STRUCTURE AND MECHANISM OF THE PHYCObILIPROTEIN LYASE CPCT

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Running title: Structure and mechanism of the phycobiliprotein lyase CpcT

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Keywords: Phycobilisome pigmentation, chromophore lyase, crystal structure

Background: Phycobiliprotein lyases catalyze covalent attachment of chromophores to cyanobacterial antenna proteins.

Results: We present crystal structures of a T-type lyase and its complex with phycocyanobilin.

Conclusion: The proposed reaction mechanism accounts for chromophore stabilization, regio- and stereospecificity.

Significance: This work sheds light on a crucial step in phycobiliprotein maturation, and provides a model for other types of phycobiliprotein lyases.

ABSTRACT

Pigmentation of light-harvesting phycobiliproteins of cyanobacteria requires covalent attachment of open-chain tetrapyrrole, bilins, to the apoproteins. Thiocyst formation via addition of a cysteine residue to the 3-ethylidene substituent of bilins is mediated by lyases. T-type lyases are responsible for attachment to Cys155 of phycobiliprotein β-subunits. We present crystal structures of CpcT (All5339) from Nostoc sp. PCC7120 and its complex with phycocyanobilin, at 1.95 and 2.50 Å resolution, respectively. CpcT forms a dimer and adopts a calyx-shaped β-barrel fold. While the overall structure of CpcT is largely retained upon chromophore binding, arginine residues at the opening of the binding pocket undergo major rotameric rearrangements anchoring the propionate groups of phycocyanobilin. Based on the structure and mutational analysis, a reaction mechanism is proposed that accounts for chromophore stabilization, and regio- and stereospecificity of the addition reaction. At the dimer interface, a loop extending from one subunit partially shields the opening of the phycocyanobilin binding pocket in the other subunit. Deletion of the loop or disruptions of the dimer interface significantly reduce CpcT lyase activity, suggesting functional relevance of the dimer. Dimerization is further enhanced by chromophore binding. The chromophore is largely buried in the dimer but in the monomer the 3-ethylidene group is accessible for the apo-phycobiliprotein, preferentially from the chromophore α-side. Asp163 and Tyr65 at the β- and α-face near the E-configured ethylidene group, respectively, support the acid-catalyzed nucleophilic Michael addition of cysteine-155 of the apoprotein to an N-acylimmonium intermediate proposed by K. Grubmayr and U.G. Wagner (Monatsh. Chem. 119, 965, 1988).

Phycobilisomes, the light-harvesting antenna complexes in cyanobacteria and red algae, are supramolecular complexes of phycobiliproteins (PBP) and linkers (1-3); they are packed with hundreds of open-chain tetrapyrrole (bilin) chromophores. These antenna pigments absorb light in the wavelength range between 480 and 660 nm; and their spatial arrangement in the phycobilisome ensures directional energy transfer with high quantum efficiency to the photosynthetic reaction centers (4,5). PBP are α,β-heterodimeric proteins, with each subunit carrying one to three bilin chromophores. Unlike chlorophylls, bilins are covalently attached to PBPs regio- and stereospecifically via thioether bonds from the C-3 atoms of bilin chromophores to highly conserved cysteines. While this attachment mode is universal to all chromophores, phycoerythrobilin (PEB) and...
Structural and Mechanistic Insights into the Phycobiliprotein Lyase CpcT

1. Introduction

Lyases are enzymes that catalyze the covalent attachment of a chromophore to a protein. Three types of lyases have been characterized: T-type, S-type, and E/F-type. T-type lyases are responsible for attaching phycocyanobilin (PCB) to a conserved cysteine (Cys-α84) in α-subunits of phycocyanin (CPC) and phycoerythrocyanin (18,19); homologous CpeY/Z catalyze addition of phycocyanobilin to the equivalent site in phycoerythrin (20). CpcE and CpcF are structurally related to armadillo proteins like α-karyopherin (21), but no crystal structure has been solved. S-type lyases are more universal, among which CpcS is best studied. Alone or in combination with CpcU (22-25), it catalyzes PCB attachment to conserved Cys-β84 sites in various biliprotein β-subunits, and to the related Cys82 sites (consensus numbering) in both subunits of allophycocyanin (22,26). CpcS transiently binds bilins such as PCB (14). The crystal structures of a cyanobacterial CpcS (11,27,28) and a cryptophyte CPES (29) have been determined in the absence of a chromophore. They both adopt a β-barrel structure similar to those of fatty acid binding proteins (FABP) (27), a sub-family of the calycin superfamily (Pfam0116) that binds a variety of small, mostly lipophilic molecules with high selectivity (30-32). The name reflects their goblet (calyx)-like structures consisting of antiparallel β-sheets that in many cases is closed by an α-helix. Interestingly, the first structure reported in the calyx family was a protein from the butterfly, Pieris brassica, which non-covalently binds a bilin chromophore, biliverdin IXγ (33). The T-type lyases constitute a third class of lyases that is distantly related to CpcS (23). They catalyze the covalent attachment of PCB or PEB to Cys-β155 sites (consensus numbering) in β-subunits of cyanobacterial (25,34) and cryptophyte biliproteins (35), and with a different stereochromy than CpcS. In Nostoc sp. PCC 7120 (Nostoc), a single protein, CpcT (All5339), is responsible for β-subunit chromophorylation with PCB at Cys-β155 in CPC and phycoerythrocyanin. Sequential binding studies indicated that this step is hindered by a preceding chromophorylation at Cys-β84 by CpcS (25).

