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The Photoheterotrophic Growth of Bacteriochlorophyll Synthase-Deficient Mutant of *Rhodobacter sphaeroides* Is Restored by I44F Mutant Chlorophyll Synthase of *Synechocystis* sp. PCC 6803

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Chlorophyll synthase (ChlG) and bacteriochlorophyll synthase (BchG) have a high degree of substrate specificity. The BchG mutant of *Rhodobacter sphaeroides*, BG1 strain, is photosynthetically incompetent. When BG1 harboring *chlG* of *Synechocystis* sp. PCC 6803 was cultured photoheterotrophically, colonies arose at a frequency of approximately 10^{-8}. All the suppressor mutants were determined to have the same mutational change, $ChlG_{I44F}$. The mutated enzyme $ChlG_{I44F}$ showed BchG activity. Remarkably, $BchG_{F28I}$, which has the substitution of F at the corresponding 28th residue to I, showed ChlG activity. The K_m values of $ChlG_{I44F}$ and $BchG_{F28I}$ for their original substrates, chlorophyllide (Chlide) *a* and bacteriochlorophyllide (Bchlide) *a*, respectively, were not affected by the mutations, but the K_m values of $ChlG_{I44F}$ and $BchG_{F28I}$ for the new substrates Bchlide *a* and Chlide *a*, respectively, were more than 10-fold larger than those for their original substrates, suggesting the lower affinities for new substrates. Taken together, I44 and F28 are important for the substrate specificities of ChlG and BchG, respectively. The BchG activity of $ChlG_{I44F}$ and the ChlG activity of $BchG_{F28I}$ further suggest that ChlG and BchG are evolutionarily related enzymes.

Keywords: *Synechocystis* sp. PCC 6803, chlorophyll synthase, *Rhodobacter sphaeroides*, bacteriochlorophyll synthase

Introduction

As protoporphyrin IX (PPn) is synthesized in a photosynthetic organism, it is chelated either with magnesium or with iron to form magnesium protoporphyrin IX (Mg-PPn) or heme, respectively. In an oxygenic photosynthetic organism, Mg-PPn is metabolized to chlorophyllide (Chlide) *a*, whose ring D is esterified with the C_{20} moiety of geranylgeranyl pyrophosphate (GGPP) by chlorophyll synthase (ChlG) (Fig. 1) to yield geranylgeranylated chlorophyll *a* (Chl a_{gg}) [31]. Chlorophyll reductase (ChlP) sequentially reduces the double bonds at positions 6, 10, and 14 of the geranylgeranyl (GG) moiety to form phytylated Chl *a* (Chl a_p or Chl *a*) [2, 6].

In anoxygenic photosynthetic organisms, Chlide *a* is further metabolized to bacteriochlorophyllide (Bchlide) *a*. Bacteriochlorophyll hydratase (BchF) hydrates the C3-vinyl group of ring A of Chlide *a* to form 3-hydroxyethyl Chlide *a*. Then, Chlide *a* oxidoreductase (COR) reduces ring B to form 3-hydroxyethyl Bchlide *a*, whose C3-hydroxyethyl group is subsequently oxidized to an acetyl group by Bchlide *a* dehydrogenase (BchC) [5]. Alternatively, the reaction of COR may precede that of BchF. Recently, a broad substrate specificity of BchC of *Chlorobaculum tepidum* [18] was found, proposing a new additional sequence of reactions in the order of BchF, BchC, and COR to synthesize Bchlide *a*. Subsequently, the ring D of Bchlide *a* is esterified with the C_{20} moiety of GGPP by bacteriochlorophyll synthase (BchG) (Fig. 1) to yield geranylgeranylated bacteriochlorophyll *a* (Bchl a_{gg}) [1]. Bacteriochlorophyll reductase (BchP) sequentially reduces the double bonds at positions 6, 10, and 14 of the GG moiety to form phytylated Bchl *a* (Bchl a_p or Bchl *a*) [3].

