Crystal structure of phytochromobilin synthase in complex with biliverdin IX$_\alpha$, a key enzyme in the biosynthesis of phytochrome

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Phytochromobilin (ΦΦB) is a red/far-red light sensory pigment in plant phytochrome. ΦΦB synthase is a ferredoxin-dependent bilin reductase (FDBR) that catalyzes the site-specific reduction of bilins, which are sensory and photosynthetic pigments, and produces ΦΦB from biliverdin, a heme-derived linear tetrapyrole pigment. Here, we determined the atomic structure of tomato ΦΦB synthase in complex with biliverdin at 1.95 Å resolution. The overall structure of tomato ΦΦB synthase was similar to that of other FDBRs, except for the addition of a long C-terminal loop and short helices. The structure further revealed that the C-terminal loop is part of the biliverdin-binding pocket and that two basic residues in the C-terminal loop form salt bridges with the propionate groups of biliverdin. This suggested that the C-terminal loop is involved in the interaction with ferredoxin and biliverdin. The configuration of biliverdin bound to tomato ΦΦB synthase differed from that of biliverdin bound to other FDBRs, and its orientation in ΦΦB synthase was inverted relative to its orientation in the other FDBRs. Structural and enzymatic analyses disclosed that two aspartic acid residues, Asp-123 and Asp-263, form hydrogen bonds with water molecules and are essential for the site-specific A-ring reduction of biliverdin. On the basis of these observations and enzymatic assays with a V121A ΦΦB synthase variant, we propose the following mechanistic product release mechanism: ΦΦB synthase-catalyzed stereospecific reduction produces 2(R)-ΦΦB, which when bound to ΦΦB synthase collides with the side chain of Val-121, releasing 2(R)-ΦΦB from the synthase.

Phytochrome is a red light-sensitive photoreceptor in plants that is involved in photoperiodic induction of flowering, chloroplast development, leaf senescence, and leaf abscission (1).

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The atomic coordinates and structure factors (codes 6KMD and 6KME) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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The chromophore of phytochrome for accepting red/far-red light is phytochromobilin (ΦΦB), which is synthesized from a heme metabolite, biliverdin IX$_\alpha$ (BV). ΦΦB synthase (EC 1.3.7.4), also known as HY2 or AUREA, which is located in chloroplasts, catalyzes the site-specific reduction of BV to produce ΦΦB using electrons supplied by ferredoxin (Scheme 1) (2, 3). After the reduction, ΦΦB can form a covalent bond with the cysteine residue of apo-phytochrome to produce holo-phytochrome (4). Several ΦΦB synthase-deficient mutants (such as hy2 in Arabidopsis thaliana and auro and yellow-green-2 in tomato) were characterized (5–7). These mutants showed distinctive features such as elongated appearance with reduced anthocyanin and chlorophyll levels, which results in pale-green—or yellow-looking plants, because of the lack of mature phytochrome.

ΦΦB synthase is a member of the ferredoxin-dependent bilin reductase (FDBR) family. The FDBR family comprises several different but closely related proteins including phycocyanobilin:ferredoxin oxidoreductase (PcyA, EC 1.3.7.5), 15,16-dihydrobiliverdin:ferredoxin oxidoreductase (PebA, EC 1.3.7.2), phycoerythrobilin:ferredoxin oxidoreductase (PebB, EC 1.3.7.3), phycoerythrobilin synthase (PebS, EC 1.3.7.6), and ΦΦB synthase (8). These enzymes are widely distributed in oxygenic phototrophs. In cyanobacteria and red algae, phycobilins, which function in light harvesting for photosynthesis and in light sensing by cyanobacteriochrome, are produced from BV by FDBR. The reaction catalyzed by FDBR consists of radical formation on bilin after one-electron reduction by ferredoxin as the electron donor (9). The reduction sites of BV differ according to the enzyme: ΦΦB synthase catalyzes the reduction of the A-ring of BV, whereas PcyA catalyzes the reductions of the vinyl group of the D-ring and A-ring of BV (10).

The X-ray structure of the BV–PcyA complex was the first reported tertiary structure of an FDBR (11, 12). PcyA has a single-domain architecture and folds into an α/β/sandwich. BV in the helical (ZZZ-all-syn or U-shaped) conformation is bound between the central β-sheet and C-terminal helices. In

2 The abbreviations used are: ΦΦB, phytochromobilin; BV, biliverdin IX$_\alpha$; 15,16-DHBV, 15,16-dihydrobiliverdin IX$_\alpha$; FDBR, ferredoxin-dependent bilin reductase; GtPEBB, phycoerythrobilin:ferredoxin oxidoreductase from Guillardia theta; ΦΦB synthase, phytochromobilin:ferredoxin oxidoreductase; SeMet-ΦΦB synthase, selenomethionine substituted ΦΦB synthase; RCCR, red chlorophyll catabolite reductase.
Structure of phytochromobilin synthase bound to substrate

Results and discussion

Characteristics of recombinant tomato PΦB synthase

We examined the enzymatic activity of tomato monomeric PΦB synthase using an assay reported for Arabidopsis PΦB synthase (24). The Q-band of BV bound to PΦB synthase was blue-shifted following the addition of NADPH, just as in the reaction catalyzed by Arabidopsis PΦB synthase (Fig. 1), indicating formation of PΦB. Before formation of PΦB, absorption at 450 and 740 nm increased and then decreased. We did not confirm radical formation by ESR measurement, but the spectral change before the formation of PΦB was similar to that reported for Arabidopsis PΦB synthase, in which radical formation was confirmed by ESR (24). Thus, the increases in absorption at 450 and 740 nm suggest the formation of radical species during the reaction. HPLC analysis of the products indicated that both 3Z-PΦB and 3E-PΦB were produced as in the reaction catalyzed by Arabidopsis PΦB synthase (Fig. 1C).