To understand the structural basis for the reaction mechanism of lyases, we have solved the crystal structure of CpcT at 1.95 Å resolution by single-wavelength anomalous dispersion (SAD) method using selenomethionine-substituted crystals. We have also determined the crystal structure of CpcT in complex with PCB at 2.5 Å resolution. Based on structural analyses and comparisons, we have further explored residues at the dimer interface and around the chromophore-binding pocket via mutagenesis. A reaction mechanism is proposed for T-type lyases that accounts for stabilizing the chromophore, as well as for the regio- and stereospecificity of the addition reaction.

2. Experimental Procedures

Constructions, expression and purification - CpcT was cloned according to standard procedures (36). Plasmids containing cpcT, ho1 and pcyA from Nostoc and mutated cpcB(C84S) from Mastigocladus laminosus were previously constructed (9,22,24,25). The CpcT used in crystallization was obtained from expression of construct pET-cpcT; it carries a His-tag at the C-terminus (Table S1).

CpcT was over-expressed in E. coli BL21(DE3). Cells were grown at 20 °C in LB medium containing kanamycin (30 µg•ml⁻¹), induced with IPTG (1 mM) at OD 600 ~ 0.5 - 0.7, and then growth was continued for an additional 12 hours after induction. Cells were harvested by centrifugation, washed twice with distilled water, and stored at -20 °C until use (22,25).

Dual plasmids were co-transformed into BL21(DE3) cells under the respective antibiotic selections (chloromycetin for pACYC-derivative, streptomycin for pCDF-derivative, kanamycin for pET-derivative). PCB-CpcB(C84S) was produced by co-expression of pET-cpcB(C84S), pACYC-ho1-pcyA and pCDF-cpcT (Table S2). Cells were grown at 20 °C in LB-medium containing kanamycin (20 µg•ml⁻¹), chloromycetin (17 µg•ml⁻¹), streptomycin (25 µg•ml⁻¹). Cells were harvested after 12 hours following induction with IPTG (1 mM) (22,25).

Cell pellets were resuspended in ice-cold potassium phosphate buffer (KB, 20 mM, pH 7.2) containing NaCl (0.5 M) and disrupted by sonication (5 min at 200 W, JY92-II, Scientz Biotechnology, Ningbo). The suspension was centrifuged at 12,000 × g for 15 minutes at 4 °C.

3. Results and Discussion

Structure and function of lyases - T-type lyases are responsible for attaching bilins such as PCB to a conserved cysteine (Cys-α84) in α-subunits of phycocyanin (CPC) and phycoerythrocyanin (18,19); homologous CpeY/Z catalyze addition of phycocyanobilin to the equivalent site in phycoerythrin (20). CpcE and CpcF are structurally related to armadillo proteins like α-karyopherin (21), but no crystal structure has been solved. S-type lyases are more universal, among which CpcS is best studied. Alone or in combination with CpcU (22-25), it catalyzes PCB attachment to conserved Cys-β84 sites in various biliprotein β-subunits, and to the related Cys82 sites (consensus numbering) in both subunits of allophycocyanin (22,26). CpcS transiently binds bilins such as PCB (14). The crystal structures of a cyanobacterial CpcS (11,27,28) and a cryptophyte CPES (29) have been determined in the absence of a chromophore. They both adopt a β-barrel structure similar to those of fatty acid binding proteins (FABP) (27), a sub-family of the calycin superfamily (Pfam0116) that binds a variety of small, mostly lipophilic molecules with high selectivity (30-32). The name reflects their goblet (calyx)-like structures consisting of antiparallel β-sheets that in many cases is closed by an α-helix. Interestingly, the first structure reported in the calyx family was a protein from the butterfly, Pieris brassica, which non-covalently binds a bilin chromophore, biliverdin IXγ (33). The T-type lyases constitute a third class of lyases that is distantly related to CpcS (23). They catalyze the covalent attachment of PCB or PEB to Cys-β155 sites (consensus numbering) in β-subunits of cyanobacterial (25,34) and cryptophyte biliproteins (35), and with a different stereochromy than CpcS. In Nostoc sp. PCC 7120 (Nostoc), a single protein, CpcT (All5339), is responsible for β-subunit chromophorylation with PCB at Cys-β155 in CPC and phycoerythrocyanin. Sequential binding studies indicated that this step is hindered by a preceding chromophorylation at Cys-β84 by CpcS (25).

