Characterization of a Plant-like Protochlorophyllide a Divinyl Reductase in Green Sulfur Bacteria

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The green sulfur bacterium Chlorobium tepidum synthesizes three types of (bacterio)chlorophyll ((B)Chl): BChl a_P, Chl a_PP, and BChl c_P. During the synthesis of all three molecules, a C-8 vinyl substituent is reduced to an ethyl group, and in the case of BChl c_P, the C-8^2 carbon of this ethyl group is subsequently methylated once or twice by the radical S-adenosylmethionine enzyme BChQ. The C. tepidum genome contains homologs of two genes, bchJ (CT2014) and CT1063, that are highly homologous to genes, bchJ and AT5G18660, and that have been reported to encode C-8 vinyl reductases in Rhodobacter capsulatus and Arabidopsis thaliana, respectively. To determine which gene product actually encodes a C-8 vinyl reductase activity, the bchJ and CT1063 genes were insertionally inactivated in C. tepidum. All three Chls synthesized by the CT1063 mutant of C. tepidum have a C-8 vinyl group. Using NADPH but not NADH as reductant, recombinant BciA reduces the C-8 vinyl group of 3,8-divinyl-protochlorophyllide in vitro. These data demonstrate that CT1063, renamed bciA, encodes a C-8 divinyl reductase in C. tepidum. The bchJ mutant produces detectable amounts of Chl a_PP, BChl a_P, and BChl c_P, all of which have reduced C-8 substituents, but the mutant cells secrete large amounts of 3,8-divinyl-protochlorophyllide a into the growth medium and have a greatly reduced BChl c_P content. The results suggest that BchJ may play an important role in substrate channeling and/or regulation of Chl biosynthesis but show that it is not a vinyl reductase. Because only some Chl-synthesizing organisms possess homologs of bciA, at least two types of C-8 vinyl reductases must occur.

Plants and bacteria convert light energy into chemical potential energy through the process known as photosynthesis. Antenna complexes harvest the light energy, and protein-pigment complexes known as reaction centers perform photochemical reactions that lead to stable charge separation. Reaction centers contain special pairs of (bacterio)chlorophyll ((B)Chl)^2 molecules that initiate these photochemical reactions (1, 2). In addition to this essential function, all known photosynthetic antenna systems rely on cyclic tetrapyrroles (Chls) or linear tetrapyrroles (bilins) for light harvesting. A variety of antenna Chls are found in nature, and these molecules carry different modifications of the tetrapyrrole macrocycle. These chemical differences help an organism to fine-tune the absorption and energy-transfer features of its light-harvesting complexes, and these differences also influence the pigment-protein and pigment-pigment interactions within the antenna system (3).

Most Chls and BChls in plants and bacteria carry an ethyl substituent, or less frequently a vinyl group, at the C-8 position of the (bacterio)-chlorin macrocycle. For example, Chls a and b occur as the 3-monovinyl (MV) derivatives in green plants, but Chl precursors sometimes accumulate as 3,8-divinyl (DV) intermediates, and the ratio between the two forms can vary depending on the species, tissue and growth conditions (4). It has been proposed that plants have parallel routes for the biosynthesis of the DV and MV precursors of Chl, and that several enzymes could thus be responsible for reducing the C-8 vinyl groups of the different precursors (5, 6). However, there is also evidence that a single enzyme acts as the DV reductase (DVR) activity in maize (7). It has not yet been determined whether one or multiple enzymes are responsible for the reduction of the DV-Chl intermediates in plants.

With the exception of some Prochlorococcus spp., which produce 3,8-divinyl-Chl a and b (8), BChls and Chls in bacteria mostly occur with an ethyl group at the C-8 position. Green sulfur bacteria (GSB) also synthesize BChls that carry reduced ethyl substituents at the C-8 position, and these organisms further methylate the C-8^2 carbon of the ethyl group in their antenna BChls to yield a mixture of BChls alkylated to different extents at the C-8 position (9).