Overall structure of tomato PΦB synthase in complex with BV

The crystal structure of tomato PΦB synthase in complex with BV, determined at 1.95 Å resolution, is shown in Fig. 2A. Consistent with other FDBRs and RCCRs, PΦB synthase folds into an α/β/α sandwich. The topology of the central β-sheet consists of a β-meander motif with helices H2–4 inserted between strands S6 and S7. The distal α-helical layer is composed of helices H2, H4, and H6–10, whereas the proximal α-helical layer is composed of helices H1, H3, and H5. BV is bound between the central antiparallel β sheet (S1–S7) and α-helices (H6 and H7) as in other FDBRs. Following the H8 helix, a long loop and short α-helices (H9 and H10) are observed in tomato PΦB synthase (50 amino acid residues). This loop and helical structure are specific to PΦB synthase. The recently reported structure of PebB from a cryptophyte (GtPEBB) also has a C-terminal extension (27), although the amino acid sequence and the structure of that C-terminal extension are not similar to those of PΦB synthase (Fig. 2C and Fig. S1). Sequence alignment of FDBRs showed that the amino acid sequence corresponding to this loop and H9 helix is not conserved in other FDBRs, whereas it is conserved in PΦB synthases (Fig. S1). The surface electrostatic potential demonstrated that the positive charge is localized near the entrance of the substrate-binding pocket as in other FDBRs and RCCRs, suggesting that acidic ferredoxin binds near the propionate groups of BV (Fig. 2B). The amino acid residues involved in the interactions between Arabidopsis PΦB synthase and ferredoxin 2 were identified using cross-linking and enzymatic assays (25). The residues corresponding to Lys-183, Arg-200, Lys-263, and Arg-264 in Arabidopsis PΦB synthase are located on the molecular surface and surrounding the entrance of BV, suggesting that they are directly involved in the interaction with ferredoxin. The Cl− ion contained in the crystallization solution is located close to the propionate group of BV and the side chain of Arg-207, which corresponds to Arg-200 in Arabidopsis PΦB.
Structure of phytochromobilin synthase bound to substrate

(A)

(B)

(C)
synthase. Upon ferredoxin binding to PΦB synthase, an acidic residue of ferredoxin may replace the Cl\textsuperscript− ion and interact with the side chain of Arg-207 and the propionate group of BV. Glu-194 and Lys-262, which correspond to Glu-187 and Lys-255 in Arabidopsis PΦB synthase, are not located on the molecular surface. These residues may be involved in the stabilization of the additional C-terminal long loop and H9 and H10 helices, which are specific to PΦB synthase and form part of the BV entrance. Thus, these two residues may be indirectly involved in the interaction with ferredoxin.

Figure 1. Enzymatic assay of tomato PΦB synthase. A, the UV-visible spectrum of BV-tomato PΦB synthase is shown as a solid line, whereas that of BV is shown as a broken line. Absorption at 640 nm increases upon binding to PΦB synthase. B, spectrum changes occurring during the conversion from BV to PΦB were monitored at 2-min intervals for 30 min. The initial and the end point spectra are shown as black bold and black dashed lines, respectively. The single-turnover reaction was initiated by the addition of NADPH. C, HPLC analysis of the product of tomato PΦB synthase. Following the single-turnover reaction shown in B, the reaction was stopped by the addition of TFA. A chromatogram obtained at 380 nm is shown. BV was purchased from Frontier Scientific.

Figure 2. Overall structure of BV-tomato PΦB synthase. A, stereo-diagram of the overall structure of tomato PΦB synthase is shown. BV is depicted as a stick model. Additional C-terminal loop and helices are shown in yellow. The N-terminal 10 residues, His tag, and C-terminal 4 residues were disordered. One Mg\textsuperscript2+ and one Cl\textsuperscript− ions are shown as spheres. B, the surface electrostatic potential was in the range of ±5 kT/e. The potential was calculated with APBS (36). C, Cα trace of BV-tomato PΦB synthase, superimposed onto that of the Synechocystis PcyA complex (cyan, PDB ID: 2D1E, left panel) and the 15,16-DHBV-GtPEBB complex (orange, PDB ID: 6QX6, right panel). Lower panels shown as dashed boxes demonstrated the zoomed-in view of the substrates. All structural figures were prepared with PyMOL (37).
**BV-binding mode in PΦB synthase**

As shown in Fig. 3A, BV bound to tomato PΦB synthase could be clearly observed. The ZZZssa conformation was observed in tomato PΦB synthase, whereas the conformation of BV bound to other FDBRs was ZZZ-all-syn (U-shaped). The conformation observed in PΦB synthase is identical to that in the Pr state of bacteriophytochrome (14–17).