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4. Conclusion

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The supernatant containing crude proteins was purified via Ni²⁺-affinity chromatography on chelating Sepharose (Amersham-Pharmacia), and eluted using buffer KPB (20 mM, pH 7.2) containing NaCl (0.5 M) containing imidazole (0.2 M). The affinity-enriched proteins were further purified via gel filtration (Amersham-Pharmacia) and were verified by SDSPAGE. The aggregation state of CpcB(C84S) and CpcT was assayed by gel filtration on Superdex 75 using KPB buffer (20 mM, pH 7.2) containing NaCl (150 mM) at an elution rate of 0.5 ml/min.

**Protein and activity assays** - Protein concentrations were determined by the Bradford method, using bovine serum albumin as standard (37). UV-VIS absorption spectra were recorded by a DU800 spectrophotometer (Beckman-Coulter). Uncorrected fluorescence spectra were recorded with a LS55 spectrofluorimeter (Perkin-Elmer). Relative enzymatic activities of CpcT mutants compared to wild type were assayed based on the fluorescence from the chromophorylated biliprotein, similar to that of CpcS (14). PCB-CpcB(C84S) was assembled in chromophorylated heme oxygenase 1 and cpcT encoding PCB:ferredoxin oxidoreductase) in plasmid pACYC184 (25). After expression, enzymatic reactions were followed by fluorescence (emission at 630 nm, excitation at 580 nm) of the PCB-CpcB(C84S) product.

To test the PCB binding, His-tagged CpcT (37 μM) was incubated with PCB (32 μM) in KPB (0.5 M, pH 7.2) containing NaCl (0.15 M) for one hour. Unbound PCB was removed by Ni²⁺-affinity chromatography with 8 volumes of the column bed of KPB buffer (0.5 M, pH 7.2) containing NaCl (0.15 M). The PCB-CpcT complex was then eluted with the same buffer containing imidazole (0.2 M). Absorption and fluorescence spectra were recorded without delay.

**Crystallization, data collection and structure determination** - Selenomethionine-substituted CpcT protein was prepared according to the standard protocol (38), and was crystallized under the condition containing MgCl₂·6H₂O (0.1 M), Bis-Tris (0.1 M, pH 5.5) and polyethylene glycol 3350 (14% w/v). The CpcT-PCB crystals were obtained by soaking in the mother liquor with additional 0.5 mM PCB chromophore (Frontier Scientific, Inc.) for 20 hours.

Both apo-CpcT and CpcT-PCB crystals were frozen with no additional cryo-protectant. All X-ray diffraction data were collected on LS-CAT beam stations at Advanced Photon Source, Argonne National Laboratory. All diffraction data were processed using HKL2000 (39). The crystal structure of apo-CpcT was determined by the single-wavelength anomalous dispersion (SAD) method (Phenix (40)) using selenomethionine-substituted crystals. The initial model was built using Coot (41) following Autobuild (Phenix) and the final structure was refined at 1.95 Å resolution (Phenix). The CpcT-PCB structure was determined by molecular replacement (Phaser in CCP4 (42)) in the space group of P1 using apo-CpcT as the search model and refined at 2.5 Å resolution. Surface areas buried at the dimer interface were calculated using the PISA server (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (43). Statistics of data collection and structural refinement are summarized in Table 1. Both coordinates and structure factor amplitudes of apo-CpcT and CpcT-PCB structures have been deposited to Protein Data Bank under accession numbers 4O4O and 4O4S, respectively.

**RESULTS**

**Crystal structure of CpcT** - The crystal structure of apo-CpcT was determined in the space group of P3₁2₁ with two molecules in the asymmetric unit using the single-wavelength anomalous dispersion (SAD) method (Fig. 1). All 199 residues of CpcT except two terminal residues (Met1 and Glu199) and the C-terminal tag have been accounted for by electron densities. The final structure was refined to 1.95 Å resolution with the final R-factor and free R-factor of 0.175 and 0.214, respectively (Table 1). The coordinates have been deposited to the Protein Data Bank under the accession code 4O4O.

In the asymmetric unit, CpcT forms a dimer in which two subunits are roughly related by a non-crystallographic 2-fold symmetry and interact with each other in a surface complementary fashion (Fig. 1). A protruding loop (residue 25-32) from one subunit partially blocks the opening of the β-barrel in the other subunit like a “plug” (Fig. 1b). The dimer interface buries a surface area of ~900 Å² (EBI-PISA (43)) that involves direct and water-mediated interactions between two subunits. Remarkably, a number of aromatic residues (Phe30, Tyr31, Tyr57, Tyr59, Tyr65, Trp175 and Phe182) lie at the dimer interface, including direct stacking interaction between two aromatic rings of Tyr59 from both subunits (Fig. 1c).
Each CpcT subunit adopts a conical, goblet shaped β-barrel with 10 anti-parallel β-strands; this fold is characteristic for the FABP subfamily of the calycin superfamily (32). Large insertions between β-strands are found at the opening of the β-barrel (Fig. 2a), where a disulfide bond between Cys116 and Cys137 brings together two adjacent strands and contributes to the overall rigidity of the β-barrel structure. A long extended structural segment (residue 87-109) folds back and docks onto the outside of the β-barrel largely via hydrophobic interactions (Fig. 2a). At the center of the β-barrel is a deep cleft with charged residues at the opening and hydrophobic residues at the bottom (Fig. 3b).