Through genetic and biochemical studies of BChl a biosynthesis in purple bacteria, it was determined that the first step after the insertion of the Mg^2+ into protoporphyrin IX and the formation of the isocyclic ring is the reduction of the vinyl group at the C-8 position of the 3,8-divinyl-protochlorophyllide (DV-PChlide) to an ethyl group (10) (Fig. 1). A mutant of Rhodobacter capsulatus with an inactive copy of bchJ, a gene found in the photosynthetic gene cluster of purple bacteria, was shown to accumulate and secrete large amounts of DV-PChide..."
by amplifying a region of the gene using the primers 5’-TACTGCTGAATTCTGTAACGGGCTCTC-3’ and 5’-ACTCTCCGGTGTTGTGCTGAGTTGATCCAC-3’ for CT1063 and 5’-GGCATACAGGAAATTCCACAGCAGCA-3’ and 5’-ACA-TCCGGATGAGATCTAGTGCCTC-3’ for CT2014 from genomic DNA. The resulting PCR products were cloned into pUC19 using EcoRI and PstI sites (underlined) engineered into the primers for CT1063 and EcoRI and BglII sites for CT2014. To insertionally inactivate these genes, the adaA gene, which confers spectinomycin/streptomycin resistance, was inserted into the ClaI site of CT1063 and the BclI site of CT2014, respectively (Fig. 2, A and B). The resulting plasmids were linearized by digestion with AhdI and used to transform wild-type C. tepidum cells as described (19). Segregation of the mutants was confirmed by PCR as described (19) (see Fig. 2, C and D).

**Pigment Analysis**—Cell cultures (25 ml) were grown at 42 °C in an anaerobic chamber for 24–36 h. The culture was diluted 1:3 (v/v) in aqueous buffer (10 mM KH2PO4, 50 mM NaCl, pH 7.0) and the whole cell absorption spectrum from 350 to 900 nm was recorded using a Genesys Spectrophotometer (ThermoSpectronic, Rochester, NY). The absorption spectrum of the extracted pigments was also recorded in methanol (1:9, v/v) in the same manner. For the HPLC and HPLC-MS analyses, cells from 1 ml of a dense culture were pelleted by centrifugation, and the pigments were extracted by sonication in acetone: methanol (7:2, v/v). The pigment extract was subjected to reverse-phase HPLC as described (20) to resolve the different BChl c species as well as BChl a and Chl a. ChemStation Rev. A.10.03 software by Agilent Technologies was used to analyze the chromatograms and the absorption spectra from the different samples and the eluants. Samples for HPLC-MS were prepared in the manner described above and were analyzed in a Micromass Quattro II mass spectrometer equipped with a Shimadzu LC10ADvp pump (Shimadzu, Columbia, MD). The resulting data were analyzed with MassLynx software (version 3.5; Micromass, Ltd., Manchester, UK).

Spent growth medium was collected by centrifuging 1 ml of a dense culture, then transferring the supernatant to a clean tube and centrifuging again to remove all suspended cells from the medium. The absorption spectrum of the medium was recorded as described for the cell cultures. The absorption in methanol was obtained by diluting the spent medium with methanol in a 1:3 (v/v) ratio. To perform HPLC analyses of the spent growth media, the clarified medium (1 ml) was dried under vacuum and extracted with 400 μl of acetonemethanol (7:2, v/v). The extract was filtered over 0.4-μm polotetrafluoroethylene filter (Whatman, Florham Park, NJ) and dried under vacuum. The pigments were extracted again with acetone:methanol (7:2, v/v) immediately before injection in the HPLC. The method used to separate these compounds was a modified version of the method described (20). Solvent A (62.5% water, 21% methanol, 16.5% acetonitrile, 10 mM ammonium acetate) was used as the stationary phase and solvent B (50% methanol, 20% acetonitrile, 30% ethyl acetate) was used as the mobile phase using the following gradient. At the time of injection, the mobile phase was 20% B at a flow rate of 0.75 ml min⁻¹; the % B increased linearly to 70% B over 10 min, at which point the flow rate was increased to 1.0 ml min⁻¹. This was followed by a