The biosynthesis of Bchl *a* has been regarded as a

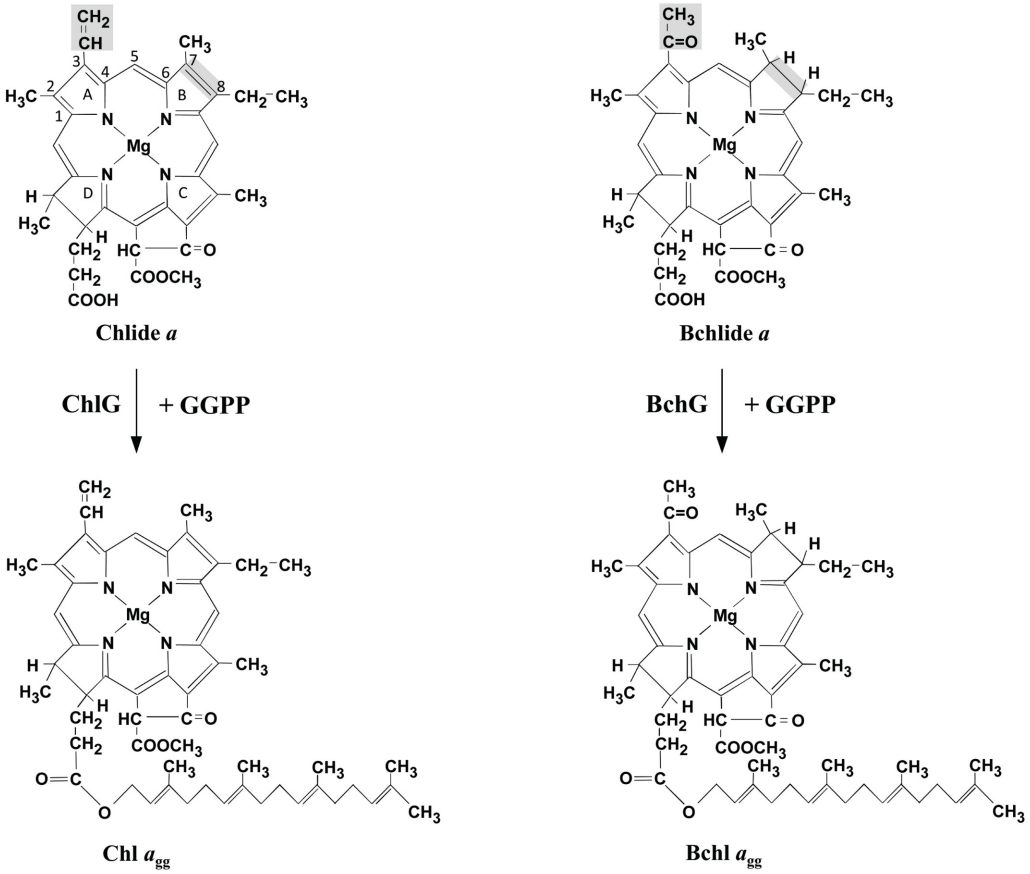


Fig. 1. Reactions of ChlG and BchG.

Chlide *a* and Bchlide *a* are esterified with GGPP by ChlG and BchG, respectively. The differences in chemical structure between Chlide *a* and Bchlide *a* are shaded. Ring names (A, B, C, and D) and carbon numbers (1 to 8) of tetrapyrrole are designated on Chlide *a*.

metabolism that existed before the emergence of the pathway to form Chl *a* [12, 33]. ChlG and ChlP have been thought to evolve through the duplication of genes coding for BchG and BchP, respectively. Previously, we showed that COR of *Rhodobacter sphaeroides*, which mediates the committing step in Bchl *a* biosynthesis, generates superoxide radicals when the reaction proceeds in the presence of low O_2 [15]. We further proposed that the superoxide-forming COR step, possibly along with the subsequent metabolic steps leading to Bchl *a*, may be degenerated as O_2 was evolved from oxygenic photosynthesis [16]. Consistent with these interpretations, the expression of COR in *Synechocystis* sp. PCC 6803 arrested photosynthetic growth unless expression of cytosolic superoxide dismutase is elevated [16].