Superposition of two subunits (A and B) within the same CpcT dimer reveals similar structures with an overall r.m.s.d. value of 0.77 Å over 197 aligned residues (Fig. 4). More prominent differences are observed in regions at the dimer interface, in particular, in a bulged segment of the β-strand (residue 177-182). Structural alignment between A/B and B/A dimers further reveals differences in relative orientations between two subunits, where the plug from subunit A is about 2.8 Å deeper into the pocket of subunit B than the other way around (Fig. 4).

Crystal structure of PCB-bound CpcT - Co-crystallization of CpcT and its substrate PCB did not generate satisfactory results. The crystal structure of CpcT bound with PCB has been obtained, however, by soaking apo-CpcT crystals with additional 0.5 mM PCB in the mother liquor for 20 hours. The soaking process apparently induced structural changes in the CpcT crystal lattice, which are subtle but sufficient to disrupt crystallographic symmetries of the apo-CpcT crystals in the space group P3_121. We had to solve the crystal structure of the CpcT-PCB complex in the space group P1 by molecular replacement using the apo-CpcT crystals in the space group P1 as the search model (Table 1). The P1 unit cell contains 12 CpcT molecules forming six dimers. Electron densities in the difference map with PCB omitted readily identified all 12 PCB binding sites, indicating sufficient accessibility and occupancy of the PCB binding (Figs. 1b, 1c). The CpcT-PCB structure was refined at 2.5 Å resolution with the final R-factor and free R-factor of 0.181 and 0.241, respectively. The coordinates have been deposited to the Protein Data Bank under the accession code 4O4S.

The PCB chromophore is located in a deep cleft at the center of the β-barrel (Fig. 2a) with both propionate groups of ring B and C pointing out to the molecular surface. Electron densities in both the simulated annealing omit Fo-Fc map and the 2Fo-Fc map clearly show that PCB adopts the ZZZsss geometry in an M-helical conformation. Although the resolution is limited (2.5 Å resolution), we were able to assign the E-configuration to the 3-ethylidene side chain of ring A based on the NCS-averaged electron densities over 12 subunits (Fig. 2b). However, we were unable to resolve the stereochemistry at C2 based on the electron density of PCB bound to CpcT (see Fig. 2b for atom numbering). The chirality at C2 has been established as R both in the educt (44) and in the product (45-47), while the chromophore structure in the CpcT-PCB complex seems to be better compatible with an in-plane geometry (Fig. 2b), perhaps due to (partial) enolization with a C1-C2 double bond. Among six dimers in the unit cell of P1, PCB consistently displays better resolved electron densities in one subunit (B) than in the other (A), likely due to the aforementioned observation that the plug from subunit A renders a tighter PCB binding pocket in subunit B (Fig. 4). The PCB binding pocket is occupied with water molecules in the apo-CpcT structure but slightly expands upon PCB binding. While the CpcT structure remains largely unchanged upon PCB binding (r.m.s.d. of ~0.33 Å in all Ca atoms between the apo-CpcT and CpcT-PCB structures), positively charged residues (Arg66, Arg68 and Arg141) undergo significant rotamer rearrangements to accommodate PCB via direct interactions with the propionate side chains (Fig. 3c). The positioning of PCB is largely determined by the polarity and shape of the protein cavity such that substituents of rings A, B and C are accessible in the CpcT monomer (Figs. 3a, 3b). However, the 3-ethylidene group of ring A, which forms a covalent bond to the Cys-B155 site of the target PBP, is shielded by the plug structure in the CpcT dimer, and only becomes accessible upon dimer dissociation (Figs. 1a, 3a).

Rings A and D of PCB are largely surrounded by hydrophobic residues including Phe18, Phe152, Phe159, Phe182, and Phe184 (Fig. 3b). Phe30 in the plug of the partner subunit provides additional shielding for ring A (Fig. 3a). The shielded C1=O and C19=O dipoles are nearly antiparallel (~140°), and probably stabilize the M-helical conformation of PCB. The pyrrole nitrogens in rings A and C form hydrogen bonds with the side chain of Arg66 and indirectly via Asp163 and the carbonyl group of ring D (Figs. 3b, 3c).
The catalytic site - CpcT catalyzes the covalent attachment of the chromophore PCB to the Cys-\(\beta\)155 site of phycobiliprotein CpcB. This reaction requires protonation (see Discussion). Our crystal structure of CpcT-PCB reveals two candidate residues near ring A that may serve as proton donors. They are Asp163 and Tyr65, which approach ring A from the opposite faces of the chromophore. The carboxyl group of Asp163 and the phenolic hydroxyl of Tyr65 are located 3.5 and 3.2 Å from the 3-ethylidene group, respectively, Asp163 is also only 3.5 Å from C5 (Fig. 3b). To explore their roles in the CpcT function, we made three single mutants, Y65F, D163V and D163A, to examine how removal of the candidate proton donor group in Y65F and D163V and/or the steric effect in D163A affect the PCB complexation with CpcT and/or its covalent attachment to the apoprotein. These mutations significantly diminish the enzymatic activities of CpcT as measured by \textit{in vivo} chromophorylation of CpcB (Fig. 5). The \textit{in vitro} assays show that these variants are still able to bind PCB (Fig. 6a,b,c). Additional features in their absorption spectra, however, indicate chromophore heterogeneities in the CpcT complexes that are yet to be characterized. We postulate that Tyr65 and Asp163 are likely to play a catalytic role in the CpcT lyase function by assisting nucleophilic attack at the C3' atom that generates a covalent bond to the Cys-\(\beta\)155 of CpcB.