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**FIGURE 1. Reduction of DV-PChlide to MV-PChlide by a DVR.** In C. tepidum, this reaction is catalyzed by the enzyme BciA.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutants**—The plating strain WT2321 of C. tepidum (16) derived from strain ATCC 49652 (17) was used in all experiments. All cultures were grown anaerobically in liquid CL medium or solid CP medium as described (18). Culture transfers and growth on solid medium were performed in a Coy Anaerobic Chamber (Grass Lake, MI) with an atmosphere composed of 10% CO2 and 10% H2 balanced with N2. The constructs for inactivating CT1063 and CT2014 were made by PCR

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linear gradient to 100% B over 30 min and kept isocratic at 100% B for another 10 min. Finally, the percentage of B was reduced to 20% with a linear gradient over 10 min and the flow rate reduced to 0.75 ml min⁻¹. Samples for mass analysis were prepared in the same way and analyzed as described above.

To calculate the protein:pigment ratios, cells from a dense culture (1 ml of a culture with A₅₅₀ nm between 1.6 and 1.8 that had been grown at a light intensity of 90 μmol photons m⁻² s⁻¹) were pelleted by centrifugation. The pellet was extracted with 1 ml of acetonemethanol (7:2, v/v) and centrifuged again to pellet the denatured protein. The pigment content was determined by absorbance assuming, for the bciA mutant extracts, that the molar extinction coefficient at 667 nm (ε₆₆₇ nm) of the 8-vinyl-BChl c was 69,717 liters mol⁻¹ cm⁻¹ (21) and that the distribution of homologs was 5% 8-vinyl-12-methyl-BChl c and 95% 8-vinyl-12-ethyl-BChl c, and that the molar extinction coefficient at 770 nm (ε₇₇₀ nm) of 8-vinyl-BChl a was 54,630 liter mol⁻¹ cm⁻¹ (22). For the extracts from the bchF mutant, the same extinction coefficients but a distribution of BChl c homologs of 67% 12-ethyl-BChl c and 33% [Pr,E]-BChl c were used. The protein content was determined using the Sigma Protein Assay Kit P5656 as described by the manufacturer. Samples were analyzed in triplicate for each strain and the results are the average of two independent determinations.

Sequence Analysis and Phylogeny—The A. thaliana protein sequence was obtained from the Entrez Protein database (NP_197367). The amino acid sequence obtained was then used as a query for tblastn analysis using the freely available software (NP_197367). The amino acid sequence obtained was then used as a query for tblastn analysis using the freely available software (Norway). The sequences for all the bacterial orthologs, except that of R. capsulatus SB1003, were obtained from the nucleotide databases revealed that one of the closest homologs of the A. thaliana protein AT5G18660 in the available sequences is the C. tepidum hypothetical protein CT1063. The translated amino acid sequence (see supplemental Fig. S1) shares 60% identity and 75% similarity with the DVR recently identified in A. thaliana (14). To verify the function of CT1063 in C. tepidum, open reading frame CT1063 was insertional inactivated as described under “Experimental Procedures.” To confirm that the bchF gene (CT2014) was not responsible for the reduction of the DV-PChlide in C. tepidum, the bchF gene (CT2014) was also insertional inactivated. As shown in Fig. 2, C and D, homozygous mutants were obtained for both genes; these data indicate that the products of CT1063 and CT2014 are not required for viability of C. tepidum cells.

RESULTS

Identification of the Divinyl Reductase—A tblastn analysis of the nucleotide databases revealed that one of the closest homologs of the A. thaliana protein AT5G18660 in the available sequences is the C. tepidum hypothetical protein CT1063. The translated amino acid sequence (see supplemental Fig. S1) shares 60% identity and 75% similarity with the DVR recently identified in A. thaliana (14). To verify the function of CT1063 in C. tepidum, open reading frame CT1063 was insertional inactivated as described under “Experimental Procedures.” To confirm that the bchF gene (CT2014) was not responsible for the reduction of the DV-PChlide in C. tepidum, the bchF gene (CT2014) was also insertional inactivated. As shown in Fig. 2, C and D, homozygous mutants were obtained for both genes; these data indicate that the products of CT1063 and CT2014 are not required for viability of C. tepidum cells.