The predicted sequence of ChlG of *Synechocystis* sp. PCC 6803 is considerably similar (35% identity) to that of *R. sphaeroides* BchG, but each enzyme has a high level of substrate specificity to distinguish its own substrate from the other [24, 28]. We previously showed that ChlG of *Synechocystis* sp. PCC 6803 is competitively inhibited by Bchlide *a*, and likewise BchG of *R. sphaeroides* is competitively inhibited by Chlide *a* [17]. Both substrates are structurally similar to each other. Thus, structural similarity would be expected between the active sites of the two enzymes.

Because the active sites of ChlG and BchG are recognized by the competitive inhibitors Bchlide *a* and Chlide *a*, respectively, we examined whether one enzyme may acquire the other enzyme activity by the mutation(s) specific to the protein. The gene *chlG* of *Synechocystis* sp. PCC 6803 was cloned into a plasmid and mobilized into the *R. sphaeroides* BchG mutant BG1, which is photosynthetically incompetent. Then, BG1 harboring *chlG* (BG1-chlG) was cultured photoheterotrophically, and mutant colonies that grew under the same conditions were obtained. All the mutants were determined to have the same I44F mutation of ChlG, and the mutated enzyme $ChlG_{I44F}$ was found to have BchG activity. We further mutated F to I at the

corresponding 28th residue of BchG, and found that BchG$_{F28I}$ has ChlG activity. Thus, one synthase acquired the other enzyme activity through the substitution of the single amino acid into the residue found at the corresponding site of the other enzyme, implying the importance of the residues I44 and F28 of ChlG and BchG, respectively, for substrate specificity.

Materials and Methods

Bacterial Strains and Growth Conditions

R. sphaeroides 2.4.1 (Table 1) was grown aerobically, anaerobically (with Dimethyl sulfoxide (DMSO)) in the dark, or photoheterotrophically at 28°C in Sistrom's succinate-based minimal medium [30] as described previously [8]. *Synechocystis* sp. PCC 6803 was grown at 30°C in BG11 medium [4] supplemented with 10 mM D-glucose as described previously [16]. *Escherichia coli* was grown at 30°C or 37°C in Luria-Bertani medium. Antibiotics were added to the cultures of *R. sphaeroides* and *E. coli* at concentrations as indicated previously [14].

Site-Directed Mutagenesis

To mutate F28 of BchG (Fig. 2A) into I, site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to the protocol supplied by the manufacturer. Plasmid pRbchG (Table 1) was used as a template for PCR with forward primer (5′-CCC ATC ACC TGG ATC CCG

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
R. sphaeroides		
2. 4. 1	Type strain	W. Sistrom
BZF1	A *bchZ-bchF* mutant; Km^r	[15]
BG1	A *bchG* mutant	[17]
BG1-chlG	A *bchG* mutant carrying pRKchlG	This study
BG1-chlG$_{I44F}$	A *bchG* mutant carrying pRKchlG$_{I44F}$	This study
BG1-bchG	A *bchG* mutant carrying pRKbchG	This study
BG1-bchG$_{F28I}$	A *bchG* mutant carrying pRKbchG$_{F28I}$	This study
Synechocystis sp.		
PCC 6803	Wild-type strain	[25]
E. coli		
DH5α	*supE*44 Δ*lacU*169 (Φ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	[10]
S17-1	C600::RP4 2-(Tc::Mu)(Km::Tn7) *thi pro hsdR hsdM*$^+$ *recA*	[29]
BL21(DE3)	*E. coli* B F$^-$ *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)	Stratagene
Plasmids		
pRK415	*ori* IncP Mob RP4 *lacZ*α; Tc^r	[13]
pRKchlG	pRK415 + 1.1 kb XbaI-EcoRI fragment containing *chlG*; Tc^r	This study
pRKbchG	pRK415 + 1.0 kb XbaI-EcoRI fragment containing *bchG*; Tc^r	This study
pRKchlG$_{I44F}$	pRK415 + 1.1 kb XbaI-EcoRI fragment containing *chlG*$_{I44F}$; Tc^r	This study
pRKbchG$_{F28I}$	pRK415 + 1.0 kb XbaI-EcoRI fragment containing *bchG*$_{F28I}$; Tc^r	This study
pRSET-A/C	Expression vector of his6-tag fusion protein; Ap^r	Invitrogen
pRchlG	pRSET-A + 1.0 kb fragment containing his6-tag fusion of *chlG*; Ap^r	[17]
pRbchG	pRSET-C + 0.9 kb fragment containing his6-tag fusion of *bchG*; Ap^r	[17]
pRchlG$_{I44F}$	pRSET-A + 1.0 kb fragment containing his6-tag fusion of *chlG*$_{I44F}$; Ap^r	This study
pRbchG$_{F28I}$	pRSET-C + 0.9 kb fragment containing his6-tag fusion of *bchG*$_{F28I}$; Ap^r	This study

[a]Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance.