Mutations that affect PCB attachment to apoprotein - To explore structural elements of CpcT that may influence the PCB attachment reaction, we specifically examined how accessibility, structural rigidity and environment of the PCB binding pocket affect the CpcT lyase activity via site-directed mutagenesis (Fig. 5).

The CpcT dimer structure reveals that the PCB binding is partially protected by the dimer interface (Figs. 1c, 3a). Not surprisingly, deletion of the plug (spanning residues 20-32) significantly reduces the lyase activity to <10% of the wild type (WT) level, and so does removal of the aromatic side chain in either Phe30 or Tyr31 at the dimer interface (Figs. 3a, 5b). Y59A, designed to disrupt the Tyr59-Tyr59 stacking interaction, also leads to reduction in the lyase activity by at least 3-fold (Figs. 1a, 5b). Taken together, we postulate that disruption of the dimer interface leads to a less protected PCB binding pocket thus reduced lyase activity.

Opposite to the dimer interface, the S-S bond between Cys116 and Cys137 structurally bridges two neighboring \(\beta\)-strands and presumably enhances the overall rigidity of the PCB binding pocket (Fig. 2a). Previous mutational studies suggested that this disulfide bond is critical for the CpcT lyase activity (48). Moreover, this disulfide bond may be functionally relevant, because the CpcT lyase reaction is inhibited by thiols (25), while other lyases, like e.g. CpcE/F (13,19) and CpcS (24) tolerate thiols at the concentrations used or, like PecE/F (15,21) are even activated. Activation has also been found for autocatalytic chromophorylation of ApcE (7). Products have been reported at very low levels, which may result from reversible addition of thiols to bilin under similar experimental conditions (17).

Before the crystal structure of CpcT was solved, Trp13, His33, Arg66, Arg97, Cys116, Cys137, Trp175 of CpcT had been identified as amino acids that are critical for the CpcT activity. Among these, mutations at His33, Cys116, Cys137 and Trp175 rendered CpcT inactive, while mutations at Trp13, Arg66 and Arg97 resulted in moderately reduced activities (48). We speculate that His33 and Trp175 at the dimer interface are required for proper positioning of the plug relative to the other subunit, thereby modulating the CpcT dimerization and PCB binding.

PCB binding and oligomeric state - Complex formation of PCB with CpcT was shown to be transient and weak at low concentration conditions, based on fluorescence and gelfiltration experiments (48). In the crystal lattice where CpcT and PCB were present at high concentrations, PCB was incorporated with sufficient occupancies that allowed assignment of pyrrole rings based on the electron density map (Fig. 2b). In order to characterize the spectroscopic properties of the CpcT-PCB complex in solution, His-tagged CpcT was incubated with PCB at high concentrations (32 \(\mu\)M) in a buffer of high ionic strength (650 mM). After removal of excessive unbound PCB with an affinity column, optical spectra of the CpcT-PCB complex were measured (Fig. 6a). Both the peak positions \((\lambda_{\text{max}} = 620\) and 370 nm) and their
absorption ratio ($Q_{\text{Vis/UV}} = 0.48$) are very similar to those of the free, unprotonated PCB chromophore that exists in a cyclic-helical conformation. This state is consistent with the crystal structure, in which no acidic residues are found near the pyrrole nitrogens of PCB. Protonation of dihydrobiliverdins causes a ~30 nm red shift of the 620 nm band and a doubling of its extinction coefficient ($Q_{\text{Vis/UV}} \sim 1$). Deprotonation leads to a much larger red-shift (>100 nm) while the extinction coefficient changes very little (49). Protonation also increases the fluorescence yield, but other factors including, in particular, the rigidity of the chromophore, also contribute to enhanced fluorescence (50,51). The yield of $\Phi_F = 0.023$ measured from CpcT-PCB is larger than that of the free chromophore, which is consistent with PCB with moderately increased rigidity in the CpcT pocket.

While the CpcT dimers are important to protect the PCB during the relatively slow lyase-catalyzed reaction, dimer dissociation is equally important for releasing PCB when CpcT is docked onto its target sites. In other words, reversible dimerization of CpcT would be crucial for its lyase function. In solution, CpcT appears to dimerize in a concentration-dependent manner, and its dimerization is further enhanced in the presence of PCB (Fig. 6e,f). At the concentration of 3 µM, CpcT is mostly monomeric in the absence of PCB, but it tends to dimerize in the presence of 0.6 µM PCB (shoulder in elution profile; Fig. 6f). For a CpcT-catalyzed reaction in E. coli (see Experimental Procedures), the concentration of CpcT is estimated to be around 150 µM. Given the cellular concentration of PCB (~3 µM in E. coli and ~2 µM in mammalian cells (52), CpcT dimerization is favorable.