One of the structural reasons why ZZZssa configuration of BV is accepted in PΦB synthase is the position of Arg-259 (Fig. 3B). If the configuration of BV bound to PΦB synthase is ZZZ-all-syn, this Arg collides with the D-ring of BV. In other words, Arg-259 acts as a dam to avoid further invasion of BV toward the interior side of the substrate-binding pocket. Also, Arg-259 interacts with the vinyl group of the D-ring of BV via a cation–π interaction to accept the ZZZssa configuration of BV. Indeed, this Arg residue is substituted by Gln in PcyA, Ser in PebA, and Met in PebS. Thus, the ZZZ-all-syn configuration of BV is accepted in other FDBRs, and BV is located on the interior side of the enzymes relative to the situation in PΦB synthase. As shown in Fig. 3 (B and C), Ser-195 forms a hydrogen bond with the lactam oxygen atom of the D-ring of BV, which is likely to stabilize the anti-conformation of the C15–C16 double bond of BV.

Another structural reason may be the substitution of the central Asp residue. In other FDBRs, an aspartic acid residue belonging to the central strand (S5), such as Asp-105 in *Synechocystis* PcyA (11), is located under the center of BV, and the carboxyl group of the aspartic acid side chain forms hydrogen bonds with the pyrrole nitrogen atoms of BV to stabilize the ZZZ-all-syn configuration. This structural feature is conserved in all other FDBRs, excluding 15,16-DHBV–bound GtPEBB, in which the substrate conformation is similar to that of tomato PΦB synthase (Fig. 2C). In tomato PΦB synthase, the corresponding residue is substituted by asparagine (Asn-140) and is not located under the center of BV but rather forms a hydrogen bond with the lactam oxygen atom of the A-ring of BV (Fig. 3B). Thus, this substitution may destabilize the syn-conformation of the C15–C16 double bond of BV in tomato PΦB synthase. This
Structure of phytochromobilin synthase bound to substrate

substitution is conserved in other higher plant PbFB synthases but not in GtPEBB (Fig. S1).

It was proposed that this aspartic acid residue in PcyA contributes to the protonation of BV upon binding to PcyA (9, 29). The light absorption shoulder observed at 740 nm in the BV–PcyA complex, which suggests that the formation of protonated BV (BVH⁺), was not observed in the BV–PbFB synthase complex (Fig. 1A), suggesting that BV bound to PbFB synthase exists in the neutral form and not as BVH⁺. This is consistent with the structural feature of PbFB synthase, in which the central Asp is substituted with Asn.

The lactam oxygen atom of the D-ring of BV also forms a hydrogen bond with a water molecule, which forms a hydrogen bond with Trp-258. The basic residue belonging to the C-terminal additional loop, Arg-316, forms a salt bridge with the propionate group of BV. Thr-192 forms a hydrogen bond with the propionate group of BV (Fig. 3B). Arg-207 and Lys-321 interact with the propionate group of BV via a water molecule.

Structural comparisons with other FDBRs

Structural comparisons with other FDBRs are shown in Fig. 2C and Fig. S2. Root-mean-square distances of Cα atoms were 1.63 Å for PcyA (116 atoms of 248 atoms were aligned), 1.21 Å for PebA (151 atoms of 244 atoms were aligned), 1.82 Å for PebS (87 atoms of 233 atoms were aligned), and 0.77 Å for GtPEBB (175 atoms of 264 atoms were aligned), respectively. BV bound to tomato PbFB synthase was flipped relative to BV bound to other FDBRs excluding GtPEBB (Fig. 2C and Fig. S2). The structure of 15,16-DHBV–bound GtPEBB, which also catalyzes the reduction of the A-ring, was recently reported (27). Indeed, the orientation of the substrate is similar to that of PbFB synthase, but the orientation of the D-ring is inverted (Figs. 2C and 3C). Phylogenetically, PebB is more closely related to PbFB synthase than to PcyA and PebA (30). Comparison of the tertiary structures of substrate-bound FDBRs also supports the idea that PbFB synthase evolved from PebB.

Furthermore, structural comparison of PbFB synthase with PcyA, PebA, and PebS revealed that the BV-binding position in PbFB synthase is ~3 Å close toward the entrance of the BV-binding pocket (Fig. 2C and Fig. S2). One of the reasons why the BV-binding site is slightly different from those in other FDBRs may be the substitution of the central Asp residue. As shown above, the central Asp residues, such as Asp-105 in Synechocystis PcyA, form hydrogen bonds with the pyrrole nitrogen atoms of BV to stabilize ZZZ-all-syn configuration. However, this Asp residue is substituted by Asn, and no residues form hydrogen bonds with the pyrrole nitrogen atoms of BV in PbFB synthase.

The corresponding Asp residue in GtPEBB forms a hydrogen bond with the lactam oxygen atom of the A-ring but not with the nitrogen atom of the A-ring. Thus, the position of 15,16-DHBV is ~1.5 Å close toward the entrance of the substrate-binding pocket (Figs. 2C and 3C). Thus, the binding position of 15,16-DHBV in GtPEBB is middle between the BV-binding position in PbFB synthase and those in other FDBRs.

Another reason may be the existence of the C-terminal extended loop. The extended C-terminal loop interacts with the propionate groups of BV in PbFB synthase. The interaction with Arg-316 especially pulls BV toward the entrance of the BV-binding pocket relative to the substrate binding position of other FDBRs and places it in the appropriate position for A-ring reduction.

