that found in *Roseiflexus* spp., occurs in this organism.

It is presently a mystery why chlorophototrophs have BciA, BciB, or both types of 8V-PChlide reductases, and why there appear to be at least three and possibly four different families of 8V-PChlide reductases. This situation is reminiscent of lycopene cyclases, for which four different families are also known to occur.[35] Although some marine cyanobacteria take advantage of the slightly enhanced blue-light absorption properties of divinyl-Chl *a* and divinyl-Chl *b*,[5,6,36] mutants of *Synechocystis* sp. PCC 6803 that synthesize divinyl-Chl *a* are more sensitive to high irradiation than wild type.[15] The results presented here demonstrate clearly that BciB uses a different reductant, reduced ferredoxin, than BciA, which uses NADPH. This difference could provide distinctive physiological advantages to organisms with the capacity to use one or the other, or both, enzymes under particular growth conditions. One obvious advantage is that BciA does not require iron and FAD for activity, which could be advantageous in nutrient-limited environments. Finally, although *Chp. thalassium* BciB has a nonessential [4Fe−4S] cluster (Cluster B) that might be sensitive to oxygen, most oxygen-evolving cyanobacteria have BciB proteins that also contain this Fe/S cluster domain. Thus, the distribution and reason(s) for the occurrence of multiple 8V-PChlide reductases remain unclear at this time. Physiological and gene expression analyses of organisms encoding both *bciA* and *bciB* genes may eventually provide an answer to these questions.

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Notes

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ABBREVIATIONS USED

8V, 8-vinyl; AI, as-isolated; BChl, bacteriochlorophyll; (B)Chl, bacteriochlorophyll and/or chlorophyll; *Cba.*, *Chlorobaculum*; Chl, chlorophyll; *Chp.*, *Chloroherpeton*; DTT, dithiothreitol; EPR, electron paramagnetic resonance; F420, 8-hydroxy-5-deazaflavin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FNR, ferredoxin−$NADP^+$ oxidoreductase; GSB, green sulfur bacterium/bacteria; HCAR, 7-hydroxymethyl Chl *a* reductase; HPLC, high-performance liquid chromatography; LB, Luria−Bertani; LC−MS, liquid chromatography mass spectrometery; ORF, open reading frame; PChlide, protochlorophyllide; PCR, polymerase chain reaction; *Ptc.*, *Prosthecochloris*

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