**DISCUSSION**

**Structure** - CpcT is a β-barrel protein with a FABP fold that is characterized by a 10-stranded structure with an N-terminal helix and a loop between the first two strands. FABPs belong to the calycin superfamily (32), in which members share low sequence homology, but exhibit conserved structures and short motifs. Seven crystal structures of bilin-binding calycins including CpcT are known, four of which contain a bound chromophore (Fig. 7). Although all bound chromophores are open-chain tetapyrroles, they differ considerably in both structure and function.

Two insect biliproteins from *Pieris brassica* (PDB ID: 1BBP) (33) and *Manduca sexta* (PDB ID: 1Z24) (53) carry biliverdin IXγ. This unusual biliverdin isomer is derived from heme cleaved at the C-15 methine bridge between rings C and D, such that the propionic acid side chains are located at the ends of the open-chain tetapyrrole. Both biliproteins exhibit narrow pockets facilitating tight chromophore binding such that biliverdin IXγ is retained during protein purification. The opening of the chromophore pocket in CpcT is somewhat wider. This may contribute to relatively weak binding of PCB that is only retained during chromatography under special conditions (such as high ionic strength and rapid work). The bound chromophore, however, adopts a similar cyclic-helical conformation in all three proteins, except that the chromophore helicity in CpcT is opposite to those in the insect biliproteins. PCB forms complexes with several CpcT variants that are catalytically inactive, but unlike the WT complex their structured spectra indicate chromophore heterogeneity, (Fig. 6), which may results from the mixture of conformers, protonation or tautomerization states; chemical reactions are unlikely to occur on the time scale of the experiment. It is possible that such heterogeneities contribute to the reduced CpcT activity, since protonation and/or tautomerization are key steps for the proposed CpcT catalyzed chromophorylation via an acylimmonium cation at ring A (see below and (54)).

Biliverdins and PCB assume a helical geometry due to steric hindrance between the carbonyl oxygens at C-1 and C-19 in a cyclical planar geometry. In solution, P- and M-configured conformers are in equilibrium (55,56). While a helical geometry is retained, CpcT selectively binds the chromophore in the M-helical configuration (Fig. 3), whereas the biliverdin IXγ chromophores are P-configured in insect biliproteins (Fig. 8). In all three structures, the propionic acid side chains are exposed, while hydrophobic rings A and D are deeply buried in the amphipatic pocket. Interestingly, the overall orientation of the chromophore largely remains the same despite phycobilins and insect bilins are derived from ring-opening at different methine bridges, that is, between rings A and D in PCB, and between rings C and D in biliverdin IXγ (Fig. 8).

The recently published bilirubin protein, UnaG from eel, is also a FABP protein (PDB ID: 4I3B (57)), in which the bound chromophore adopts a structure considerably different from
that of CpcT. In UnaG, the bilirubin chromophore has an extended conformation, which is completely buried and shielded by two helices at the opening of the goblet (Figs. 7, 8). Unlike biliverdins and PCB, isolated bilirubin is very hydrophobic and strong internal H-bonds (58,59) must be disrupted inside a chromoprotein and stabilized by hydrogen bonds to buried residues (57).

Three other bilin binding calycins have been crystallized in the absence of any chromophore. The dinoflagellate luciferase (PDB ID: 1VPR) contains a β-barrel domain that is decorated with several α-helices; it binds a chlorophyll-derived bilin cleaved at the C20 methine bridge (60) (Fig. 7). CpcS from Thermosynechococcus elongatus (PDB ID: 3BDR (27,28)) is another PBP lyase that shares 12% sequence identity with CpcT. CpcS exhibits a β-barrel structure very similar to CpcT with r.m.s.d. of 2.7 Å over 116 aligned residues (12,23). Residues 19-38 of CpcS adopt an extended anti-parallel β-strand conformation in contrast to the equivalent plug structure of CpcT that contains two helical turns (Fig. 7). CPES from the cryptophyte, Gaillardia theta, has a very similar structure but differs from CpcT in the chromophore specificity (29). The putative chromophore binding pocket in CpcS would be sufficiently spacious for PCB binding without significant changes in the protein structure (Fig. 7) (27,28), but the absorption spectrum of PCB-loaded CpcS indicates an extended chromophore geometry (61). Ligand binding did not require dramatic changes in the protein structure of a lipocalin engineered to bind digitonin (62). CPES shows a tighter cavity (57).

Among all lyases, only CpcT is inhibited markedly by thiols (25). We speculate that this property is pertinent to the disulfide bond between Cys116 and Cys137 in CpcT and provides another level of regulation for chromophorylation (63), since the higher reduction state in a photosynthetic organism, the less demand for light harvesting.