As shown above, the substrate binding modes of PbFB synthase and GtPEBB are similar to each other, but the D-ring is in opposite orientations between GtPEBB and PbFB synthase. Ser-195, which forms a hydrogen bond with the lactam oxygen atom of the D-ring, is replaced by Ala in GtPEBB (Fig. 3C). The lactam oxygen atom of the D-ring of 15,16-DHBV bound to GtPEBB forms a hydrogen bond with Gln-114, which is substituted by Asn in PbFB synthase (Fig. 3C) (27). Thus, the D-ring is in opposite orientations between GtPEBB and PbFB synthase.

Implications of the reaction mechanism

Asp-263 interacts with the lactam oxygen atom of the A-ring of BV via a water molecule, Wat-1. Another water molecule, Wat-3, is located 4.2 Å from the C2 atom of the A-ring of BV (Fig. 3). Wat-3 forms a hydrogen bond with the water molecule Wat-2, and Wat-2 forms a hydrogen bond network with Asp-263 and Asp-123. These two acidic residues appear to be involved in A-ring reduction and are conserved in plant PbFB synthases (Fig. S1). To test this hypothesis, we prepared the mutated enzymes D123N and D263N, in which Asp-123 or Asp-263 are replaced by asparagine residues. Enzymatic assays showed that the D123N and D263N mutant enzymes retained BV-binding activity and radical formation activity, whereas the PbFB formation activity was negligible in the D123N or D263N mutant (Fig. 4). Similar results were reported in Arabidopsis PbFB synthase, in which mutations introduced in the corresponding acidic residues, Asp-116 and Asp-256, severely reduced the enzymatic activity (24). The two acidic residues and the water molecules bound to these two residues thus donate protons to the A-ring. Asp-263 functions as a first proton donor, and Asp-123 functions as a second proton donor because Asp-263 is geometrically closer to the A-ring than Asp-123, and the enzymatic activity is reduced more severely by the D263N mutation. Arg-259, of which the corresponding residue is essential for the GtPEBB reaction by forming a salt bridge with the catalytic aspartic acid residue (27), forms a salt bridge with the essential Asp-263. The water molecules, Wat-1 to Wat-3, are located between the H7 helix and BV (Fig. 3). Thus, a hydrogen atom, which is predicted to bind to the C2 atom of BV, is derived from the H7 helix side, resulting in the formation of 2(R)-PbFB. Following the formation of PbFB, the methyl group of the A-ring may move toward the central strands. The conformational change would promote the release of PbFB because of the collision between the methyl group of the A-ring of PbFB and the side chain of the conserved Val-121, which is located at a distance of 3.5 Å from the methyl group of the A-ring of BV before the reaction (Fig. 3 and Fig. S1). To confirm this hypothesis, steady-state and single-turnover analyses of V121A mutated tomato PbFB synthase were performed. Single-turnover analysis demonstrated that the V121A mutated protein was slightly slower, although it produced 3Z/E-PbFB, as well as native tomato PbFB synthase (Fig. 5, B–D), whereas no activity was detected in the V121A mutated protein in the steady-state analysis (Fig. 5E). The activity of the V121A mutated protein was not detected, even when the amount of
Structure of phytochromobilin synthase bound to substrate

(A) D123N

(B) D263N

(C) BV

Retention time (min)
enzyme used in the steady-state assay was increased by 2-fold. That indicates that V121A can reduce the A-ring, although the turnover rate was low. The visible spectrum of BV bound to the V121A mutated protein was similar to that of BV bound to tomato PΦB synthase, suggesting that the substrate-binding activity of the V121A mutant was normal (Fig. 5A). Therefore, the V121A mutated protein is difficult to dissociate from the product because of the lack of collision between product and enzyme.

A recently discovered FDBR enzyme from streptophyte algae (KflaHY2) is phylogenetically related to PΦB synthase but can reduce the vinyl group of the D-ring and the A-ring of BV, which is similar to PsyA (30). Homology modeling of KflaHY2 based on the current tomato PΦB synthase structure (Fig. S3) suggests that the residue corresponding to Asp-263 in tomato PΦB synthase is conserved in KflaHY2, whereas the residue corresponding to Asp-123 in tomato PΦB synthase is substituted by Asn, and the residue corresponding to Asn-140 in tomato PΦB synthase is substituted by Asp, which is identical to Synechocystis PsyA. Arg-259 and Ser-195 in tomato PΦB synthase, which appear to be important for stabilizing the flipped ZZŻsa conformation of BV, are also conserved in KflaHY2. Thus, the flipped ZZŻsa conformation of BV is probably conserved in KflaHY2 (Fig. S3). If the BV-binding mode of KflaHY2 is similar to that of tomato PΦB synthase, Asp residues corresponding to Asn-140 and Asp-256 in tomato PΦB synthase may be involved in the enzymatic reaction. In the Synechocystis PsyA reaction, another acidic residue, Glu-76, which is adjacent to the vinyl group of the D-ring, is required for the reduction of the vinyl group of the D-ring (8, 31). In the homology model of KflaHY2, the acidic residue adjacent to the vinyl group of the D-ring could not be identified. Therefore, the BV-binding mode of KflaHY2 may be different from that of tomato PΦB synthase and other FDBR enzymes; alternatively, a different mechanism underlying the reduction of the vinyl group of the D-ring in KflaHY2 may be involved.