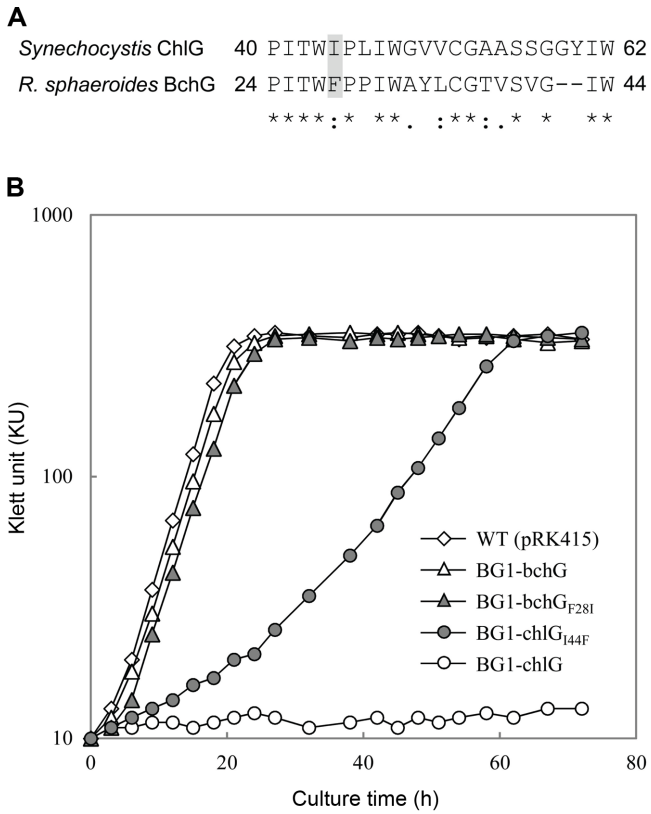


Fig. 2. Aligment of the ChlG and BchG sequences, and photoheterotrophic growth of BG1-bchG, BG1-bchG$_{F28I}$, BG1-chlG, and BG1-chlG$_{I44F}$.

(**A**) The sequence of the first predicted transmembrane domain of ChlG of *Synechocystis* sp. PCC 6803 was compared with that of BchG of *R. sphaeroides*. I44 of ChlG and F28 of BchG are shaded. Keys denoting conserved sequence (*), conservative mutations (:), and semi-conservative mutations (.) are illustrated below the enzyme sequences. (**B**) Cells were grown under photoheterotrophic growth conditions at 10 W/m^2. WT cells containing pRK415 were included as a control. The experiments were independently repeated three times; data shown are one of three representative experiments.

CCG ATC TG-3′: mutated sequence is underlined, unless noted otherwise) and reverse primer (5′-CAG ATC GGC GGG ATC CAG GTG ATG GG-3′) to yield pRbchG$_{F28I}$ (Table 1).

Construction of Plasmids

Expression of ChlG, BchG, ChlG$_{I44F}$, and BchG$_{F28I}$ in *R. sphaeroides*. A 1.1 kb XbaI-EcoRI DNA fragment of pRchlG (Table 1) was cloned into the XbaI-EcoRI sites of pRK415 (Table 1) to yield pRKchlG (Table 1). Likewise, a 1.0 kb XbaI-EcoRI DNA fragment of pRbchG (Table 1) was cloned into the XbaI-EcoRI sites of pRK415 to yield pRKbchG (Table 1). The recombinant plasmid pRKchlG$_{I44F}$ containing the I44F mutation of ChlG (Table 1) was constructed with a 1.1 kb XbaI-EcoRI DNA fragment containing *chlG*$_{I44F}$, which was PCR-amplified from the genome of the suppressor mutant of BG1-chlG (Table 1). Likewise, a 1.0 kb DNA fragment containing *bchG*$_{F28I}$ of pRbchG$_{F28I}$ was digested with XbaI and EcoRI and cloned into the XbaI-EcoRI sites of pRK415 to generate pRKbchG$_{F28I}$ (Table 1).