Site-specific interaction with CpcB - As a chromophore chaperone, CpcT must balance two aspects of its lyase function. On the one hand, tight chromophore binding would be favorable given the low concentration of free PCB in cyanobacteria, the chemical reactivity of PCB, and the slow reaction rates of CpcT (25) in solution. On the other hand, CpcT as lyase must present and release the chromophore for covalent attachment to Cys-β155. We postulate that the observed CpcT dimerization plays an important role for its chaperone function. In particular, the plug-like structures at the dimer interface presumably shield and protect the reactive and labile chromophore from unwanted reactions with, for example, other thiols or with oxygen. The CpcT dimer has to dissociate upon docking onto the bilin-accepting apoprotein, CpcB.

In the absence of a crystal structure for any apo-PBP, we used a fully assembled PBP structure (PDB ID: 1PCP (45)) to examine structural features surrounding the target site. Cys-β155 is located at the outer rim of the (αβ)_3 trimer with the propionate side chains of the bound chromophore exposed (Fig. 9). A few highly conserved structural features are found in the vicinity of Cys-β155. These include the charged face of a long, bent helix (spanning residue 20-62 in the β-subunit of 1PCP) that crosses over the PCB in an extended ZZZasa configuration, and the anchor cysteine containing a GDC sequence motif in a helical hairpin loop (Figs. 9, 10). Surface analysis suggests that both surface complementarity and electrostatic interactions between charged patches are responsible for guiding specific docking of CpcT onto Cys-β155 (Fig. 9). Specifically, two large loop insertions of CpcT (residues 87-109 and 56-65) (Fig. 1a) may be directly involved in docking onto the concave surface near Cys155 (Fig. 9). Furthermore, interactions between positively charged residues at the opening of the PCB binding pocket in CpcT (Arg66, Arg68 and Arg141 and the negative patch of CpcB (Asp33,
Asp39 and Asp154) might contribute to precise positioning between the donor and target sites. Such docking via loop insertions is expected to disrupt the dimer interface of CpcT, thereby allowing access to the chromophore. Kronfel et al. (28) pointed out the difference in charges between the two binding sites of the β-subunit and suggested its role in assisting docking of CpcS to the Cys-β84 binding site. Overkamp et al. (29) also concluded preferential docking of CPES to the β-subunit of the cryptophyte biliprotein, PE545, which is structurally and phylogenetically related to cyanobacterial biliprotein β-subunits. By the same token, docking of the positively charged rim of the CpcT pocket will preferentially dock to the negatively charged Cys-155 binding site of C-PC β-subunits.

Catalytic action - In the fully chromophorylated PBP trimer, the binding Cys-β155 is located in a loop that interacts intimately with rings A and B of the bound chromophore (Fig. 9). We speculate that this loop is less structured in the non-chromophorylated PBP, and is thus flexible enough to act as a “fishing line”. Similar to the “plug” in the dimer, it reaches into the cleft of CpcT and allows the covalent attachment of PCB. Such specificity may be guided by electrostatic interactions between charged surface patches of CpcB and CpcT (Figs. 1d, 9, 10). Approach of the cysteine sulfhydryl probably occurs for steric reasons from the more open α-face of the chromophore. The subsequent binding reaction (Fig. 11) is rationalized on the basis of the mechanism involving an acylimmonium intermediate proposed by Grubmayr and Walter (54). It starts by protonation of the chromophore at C5 by the Cys155-sulfhydryl of the approaching apoprotein, assisted by hydrogen bonding to Tyr65, possibly via transient protonation of a Tyr65 anion, which is also located on the more open α-face of the chromophore. The resulting N21-acylimmonium-cation is stabilized by the negatively charged carboxylate group of Asp163 on the sterically hindered β-face of the chromophore. The C-S bond is then formed by nucleophilic attack of the thiolate at C31 of the chromophore from the less hindered α-side, resulting in the 31S configuration that distinguishes the β155 chromophore from those attached to other sites (46,47,64). Protonation at C3 from the α-face would then result in the correct 3R-configuration, with the C2 and C3 substituents in trans-position. This protonation is either intramolecular (as shown in Fig. 11), but possibly assisted again by Tyr65 that is located on the proper face. Consistent with this proposal, single mutations at either position (D163A, D163V and Y65F) largely abolish CpcT activity (Fig. 5b).

The proposed catalytic mechanism rationalizes current knowledge about the CpcT action. The CpcT dimer protects the labile chromophore. By docking of CpcB, the CpcT dimer dissociates. The anchor cysteine of CpcB is then allowed to access C31 of the chromophore for proper chromophore attachment. The mechanism implies that the attachment occurs while the chromophore is still in the CpcT pocket in the ZZZsasa geometry. Structural transformation to the final ZZZsasa geometry, together with local rearrangements in the binding site of CpcB, is expected to occur during or after release from CpcT following the covalent bond formation. This sequence of events does not contradict spontaneous additions, in which PBPs bind chromophores in an extended conformation, although with low stereochemical fidelity and side reactions due to lack of protection (10,13,14,21,61,65). It is not clear if this reaction mechanism also applies to CpcS. A chromophore has been modeled into the structure of the empty lyase, CpcS, in an extended, bilirubin-like ridge-tile conformation that retains the Zs geometry at both the C5 and C15 methine bridges (28). Similarly, phycoerythrobilin in an extended geometry fits in the cavity of an eukaryotic CPES (29). This would also require subsequent rotation at both bridges. A more extended chromophore conformation in CpcS than in CpcT would be in line with the red-shift and increase of the long-wavelength band in the absorption spectra of PCB bound to CpcS (61) and of phycoerythrobilin bound to CPES (29). A twisting of the two halves by 90° at the central methine bridge, as in bilirubin (57,66), would, however, interrupt the conjugation and result in a strong blue-shift (50).