HPLC and HPLC-MS of Chls in the CT1063::aadA and bchF::aadA Mutant Cells—An HPLC chromatogram of a pigment extract from C. tepidum wild-type cells recorded at 667 nm, the absorbance maximum of BChl c, shows four main peaks that elute between 18 and 24 min and that correspond to the differently methylated homologs of BChl c (Fig. 3B; see Ref. 9). The HPLC chromatogram recorded at 667 nm of the pigment extract from the CT1063::aadA mutant revealed only two BChl c₇-related peaks, indicating that methylation was only occurring at the C-12¹ position. The retention times for these peaks were ~30 s longer than those for the corresponding peaks in the extract from wild-type cells (compare Fig. 3, A and B). The absorbance spectra of the two BChl c₇-related compounds from the CT1063::aadA mutant showed features similar to those of BChl c₆ with a Qₐ maximum at 667 nm, but the Soret absorption peak was red-shifted by 10 nm from 435 to 445 nm (Fig. 3C). The red-shifted Soret absorbance maximum is consistent with the presence of a vinyl group attached to the chlorin macrocycle, and from the absence of C-8² methylation, it can be inferred that the vinyl group must occur at the C-8 position. HPLC-MS analyses confirmed that the two BChl c₇ derivatives...
in the CT1063::aadA mutant were 2 mass units lighter (i.e. 790 and 804 Da) than their counterparts in wild-type cells (792 and 806 Da). These data confirm the presence of a vinyl group on these two molecules (Fig. 3, A and B).

Insertional inactivation of open reading frame CT1063 also altered the properties of Chl a PD and BChl a. The absorption spectrum of Chl a PD from the CT1063::aadA mutant also had a Soret peak whose maximal absorbance was red-shifted by ~10 nm from 432 to 443 nm (Fig. 4B), and the mass of this Chl a PD derivative was 2 Da less (888 Da) than that of Chl a PD from wild-type cells (890 Da) (Fig. 4A). Similarly, BChl a p from the CT1063::aadA mutant had a mass of 908.5 Da, which is 2 Da less than the mass of BChl a p from the wild-type cells (910.5 Da) (Fig. 4C). However, the Soret absorption maxima for BChl a p from the two strains were identical (Fig. 4D). These results further support the assignment of the vinyl group to the C-8 position. BChl a, a bacteriochlorella of the C-7 and C-8 carbons, in contrast to BChl c and Chl a PD, which are both chlorins, which have a double bond joining the C-7 and C-8 carbons. From these data, it can be concluded that open reading frame CT1063, which has been renamed bciA in accordance with established bacterial gene nomenclature rules (24, 25), encodes the only C-8 vinyl reductase in C. tepidum and that the BciA enzyme is responsible for the reduction of the C-8 vinyl groups of Chl a, BChl a, and BChl c to the corresponding ethyl groups.

HPLC analyses of the bchJ::aadA mutant revealed that the BChl c F homologs had identical elution times and masses (Fig. 5A) and absorption properties (Fig. 5B) as the BChl homologs of
the wild-type strain. Similarly, BChl $a_p$ and Chl $a_{(r)}$ from the bch$J$:aad$A$ mutant had retention times and masses (data not shown) and absorbance spectra (Fig. 5, C and D) that were identical to those of these compounds from the wild-type strain. However, the total BChl $c$ content in the bch$J$ mutant was severely reduced (Fig. 5A and Table 1). These results, together with the presence of DV-BChls in the bci$A$ mutant establish that bch$J$ does not encode the C-8 vinyl reductase in C. tepidum.