Expression of ChlG, BchG, ChlG$_{I44F}$, and BchG$_{F28I}$ in *E. coli*. Plasmids pRchlG and pRbchG (Table 1) were used for the expression of ChlG and BchG in *E. coli*, respectively, and plasmids pRchlG$_{I44F}$ and pRbchG$_{F28I}$ (Table 1) were used for the expression of ChlG$_{I44F}$ and BchG$_{F28I}$ in *E. coli*, respectively. Plasmid pRchlG$_{I44F}$ was constructed by cloning a 1.1 kb XbaI-EcoRI DNA fragment encompassing the *chlG*$_{I44F}$ from pRKchlG$_{I44F}$ into the XbaI-EcoRI sites of pRSET-A (Table 1).

Determination of Light-Harvesting Complexes

R. sphaeroides was grown anaerobically (with DMSO) in the dark, and cell-free lysates were prepared as described previously [19]. Absorption spectra of the equivalent cell-free lysates (400 μg protein) were examined with a UV 2550-PC spectrophotometer (Shimadzu, Japan). Protein levels were determined by a modified Lowry method as described previously [20]. The amount of B800-850 complex was calculated from the spectrophotometric profile by $A_{849\text{-}900}$, using an extinction coefficient (ε) of 96 mM^{-1} cm^{-1}, while the amount of B875 complex was determined by $A_{878\text{-}820}$ with ε of 73 mM^{-1} cm^{-1} [22].

Purification and Determination of Bchl *a*

Bchl *a* was purified from *R. sphaeroides* as described previously [7, 11]. Bchl *a* was collected in *n*-hexane and its level was determined with ε of 83.9 mM^{-1}cm^{-1} at 771 nm [32].

Purification of Chlide *a* and Bchlide *a*

Chlide *a* and Bchlide *a* were purified from culture supernatant of BZF1 and BG1 (Table 1), respectively, as described previously [23]. Their levels were determined with ε of 77.1 mM^{-1}cm^{-1} at 663 nm for Chlide *a* [23] and ε of 42.1 mM^{-1}cm^{-1} at 773 nm for Bchlide *a* [32]. Alternatively, the C_{20} moieties of Chl *a* and Bchl *a* were removed by chlorophyllase to yield Chlide *a* and Bchlide *a*, respectively, as described previously [21, 24]. Chl *a* was purchased from Sigma-Aldrich and Bchl *a* was purified from *R. sphaeroides* as described above.

Assays of ChlG, BchG, ChlG$_{I44F}$, and BchG$_{F28I}$

E. coli strain BL21(DE3) (Table 1) was transformed with the recombinant plasmids pRchlG, pRbchG, pRchlG$_{I44F}$, and pRbchG$_{F28I}$, and each recombinant strain was cultured and harvested as described previously [17]. Reactions were performed at 30°C and stopped by acetone as described previously [24]. The levels of Chl *a* and Bchl *a* were determined by HPLC.

HPLC Analysis

Reverse-phase HPLC analyses were performed on a LC6-AD system (Shimadzu, Japan) equipped with a Gemini C18 column

(Phenomenex, Torrance, CA, USA; particle size, 5 μm; column length × diameter, 250 mm × 4.6 mm), a RF-20A fluorescence detector, and a SPD-M20A diode array detector as described previously [17]. The fluorescence detector was set at 405 nm for excitation and at 675 nm for emission. Chl *a* and Bchl *a* were used as standards.