In summary, we determined the crystal structure of tomato PΦB synthase in complex with BV at 1.95 Å resolution. The structure of the PΦB synthase-specific sequence was determined. The configuration and orientation of BV bound to PΦB synthase are completely different from those of other known BV structures bound to FDBRs. Relative to the BV-binding position in other FDBRs, BV is slightly close toward the entrance of the BV-binding pocket because of the loss of the interaction with the pyrrole nitrogens of BV and the central aspartic acid residue and the gain of interaction with Arg-316, which is specific to PΦB synthase. The present structural and enzymatic analyses suggest that two aspartic acid residues function as acidic catalysts for the reduction of the A-ring. Water molecules between the A-ring and H7 helix function as a proton relay system to donate protons. Following the reduction, the methyl group of the A-ring would collide with the side chain of Val-121 to release the product.

**Experimental procedures**

**Construction of tomato PΦB synthase expression plasmids**

Amino acid sequence data for PΦB synthase from Solanum lycopersicum (tomato) (Q588D6) were retrieved from UniProt (32). The nucleotide sequence corresponding to the amino acid sequence of tomato PΦB synthase (aurea), which was optimized for expression in E. coli and cloned into pUC57-Kan, was purchased from GENEWIZ (South Plainfield, NJ). Single mutation of T116S was unintentionally introduced during the plasmid design. Thr-116 is not conserved in PΦB synthases (Fig. S1) and is distant from the active site; furthermore, the resulting enzyme is active as shown in Fig. 1. To remove the chloroplast transition peptide and add a His tag at the N terminus, the ORF of tomato PΦB synthase (Met-45–Val-342) was amplified by PCR using primers 1 and 2 (Table S1). The resulting amplified fragment was fused into linearized pET-15b (Novagen) using the In-Fusion HD cloning kit (Takara Bio), creating pET-15b-aurea. The sequence of the ORF region of pET-15b-aurea was verified.

Site-directed mutagenesis of tomato PΦB synthase was performed using the KOD Plus mutagenesis kit (Toyobo) and plasmid pET-15b-aurea as the template. The oligonucleotides shown in Table S1 were used to introduce mutations. Sequence analysis verified that the construct was free of errors.

**Expression and purification of tomato PΦB synthase**

*E. coli* BL21(DE3) (Novagen) was transformed with pET-15b-aurea. The transformant was grown in TB medium with ampicillin (100 μg/ml) at 28 °C. After induction with 500 μM isopropyl-β-d-thiogalactopyranoside, the cells were grown for 16 h, harvested by centrifugation, and stored at −30 °C. The following steps were performed at 4 °C or on ice. Frozen cells were thawed and suspended in 50 ml of lysis buffer (150 mM KCl and 20 mM Tris·HCl, pH 8.0) and sonicated. The membrane fraction was removed by centrifugation at 27,000 × g for 30 min. The supernatant was loaded onto a Ni-NTA-agarose column (FUJIFILM Wako Pure Chemical Corp.), equilibrated with 150 mM KCl and 20 mM Tris·HCl, pH 7.4. The column was washed with the same buffer, and the protein was eluted with an increasing linear gradient of 0 to 200 mM imidazole. Fractions containing PΦB synthase were collected and loaded onto a Hitrap Q HP column (GE Healthcare) equilibrated with 20 mM Tris·HCl, pH 7.4. The column was washed with the same buffer, and the protein was eluted with an increasing linear gradient of 0–500 mM KCl. Fractions containing PΦB synthase were concentrated, loaded onto a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare) equilibrated with 150 mM KCl in 20 mM Tris·HCl (pH 7.4), and eluted with the same buffer. Chromatographic analysis was performed using the ÄKTA prime plus system (GE Healthcare). Fractions containing PΦB synthase were monitored by SDS-PAGE. During purification, PΦB synthase separated into two peaks in anion-
Structure of phytochromobilin synthase bound to substrate

(A) 

(B) 

(C) 

(D) 

(E) WT

3E-PφB

3Z-PφB

V121A

0.00 5.00 10.00 15.00 20.00 25.00 30.00 35.00 40.00

Retention time (min)

Retention time (min)

Retention time (min)
exchange chromatography. Size-exclusion chromatography showed that the PφB synthase monomer was contained in the major peak of anion-exchange chromatography, and the PφB synthase homodimer was contained in the minor peak of anion-exchange chromatography. Because of the quantity and purity of the fraction, the monomer fraction was collected and used for further biochemical analyses including crystallization. Purified PφB synthase was concentrated to 30 mg/ml using Amicon Ultra (Merck–Millipore) for crystallization. Selenomethionine-substituted PφB synthase (SeMet-PφB synthase) was expressed and purified as described for native PφB synthase. Typical yields of PφB synthase and SeMet-PφB synthase were 30 mg from 1-liter culture medium. The D123N, D256N, or V121A mutant proteins were expressed and purified as described for native PφB synthase.