### Pigment Content of Whole Cells

The cells of the bci$A$:aad$A$ mutant were dark green in color and similar in appearance to wild-type cells. However, it should be noted that the absorption maximum of the BChl $c$ aggregates in the bci$A$:aad$A$ mutant was blue-shifted to 724 nm (Fig. 6A). This blue-shifted absorption maximum is similar to that observed in a bch$Q$ mutant, which like the bci$A$ mutant synthesizes BChl $c$ that is not methylated at the C-8 position. In contrast, the bch$J$:aad$A$ cells were orange in color, which suggested that these cells had a severely reduced BChl $c$ content (see Refs. 19). The whole cell absorption spectrum of the mutant and wild-type cells confirmed this observation (Fig. 6A). To determine the amounts of BChl $c$, BChl $a$, and carotenoids in the bci$A$ and bch$J$ mutant cells with respect to those of the wild-type cells, the pigment to protein ratios were determined as described under “Experimental Procedures.” These analyses confirmed that the bch$J$:aad$A$ mutant contained only 9% of the BChl $c$ found in the wild-type cells. In contrast, bci$A$:aad$A$ mutant cells, which contain 8-vinyl-BChl $c$, accumulated 23% more BChl $c$ than the wild type when grown under the same growth conditions. The BChl $a$ content of the bch$J$:aad$A$ mutant cells was also reduced to only 41% of that of the wild type; however, the BChl $a$ content of the bci$A$:aad$A$ mutant cells was only 6% lower than that of the wild type (Table 1). Carotenoids were more abundant in both mutants than in the wild type. A large difference was vis-

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Table 1

| Mutant  | BChl a | BChl c | Carotenoids |
|---------|--------|--------|-------------|
| WT      | 8.4 ± 0.7 | 89.5 ± 2.9 | 9.3 ± 0.2 |
| bciA    | 7.9 ± 1.1 | 110.8 ± 4.2 | 10.3 ± 0.5 |
| bchJ    | 3.5 ± 0.7 | 8.4 ± 0.6  | 13.5 ± 0.3 |

PChlide a and its demetallated analog, MV-protopheophorbide a (MV-Protopheo a; also known as pheoporphyrin a5). HPLC analyses showed that there were indeed two compounds in the spent medium (Fig. 8, top) with peaks resembling MV-protopheophorbide (MV-PChlide) (Fig. 8B) and MV-Protopheo a (Fig. 8A). The HPLC-MS analysis of the spent medium showed that the two compounds had masses of 610 and 588 Da, which are 2 mass units lighter than the masses of MV-PChlide (612 Da) and MV-Protopheo a (590 Da). These data establish that the substance excreted into the media by the bchJ::aadA mutant cells is DV-PChlide.

Activity Assay—BciA was heterologously expressed in E. coli BL21(DE3) cells. When the clarified whole cell extract from E. coli cells expressing bciA was mixed with DV-PChlide in the presence of NADPH, the absorbance of the Soret peak of the resulting product was blue-shifted ~5 nm with respect to the DV-PChlide. In the absence of the cell extract or NADPH, there was no change in the absorbance (Fig. 9). NADH was unable to substitute for NADPH in the reaction. The 5-nm blue-shift was not observed when DV-PChlide was incubated together with NADPH and a control whole cell extract from an E. coli strain that were not expressing the bciA gene (Fig. 9B). No shift was observed when NADH was used as a cofactor. Under all experimental conditions tested, no shift was observed in the absorbance spectrum corresponding to DV-Protopheo a (Fig. 9A). These results are consistent with a modification of the porphyrin macrocycle and confirm that BciA has C-8 vinyl reductase activity. These results further show that BciA uses NADPH as a cofactor and requires the presence of the central Mg2+ for activity.