Results

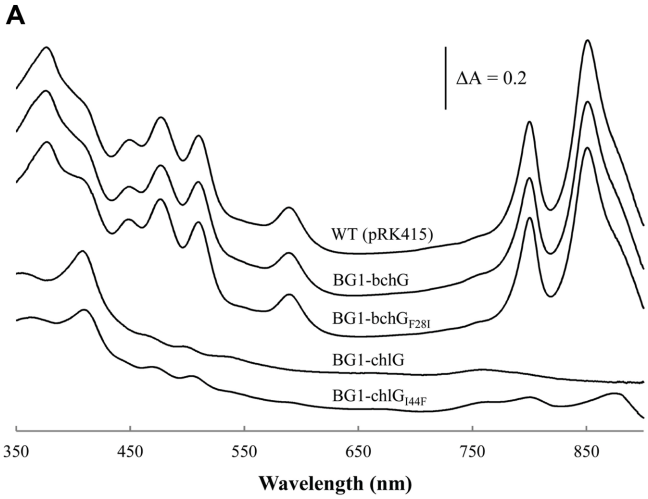
ChlG$_{I44F}$ of *Synechocystis* sp. PCC 6803 Supports the Photoheterotrophic Growth of *R. sphaeroides* BchG Mutant BG1

ChlG of *Synechocystis* sp. PCC 6803 and BchG of *R. sphaeroides* are competitively inhibited by Bchlide *a* and Chlide *a*, respectively, and the two Mg-tetrapyrroles are structurally similar to each other [17]. Thus, the active sites of the two enzymes are thought to have similar structures, but both enzymes exhibit a high degree of substrate specificities [17, 24, 28]. In this work, we tried to find out the residue(s) that confer substrate specificity, and further examined whether one enzyme can acquire the other enzyme activity by mutation(s).

R. sphaeroides BG1 [17] does not grow photoheterotrophically. Both the growth (Fig. 2B) and spectral complex formation (Fig. 3) of BG1 under anaerobic conditions were restored to wild-type (WT) levels if pRKbchG, a recombinant pRK415 carrying *R. sphaeroides bchG*, was provided in trans. Separately, a DNA fragment containing *chlG* of *Synechocystis* sp. PCC 6803 was cloned into pRK415 to generate pRKchlG and it was mobilized into *R. sphaeroides* BG1. The BG1-harboring pRKchlG (BG1-chlG) did not grow photoheterotrophically (Fig. 2B), but colonies that grew under these conditions arose at a frequency of approximately 10^{-8}. Approximately 20 of these suppressor mutants were randomly selected from several independent experiments. Although both *trans*-acting (chromosomal) and *cis*-acting (plasmid) spontaneous mutations were expected, all the mutants showing photoheterotrophic growth were found to have *cis*-acting mutations. Through DNA sequencing of the plasmids from mutants, all the plasmids were determined to have the same mutational change from isoleucine (ATT) to phenylalanine (TTT) at the 44th residue of ChlG (Fig. 2A). This residue is located on the first predicted transmembrane domain of ChlG.

ChlG$_{I44F}$ of *Synechocystis* sp. PCC 6803 Shows BchG Activity

A DNA fragment containing *chlG*$_{I44F}$ was restricted from the plasmid of suppressor mutants of BG1-chlG, and re-cloned into pRK415 to generate pRKchlG$_{I44F}$. The recombinant plasmid supported the photoheterotrophic growth of BG1 (Fig. 2B), confirming ChlG$_{I44F}$ indeed as a suppressor. The BG1-harboring pRKchlG$_{I44F}$ (BG1-chlG$_{I44F}$) grew slowly compared with WT cells (Fig. 2B). The spectral complexes of BG1-chlG$_{I44F}$ were examined after growth under anaerobic (with DMSO) and dark conditions (Fig. 3A). The B875 complex level of BG1-chlG$_{I44F}$ was approximately 20% of WT level, whereas the amount of B800-850 complex was



B

Strains	Levels of light-harvesting complexes	
	B875	B800-850
WT (pRK415)	7.2 ± 0.5[a]	22.9 ± 1.3
BG1-bchG	6.9 ± 0.6	20.7 ± 1.8
BG1-bchG$_{F28I}$	6.8 ± 0.9	19.9 ± 2.1
BG1-chlG	ND[b]	ND
BG1-chlG$_{I44F}$	1.5 ± 0.4	2.2 ± 0.7

[a]nmole/mg protein.
[b]Not detectable.