Enzymatic assay

Single-turnover assays of PφB synthase activity were performed as described by Tu et al. (24) with the following modifications: The assay solution was prepared in an anaerobic chamber and contained 10 μM PφB synthase or mutated proteins, 5 μM ferredoxin, 15 nm ferredoxin:NADPH oxidoreductase, and 10 μM BV in 25 mM TES-KOH buffer (pH 8.0). The reaction was initiated by adding 100 μM NADPH (final concentration) to this solution in a capped quartz cuvette on a laboratory bench. The reaction mixture was incubated at 293 K in a capped cuvette, and absorption spectra were monitored every 2 min using a SHIMADZU UV-2550 spectrophotometer. After 30 min, TFA was immediately added to the samples to stop the reaction. Steady-state assays of PφB synthase activity were also performed as described by Tu et al. with the following modifications: the assay solution was prepared in an anaerobic chamber and contained 0.1 μM PφB synthase or mutated proteins, 5 μM ferredoxin, 15 nm ferredoxin:NADPH oxidoreductase, and 10 μM BV in 25 mM TES-KOH buffer (pH 8.0). The reaction was initiated by adding 100 μM NADPH (final concentration) to this solution. The reaction mixture was incubated at 303 K for 30 min, and the reaction was terminated by addition of TFA.

The reaction mixtures resulting from single-turnover and steady-state analyses were analyzed by HPLC. The samples were pretreated with Sep-Pak C18 (Millipore) and concentrated. Crude bilins were diluted using 150 μl of mobile phase solution (50% (v/v) of acetone/10 mM formic acid in water). A volume of 100 μl of diluted crude bilins was applied onto a HPLC column (InertSustain Swift C18, GL Sciences) connected to the Alliance HPLC system (Waters).

Crystallization of the BV–PφB synthase complex

The BV–PφB synthase complex was prepared by equimolar incubation of PφB synthase and BV for 1 h on ice. The crystallization conditions for the BV–PφB synthase complex were screened by the sitting-drop vapor-diffusion method using Mosquito at 277 K. Tiny needle-shaped crystals of the BV–PφB synthase complex were obtained with a reservoir solution containing 20% (w/v) PEG 8000, 0.1 M Tris-HCl, pH 8.5, and 0.2 M MgCl2. Isomorphous crystals were also obtained with a reservoir solution containing 30% (w/v) PEG 4000, 0.1 M Tris-HCl, pH 8.5, and 0.2 M MgCl2. The BV–SeMet–PφB synthase complex was crystallized using the same method.

Data collection and structure determination

BV–PφB synthase and BV–SeMet–PφB synthase crystals were soaked in crystallization solution containing glycerol up to 20% (v/v) as a cryo-protectant and frozen in liquid nitrogen. Diffraction data of the native crystal were collected at 100 K using Synchrotron radiation (λ = 1.000 Å) from the BL32XU Beamline at SPring-8 and EIGER X 9M hybrid photon counting detector (Dectris). A 10 μm × 10 μm microfocused X-ray beam was used to obtain diffraction data from 10–15 μm width crystals. Diffraction data for the BV–SeMet–PφB synthase crystal were collected at 100 K using Synchrotron radiation (λ = 0.9750 Å) from the BL32XU Beamline at SPring-8. Diffraction data from 50 crystals were merged for BV–PφB synthase to improve data quality and resolution, whereas diffraction data from a single BV–SeMet–PφB synthase crystal was used for phase determination to reduce systematic error caused by nonisomorphism between crystals. All diffraction data were processed, merged, and scaled with KAMO (33). Crystallographic statistics are summarized in Table 1. Rmerge and the redundancy of the native data set were relatively high because diffraction data from many crystals were merged.

Phased determination and auto-model building of BV–SeMet–PφB synthase were performed using Phenix (34). The resulting automatically built model was improved manually with COOT including the addition of BV, magnesium, and chloride ions, water molecules, and several multiple conformers. The structure of native PφB synthase was determined by Fourier synthesis using BV–SeMet–PφB synthase. Both models were further refined and manually adjusted using Phenix (34) and COOT (35). Refinement statistics are summarized in Table 1.
Table 1

| Crystallographic data set | Native PbF synthase | SeMet PbF synthase |
|---------------------------|---------------------|--------------------|
| Wavelength (Å)            | 1.0000              | 0.9750             |
| Space group               | P42,2               | P42,2              |
| Unit cell (a, c, Å)       | 64.13               | 63.93              |
| Diffraction statistics    |                     |                    |
| Maximum resolution (Å)    | 1.95 (2.03–3.03)    | 2.2 (2.33–3.88)    |
| Redundancy (%)            | 95.0 (91.3)         | 6.97 (6.80)        |
| Completeness (%)          | 100 (100)           | 99.8 (98.9)        |
| Mean I/σ(I)              | 17.6 (3.84)         | 13.21 (3.88)       |
| Rmerge (%)                | 53.4 (422.3)        | 11.0 (56.4)        |
| Rp (%)                    | 5.5 (44.7)          | 3.5 (17.1)         |
| CC1/2 (%)                 | 0.997 (0.959)       | 0.997 (0.864)      |

Phasing
Selenium sites 7
Figure of merit 0.304
Refinement statistics
Rfactor (%) 16.2 17.8
Rfree (%) 20.7 21.2
Number of atoms
Protein 2307 2265
Ligands 45 46
Water 246 176
Average B-factors (Å²)
Protein 32.51 32.50
Ligands 27.78 26.80
Water 40.09 37.26
Root-mean-square deviation from ideal values
Bond lengths (Å) 0.007 0.004
Bond angles (°) 0.835 0.641
Ramachandran plot
Preferred (%) 97.16 96.42
Allowed (%) 2.84 3.58
PDB code 6KME 6KMD

* The values in parentheses correspond to the highest-resolution shell.
* Rmerge = Σhkli|Ihkli|−|<Ihkli>|/Σhkli|Ihkli|, Rfree = Σhkli|Ihkli|−|<Ihkli>|/Σhkli|Ihkli|, where n is the redundancy of the data.
* R factor = Σhkli|Ihkli|−|<Ihkli>|/Σhkli|Ihkli|.
* Rfree is the R factor calculated for 5% of the data not included in the refinement.