Comparative Genomic Analysis—The family of BciA protein is very well conserved among various species. The C. tepidum BciA has 60% identity and 75% similarity to the A. thaliana divinyl reductase (see supplemental Fig. S1). BciA is conserved to a much higher degree within the GSB (see supplemental Fig.
S2). Interestingly, however, \( \text{bciA} \) is not universally distributed among GSB. In fact, among the genomes of photosynthetic bacteria that are publicly available, only 9 of the 11 genomes of GSB, 5 of 13 purple bacterial genomes, and 3 of 12 cyanobacterial genomes (excluding genomes of \( \text{Prochlorococcus} \) spp., all of which produce 8-vinyl-Chl \( \alpha \) and \( \beta \)) encode a homolog of \( \text{bciA} \). \( \text{BchJ} \), on the other hand, is much more highly conserved and widely distributed among GSB and purple bacteria. All of the green sulfur and purple bacterial genomes available encode an ortholog of \( \text{bchJ} \). However, neither plants nor cyanobacteria encode a homolog of \( \text{bchJ} \) in their genomes.

**DISCUSSION**

Both the genetic and biochemical data indicate that \( \text{bciA} \) (CT1063) and not \( \text{bchJ} \) (CT2014) encodes the C-8 vinyl (divinyl)
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reductase in *C. tepidum*. For the bciA (CT1063) mutant, the red shift observed in the absorption spectrum of the Soret maximum of the two chlorins (Chl \(a_{PD}\) and BChl \(c_{c}\)) and the absence of an absorption shift in the spectrum of the bacteriochlorin (BChl \(a_{p}\)) clearly indicate that the reduction is taking place at the C-8 position of the (B)Chl macrocycle. The masses determined for the various Chl derivatives also confirm that the bciA mutant lacks the reductase activity. It is interesting to note that all of the Chls and BChls can be replaced with their divinyl homologs without causing any major growth defect for the organism. Marine *Prochlorococcus* sp. use the divinyl derivatives of Chl \(a\) and Chl \(b\) to enhance their ability to absorb light in the blue (8, 26). Green sulfur bacteria on the other hand synthesize BChl \(e\), in which the methyl group of BChl \(e\) at the C-7 position is oxidized to a formyl group, to enhance blue light absorption for photosynthesis (27). The presence of a vinyl group at the C-8 position could enhance blue light absorption, but because it prevents methylation of the C-8\(^2\) carbon, which might adversely affect BChl aggregate formation in chlorosomes, it is perhaps not surprising that no green sulfur bacterium has yet been identified that produces divinyl-BChl \(c, d,\) or \(e\).

The identification of BciA as the C-8 vinyl reductase naturally raised an obvious question concerning the function of BchJ, a gene product previously suggested to be a C-8 vinyl reductase (11). The phenotype of the *C. tepidum bchJ* mutant is very similar to the phenotype of the *R. capsulatus bchJ* mutant observed by Suzuki and Bauer (11), who reported that *bchJ* mutant cells have reduced amounts of BChl \(a\) and excrcte large amounts of DV-PChlide into the medium. However, both the *C. tepidum* and *R. capsulatus bchJ* mutants can synthesize wild-type BChls that are reduced at the C-8 position (Ref. 11 and this work). This evidence suggests that enzymes other than BchJ is responsible for the reduction of the C-8 vinyl of DV-PChlide, but that BchJ likely regulates the biosynthesis of BChl at the level of DV-PChlide. In fact, the presence of a highly conserved bciA gene homolog in *R. capsulatus* (see supplemental Fig. S2) suggests that BciA is responsible for the reductase in the DV-PChlide in this organism as well. Although the precise role of BchJ in photosynthetic bacteria is unknown, an interesting possibility is that BchJ acts as a substrate chaperone or substrate delivery protein, without which there is disruption of normal flux of intermediates through this BChl biosynthetic pathway. Because small amounts of BChl \(a\), BChl \(c\), and Chl \(a\) are produced in its absence, it is clear that BchJ does not play an essential role in the synthesis of these Chls in *C. tepidum*.