Fig. 3. Absorption spectra and levels of light-harvesting complexes of BG1-bchG, BG1-bchG$_{F28I}$, BG1-chlG, and BG1-chlG$_{I44F}$.
Cells were grown anaerobically (with DMSO) in the dark in order to minimize the occurrence of suppressor mutations rescuing the growth of BG1-chlG under photoheterotrophic conditions. Absorption spectra (**A**) were illustrated with the levels of B800-850 complex and B875 complex (**B**). WT cells containing pRK415 were included as a control. The experiments were independently repeated three times; data shown are one of three representative experiments, and the average values of light-harvesting complexes are shown with standard deviation.

only 10% that of WT cells (Fig. 3B). The carotenoid level was also reduced relative to WT cells (Fig. 3A). The cellular Bchl *a* content, which amounted to 3.7 nmol/mg protein, was approximately 15% of the WT level. The results indicate that the BchG activity of $ChlG_{I44F}$ was not enough to synthesize WT levels of Bchl *a* under the conditions examined (Fig. 3B).

ChlG and $ChlG_{I44F}$ of *Synechocystis* sp. PCC 6803 were overexpressed in *E. coli* and used as enzyme sources. The K_m of $ChlG_{I44F}$ for Chlide *a* was not different from that of ChlG, indicating no change in the affinity for Chlide *a* by the mutation of I44F (Table 2). However, the K_m for Bchlide *a* was more than 10-fold larger than for Chlide *a* (Table 2). Thus, although $ChlG_{I44F}$ has BchG activity, its affinity for Bchlide *a* appears to be significantly lower than for its original substrate Chlide *a*.

The K_m of $ChlG_{I44F}$ for Bchlide *a* was further compared with that of BchG for the same substrate. *R. sphaeroides* BchG was overexpressed in *E. coli* and used as an enzyme source. The K_m of BchG for Bchlide *a* was approximately 10 times smaller than that of $ChlG_{I44F}$ (Table 2). Therefore, $ChlG_{I44F}$ appears to have lower affinity for Bchlide *a* than BchG. The results may explain the slower growth (Fig. 2B) and the reduced formation of spectral complexes (Fig. 3) of BG1-$chlG_{I44F}$ compared with WT cells.

$BchG_{F28I}$ of *R. sphaeroides* Shows ChlG Activity

Because $ChlG_{I44F}$ of *Synechocystis* sp. PCC 6803 showed BchG activity, it was examined whether ChlG activity could be shown by $BchG_{F28I}$. The amino acid F (TTC) at the 28^{th} residue of BchG, which corresponds to the 44^{th} of ChlG, was substituted to I (ATC). $BchG_{F28I}$ was overexpressed in *E. coli* and used as an enzyme source. Remarkably, $BchG_{F28I}$ of *R. sphaeroides* showed ChlG activity (Table 2), but the K_m of $BchG_{F28I}$ for Chlide *a* was more than 15-fold larger than that for Bchlide *a* (Table 2). Thus, although $BchG_{F28I}$ has ChlG activity, its affinity for Chlide *a* appears to be significantly lower than for its original substrate Bchlide *a*. The K_m of $BchG_{F28I}$ for Bchlide *a* was not different from that of BchG, indicating no change in the affinity for Bchlide *a* by the mutation of F28I. In accordance with the results, no difference in photoheterotrophic growth (Fig. 2B) and the spectral complexes (Fig. 3) was observed between WT cells and BG1-containing pRKbchG_{F28I} (BG1-bchG_{F28I}), which is a recombinant pRK415 carrying *bchG*$_{F28I}$ (Table 1).

In summary, the mutated enzymes $ChlG_{I44F}$ and $BchG_{F28I}$ showed new activities of BchG and ChlG, respectively, but the affinities for the new substrates were significantly lower than for their original ones. Thus, I44 and F28 residues are important for the substrate specificity of ChlG and BchG, respectively.

Table 2. K_m values of ChlG, BchG, and their mutated enzymes $ChlG_{I44F}$ and $BchG_{F28I}$ for Chlide *a* and Bchlide *a*[a].