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References
1. Schafer, E., and Bowle, C. (2002) Phytochrome-mediated photoperception and signal transduction in higher plants. EMBO Rep. 3, 1042–1048 CrossRef Medline
2. Frankenberg, N., Mukougawa, K., Kohchi, T., and Lagarias, J. C. (2001) Functional genomic analysis of the HY2 family of ferredoxin-dependent bilin reductases from oxygenic photosynthetic organisms. Plant Cell 13, 965–978 CrossRef Medline
3. Kohchi, T., Mukougawa, K., Frankenberg, N., Masuda, M., Yokota, A., and Lagarias, J. C. (2001) The Arabidopsis HY2 gene encodes phytochromobilin synthase, a ferredoxin-dependent biliverdin reductase. Plant Cell 13, 425–436 CrossRef Medline
4. Lagarias, J. C., and Lagarias, D. M. (1989) Self-assembly of synthetic phytochrome holoprotein in vitro. Proc. Natl. Acad. Sci. U.S.A. 86, 5778–5780 CrossRef Medline
5. Terry, M. J., and Kendrick, R. E. (1996) The aurea and yellow-green-2 mutants of tomato are deficient in phytochrome chromophore synthesis. J. Biol. Chem. 271, 21681–21686 CrossRef Medline
6. Kendrick, R. E., Kerchkoffs, L. H. J., Pundses, A. S., Van Tuinuen, A., Koornneef, M., Nagatanii, A., Terry, M. J., Tretyn, A., Cordonnier-Pratt, M. M., Hauser, B., and Pratt, L. H. (1994) Photomorphogenic mutants of tomato. Euphytica 79, 227–234 CrossRef
7. Koornneef, M., Rolfseh, E., and Spruit, C. J. P. (1980) Genetic control of light-inhibited hypocotyl elongation in Arabidopsis thaliana (L.) heynh. Zeitschrift für Pflanzenphysiologie 100, 147–160
8. Tu, S. L., Rockwell, N. C., Lagarias, J. C., and Fisher, A. J. (2007) Insight into the radical mechanism of phycoerythrobilin-ferredoxin oxidoreductase (PcyA) revealed by X-ray crystallography and biochemical measurements. Biochemistry 46, 1484–1494 CrossRef Medline
9. Tu, S. L., Gunn, A., Toney, M. D., Britt, R. D., and Lagarias, J. C. (2004) Biliverdin reduction by cyanobacterial phycocyanobilin:ferredoxin oxidoreductase (PcyA) proceeds via linear tetrapyrrole radical intermediates. J. Am. Chem. Soc. 126, 8682–8693 CrossRef Medline
10. Sugishima, M., Wada, K., Unno, M., and Fukuyama, K. (2019) Bilin-metabolizing enzymes: site-specific reductions catalyzed by two different type of enzymes. Curr. Opin. Struct. Biol. 59, 73–80 CrossRef Medline
11. Hagiwara, Y., Sugishima, M., Takahashi, Y., and Fukuyama, K. (2006) Crystal structure of phycoerythrobilin:ferredoxin oxidoreductase in complex with biliverdin IXa, a key enzyme in the biosynthesis of phycoerythrobilin. Proc. Natl. Acad. Sci. U.S.A. 103, 27–32 CrossRef Medline
12. Unno, M., Sugishima, M., Wada, K., and Fukuyama, K. (2013) Structure–function relationships of ferredoxin-dependent bilin reductases. In Integrating Approach to Photofunctional Hybrid Materials for Energy and the Environment (Akitsu, T., ed) pp. 47–68, Nova Science Publishers, Inc., New York
13. Duerring, M., Schmidt, G. B., and Huber, R. (1991) Isolation, crystallization, crystal structure analysis and refinement of constitutive C-phycocyanin from the chromatically adapting cyanobacterium Frenyrella diplophoton at 1.6 Å resolution. J. Mol. Biol. 217, 577–592 CrossRef Medline
14. Wagner, I. R., Zhang, J., Brunzelle, J. S., Vierstra, R. D., and Forest, K. T. (2007) High resolution structure of Deinococcus bacteriochlorophyll yields new insights into photochrome architecture and evolution. J. Biol. Chem. 282, 12298–12309 CrossRef Medline
15. Yang, X., Kuk, J., and Moffat, K. (2008) Crystal structure of Pseudomonas aeruginosa bacteriophytochrome: photoconversion and signal transduction. Proc. Natl. Acad. Sci. U.S.A. 105, 14715–14720 CrossRef Medline
16. Wagner, I. R., Brunzelle, J. S., Forest, K. T., and Vierstra, R. D. (2005) A light-sensing knot revealed by the structure of the chromophore-binding domain of photochrome. Nature 438, 325–331 CrossRef Medline
17. Yang, X., Stojkovic, E. A., Kuk, J., and Moffat, K. (2007) Crystal structure of the chromophore binding domain of an unusual bacteriophytochrome, RpBphP3, reveals residues that modulate photoconversion. Proc. Natl. Acad. Sci. U.S.A. 104, 12571–12576 CrossRef Medline
18. Busch, A. W., Reijerse, E. J., Lubitz, W., Frankenbank-Dinkel, N., and Hofmann, E. (2011) Structural and mechanistic insight into the ferredoxin-mediated two-electron reduction of bilins. Biochem. J. 439, 257–264 CrossRef Medline
19. Dammeyer, T., Hofmann, E., and Frankenbank-Dinkel, N. (2008) Phycoerythrobilin synthase (PebS) of a marine virus: crystal structures of the biliverdin complex and the substrate-free form. J. Biol. Chem. 283, 27547–27554 CrossRef Medline
20. Ledermann, B., Schwam, M., Sonnerkamp, J. A., Hofmann, E., Béja, O., and Frankenbank-Dinkel, N. (2018) Evolution and molecular mechanism of four-electron reducing ferredoxin-dependent bilin reductases from oceanic phases. FEBS J. 285, 339–356 CrossRef Medline
21. Sugishima, M., Kitamori, Y., Noguchi, M., Kohchi, T., and Fukuyama, K. (2009) Crystal structure of red chlorophyll catabolite reductase: enlargement of the ferredoxin-dependent bilin reductase family. J. Mol. Biol. 389, 376–387 CrossRef Medline
Structure of phytochromobilin synthase bound to substrate