It is evident from the analysis of the BChls extracted from the bciA mutant that *C. tepidum* can synthesize 8-vinyl-BChl \(a_{PD}\), 8-vinyl-Chl \(a_{PD}\), and 8-vinyl-BChl \(c_{c}\) at levels nearly equivalent to those of the corresponding reduced pigments in the wild type. Two mechanisms could explain this observation in *C. tepidum*. First, with the obvious exception of the C-8\(^2\) methyltransferase (BchQ), the presence of a vinyl group at the C-8 position has little or no effect on the enzymes that catalyze the downstream steps in the Chl biosynthetic pathways. Alternatively, it is possible that the C-8 vinyl reductase does not have high substrate specificity but exerts its activity upon a wide range of substrates that are generated after the branching of the BChl biosynthetic pathways (see Ref. 28 for details). However, there are two observations that support the former hypothesis. First, methylation at the C-8\(^2\) carbon occurs before the hydration of the C-3\(^3\) carbon and has a direct effect on the chirality of the BChl \(c\) molecule (29). This suggests that the reduction of the C-8 vinyl group precedes the hydration of the C-3\(^3\) carbon in BChl \(c\) biosynthesis, and would presumably also be the case in BChl \(a\) biosynthesis. Second, a highly specific divinyl reductase might be required to avoid unwanted reduction of other vinyl tetrapyrroles (e.g. heme) in the cell.

Given the fact that *C. tepidum* has only one type of light-independent PChlide reductase (DPOR) and no light-dependent PChlide reductase (LPOR) (12), the wild-type levels of 8-vinyl BChls in the bciA mutant indicate the DV-PChlide is a good substrate for the DPOR. These results are consistent with a recent report that LPOR from *R. capsulatus* has no preference for either DV-PChlide and MV-PChlide *in vitro* (30). These data suggest that by having only one gene product responsible for the reduction of the DV-PChlide to MV-PChlide, an organism can control the ratio of the two by regulating the activity of the DVR.

Before the identification of the DVR in *A. thaliana*, it had been proposed that several enzymes, or a multisubunit enzyme complex could be responsible for the reduction of the C-8 vinyl of the DV-PChlide (5). Nagata *et al.* (14) showed that the *A. thaliana* DVR and NADPH were the only requirements for the reduction of DV-PChlide. In the studies reported here, we have shown that when the *C. tepidum* BciA was heterologously expressed in *E. coli*, there was no requirement for additional proteins to reduce DV-PChlide *in vitro*. This strongly suggests that BciA is the only gene product responsible for the DVR activity. However, these results do not exclude the possibility that BciA and POR might require another protein (such as BchJ) for optimal, *in vivo* reduction of DV-PChlide and MV-PChlide, respectively.

The absence of a bciA homolog in two green sulfur bacterial genomes, some purple bacterial genomes, and most cyanobacterial genomes implies that there must be at least two structural classes of divinyl reductases: BciA and an as yet unidentified cyanobacterial-type DVR, which could also be found in some other Chl-synthesizing phototrophs. The absence of a bchJ homolog in plants and cyanobacteria suggests that they probably regulate the biosynthesis of Chl \(a\) in a different way than GSB and purple bacteria, perhaps by controlling the relative amounts of LPOR and DPOR. If BchJ were to act as a substrate channeling protein, then these organisms must no longer require this activity or have evolved another way to perform this function.

In 1995, when Suzuki and Bauer (11) proposed that *R. capsulatus* BchJ was probably the C-8-vinyl reductase, all the available evidence at the time supported this hypothesis. However, this proposed function of BchJ failed to explain the accumulation in *R. capsulatus bchJ* mutant cells of small amounts of BChl \(a\) carrying an ethyl group at the C-8 position and implied that DV-PChlide could be reduced spontaneously or by another enzyme. It also required the *R. capsulatus* DPOR to have a low affinity for the DV substrate, and organisms like *Prochlorococcus* spp. to have a specialized (D/L)POR, which could efficiently reduce DV-PChlide to DV-Chlide (11). The identification of
BciA as the DVR has at last resolved both of these issues, but it has opened a new set of questions regarding the regulation of BChl biosynthesis and the possibility that protein chaperones may function in this highly complex biosynthetic pathway.

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