Enzyme[b]	K_m (mM)	
	Chlide *a*	Bchlide *a*
ChlG	0.09 ± 0.01	NA[c]
$ChlG_{I44F}$	0.11 ± 0.02	1.42 ± 0.21
BchG	NA	0.13 ± 0.02
$BchG_{F28I}$	2.87 ± 0.35	0.16 ± 0.03

[a]The experiments were independently repeated three times; data are shown as the mean ± SD.

[b]Enzymes were expressed as his-tagged proteins in *E. coli* BL21(DE3).

[c]Not applicable.

Discussion

Chlide *a* and Bchlide *a* are structurally similar to each other except for the C3 functional group (vinyl and acetyl, respectively) of ring A and the redox state of the C7-C8 bond (oxidized and reduced, respectively) of ring B (Fig. 1). These structural differences between the two substrates may be prerequisites for ChlG and BchG to esterify ring D of their substrates with the C_{20} moiety that had been covalently attached to the enzyme using GGPP. However, the structural differences between Chlide *a* and Bchlide *a* are ignored if they bind to the active sites of BchG and ChlG, respectively, as competitive inhibitors [17].

Both ChlG and BchG are predicted to transverse the cell membrane nine times [27]. Although a conserved region ([ND]-x(3)-[DE]-x(3)-D) for the binding site of polyprenyl diphosphate (GGPP and phytyl pyrophosphate [PPP]) is found at a loop between the second and third transmembrane segments [27], the residue responsible for prenylation and the domains for the binding of Chlide *a* or Bchlide *a* have not yet been determined. The results in this work clearly demonstrate that I44 of ChlG and F28 of BchG, which are located on the first transmembrane domain, are important for the substrate specificity for Chlide *a* and Bchlide *a*, respectively. This interpretation was further corroborated by new activities of BchG and ChlG by $ChlG_{I44F}$ and $BchG_{F28I}$, respectively. A detailed understanding of the mechanism by which substrates interact with the I44 of ChlG and the F28 of BchG must await the determination of each enzyme structure.

The affinity of $ChlG_{I44F}$ for the new substrate Bchlide *a* appears to be lower than that of BchG. The same is true for

the affinity of $BchG_{F28I}$ for the new substrate Chlide *a*. It remains to be determined whether the mutated enzymes $ChlG_{I44F}$ and $BchG_{F28I}$ can be further developed through additional mutation(s) to show the WT-level affinity for the new substrates.

Interestingly, most cyanobacterial ChlG sequences that are available in the protein database have an I at the 44th residue, but the corresponding amino acid of BchG in most purple and green phototrophic bacteria is F at this position. *Chlorobaculum tepidum*, a green sulfur bacterium, contains two BchG enzymes; one has F at the site corresponding to the 28th residue of *R. sphaeroides* BchG, whereas the other has I at the corresponding site. The two enzymes harboring F and I residues may reflect the biosynthesis of Bchl *a* (approximately 3% of total Chl and Bchl) and Chl *a* (approximately 0.3%), respectively, in addition to the major pigment Bchl *c* (approximately 97%) [9]. Bchl *c* is mainly esterified with C_{15} farnesol by Bchl *c* synthase (BchK) [26] that is paralogous to BchG. The predicted sequence of BchK illustrates approximately 50% similarity to that of BchG of *R. sphaeroides* as well as to that of ChlG of *Synechocystis* sp. PCC 6803. As observed for ChlG, BchK harbors I at the site corresponding to F28 of BchG. It remains to be determined whether BchK activity can be affected by Bchlide *a*.

The eukaryotic green alga *Chlamydomonas reinhardtii* has I at the site corresponding to the 44th residue of ChlG of *Synechocystis* sp. PCC 6803, whereas ChlG of plants such as *Avena sativa*, *Oryza sativa*, *Nicotiana tabacum*, and *Arabidopsis thaliana* contain proline at this site. Thus, it will be interesting to determine whether this residue also confers the high levels of substrate specificity as observed for ChlG of *Synechocystis* sp. PCC 6803.

Taken together, I44 of ChlG and its corresponding residue F28 of BchG are important in determining substrate specificity. The results in this work further reinforce the notion that ChlG and BchG are evolutionarily related enzymes. As far as we know, this is the first report on the BchG and ChlG activities of $ChlG_{I44F}$ and $BchG_{F28I}$, respectively.

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