22. Sugishima, M., Okamoto, Y., Noguchi, M., Kohchi, T., Tamiaki, H., and Fukuyama, K. (2010) Crystal structures of the substrate-bound forms of red chlorophyll catabolite reductase: implications for site-specific and stereospecific reaction. *J. Mol. Biol.* **402**, 879–891 CrossRef Medline

23. Hörtensteiner, S., and Kräutler, B. (2011) Chlorophyll breakdown in higher plants. *Biochim. Biophys. Acta* **1807**, 977–988 CrossRef Medline

24. Tu, S. L., Chen, H. C., and Ku, L. W. (2008) Mechanistic studies of the phytochromobilin synthase HY2 from *Arabidopsis*. *J. Biol. Chem.* **283**, 27555–27564 CrossRef Medline

25. Chiu, F. Y., Chen, Y. R., and Tu, S. L. (2010) Electrostatic interaction of phytochromobilin synthase and ferredoxin for biosynthesis of phytochrome chromophore. *J. Biol. Chem.* **285**, 5056–5065 CrossRef Medline

26. Muramoto, T., Kami, C., Kataoka, H., Iwata, N., Linley, P. J., Mukougawa, K., Yokota, A., and Kohchi, T. (2005) The tomato photomorphogenic mutant, aurea, is deficient in phytochromobilin synthase for phytochrome chromophore biosynthesis. *Plant Cell Physiol.* **46**, 661–665 CrossRef Medline

27. Sommerkamp, J. A., Frankenberg-Dinkel, N., and Hofmann, E. (2019) Crystal structure of the first eukaryotic bilin reductase GtPEBB reveals a flipped binding mode of dihydrobiliverdin. *J. Biol. Chem.* **294**, 13889–13901 CrossRef Medline

28. Liebschner, D., Afonine, P. V., Moriarty, N. W., Poon, B. K., Sobolev, O. V., Terwilliger, T. C., and Adams, P. D. (2017) Polder maps: improving OMIT maps by excluding bulk solvent. *Acta Crystallogr. D Struct. Biol.* **73**, 148–157 CrossRef Medline

29. Unno, M., Ishikawa-Suto, K., Kusaka, K., Tamada, T., Hagiwara, Y., Sugishima, M., Wada, K., Yamada, T., Tomoyori, K., Hosoya, T., Tanaka, I., Niimura, N., Kuroki, R., Inaka, K., Ishihara, M., et al. (2015) Insights into the proton transfer mechanism of a bilin reductase PcyA following neutron crystallography. *J. Am. Chem. Soc.* **137**, 5452–5460 CrossRef Medline

30. Rockwell, N. C., Martin, S. S., Li, F. W., Mathews, S., and Lagarias, J. C. (2017) The phycocyanobilin chromophore of streptophyte algal phycocyanin is synthesized by HY2. *New Phytol.* **214**, 1145–1157 CrossRef Medline

31. Hagiwara, Y., Sugishima, M., Khawn, H., Kinoshita, H., Inomata, K., Shang, L., Lagarias, J. C., Takahashi, Y., and Fukuyama, K. (2010) Structural insights into vinyl reduction regiospecificity of phycocyanobilin: ferredoxin oxidoreductase (PcyA). *J. Biol. Chem.* **285**, 1000–1007 CrossRef Medline

32. UniProt Consortium (2019) UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* **47**, D506–D515 CrossRef Medline

33. Yamashita, K., Hirata, K., and Yamamoto, M. (2018) KAMO: towards automated data processing for microcrystals. *Acta Crystallogr. D Struct. Biol.* **74**, 441–449 CrossRef Medline

34. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L.-W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D* **66**, 213–221 CrossRef Medline

35. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of COOT. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501 CrossRef Medline

36. Baker, N. A., Sept, D., Joseph, S., Holst, M. J., and McCammon, J. A. (2001) Electrostatics of nanosystems: application to microtubules and the ribosome. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10037–10041 CrossRef Medline

37. DeLano, W. L. (2010) The PyMOL Molecular Graphics System, version 1.3r1, Schrödinger, LLC, New York