Although S- and T-type bilin lyases are distantly related, they may differ in mechanistic details, particularly the conformation and protonation state in which the chromophore is presented. This would account for their differences in stereochemistry of the respective binding sites as well as in chromophore selectivity. Nonetheless, both lyases carry out three main functions: 1) assisting site selectivity in the apo-phycobiliprotein, 2) protecting the chromophore, 3) ensuring the regio- and stereoselectivity of the addition, since both do
not seem to present the chromophore in the final \textit{ZZZasa} geometry. The reaction of the phylogenetically unrelated E/F type lyases may still be different. Last but not least, it should be noted that deprotonation of the cationic acylimmonium intermediate (Fig. 11) at C2 rather than C5 would result in the conversion of PCB to a phycoviobilin chromophore; such isomerization occurs in E/F type lyases (15,16,67) as well as in several cyanobacteriochromes during autocatalytic chromophore attachment (68-70).

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FOOTNOTES

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1) This work is supported by the National Natural Science Foundation of China (grants 31110103912 and 31270893 to KHZ, 31370777 to MZ), and Key State Laboratory of Agricultural Microbiology. XY acknowledges supports from Dept. of Biochemistry and Molecular Biology, the University of Chicago and National Institute of Health grant R01EY024363. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-06CH11357.
2) The abbreviations used are: CPC, cyanobacterial phycocyanin; CpcB, apoprotein of CPC β-subunit; *E.* Escherichia; FABP, fatty acid binding protein; KPB, potassium phosphate buffer; *Nostoc* Nostoc (Anabaena) sp. PCC 7120; PBP, Phycobiliprotein; PCB, phycocyanobilin; PEB, phycoerythrobilin.

**FIGURE LEGENDS**

**FIGURE 1.** Crystal structure of the CpcT dimer. a) Surface representation reveals a CpcT dimer with an intimate dimer interface. b) Ribbon diagram shows protruding loops from one subunit extending to the other subunit like a plug. Bulky aromatic side chains at the interface are highlighted in yellow for subunit A and cyan for subunit B. c) A zoom-in view of the interface reveals stacking interaction between two Tyr59 side chains. Other Tyr residues lining at the interface are also labeled.

**FIGURE 2.** The CpcT structure bound with the PCB chromophore. a) PCB (in cyan) is located to a cavity enclosed by 10-stranded β-barrel that is stabilized by a disulfide bond (yellow) between Cys116 and Cys137. Three large insertions between β-strands are colored in magenta. b) The 2Fo-Fc map (NCS-averaged over 12 subunits; contoured at 3.5σ level) of PCB show that the chromophore adopts the ZZZsss conformation (left) with an M-configured cyclic-helical structure (right: bottom view from left). c) Molecular surface of CpcT shows a deep but accessible chromophore binding pocket (marked by a red arrow), which is partially shielded by the plug structure of the partner subunit (in green). d) Electrostatic potential surface representation reveals a positively charged patch (in blue) at the rim of the PCB binding site (indicated by a red arrow).

**FIGURE 3.** Protein environment in the PCB binding pocket. a) The propionate side chains of PCB (in cyan) are exposed to the molecular surface. Access to the ethylenedene group of ring A is hindered by bulky or hydrophobic residues from both subunits (A in green and B in cyan) at the dimer interface. The Cys116-Cys137 disulfide bond (yellow) is located opposite to the dimer interface. b) The opening of the PCB pocket is lined with positively charged residues while non-polar residues are clustered at the bottom of the pocket. Red dashes mark the distances between the C31 atom of PCB and two potential proton donors (Tyr65 and Asp163). The side view of PCB shows that ring A is located below ring D (standard orientation of formula), corresponding to M-helicity. c) A top view from the pocket opening shows structural rearrangements in Arg66, Arg68 and Arg141 before (yellow) and after (grey) the PCB (cyan) binds.

**FIGURE 4.** a) Structural comparisons between the A/B (light blue) and B/A (green) CpcT dimers. While subunits A and B as monomers (left) are very well superimposed (with an overall r.m.s.d. value of ~0.77 Å), subunits on the right of the dimer are not aligned, suggesting differences in relative positioning between subunits A and B. Specifically, the plug of the subunit A is deeper into B by a distance of 2.8 Å. b) Structural superposition between the dimers of apo-CpcT (light blue) and the CpcT-PCB complex (dark blue). Incorporation of the chromophore (cyan) slightly altered molecular packing in the crystal lattice, which disrupted the symmetries of space group P3121 for apo-CpcT and led to P1 for CpcT-PCB.

**FIGURE 5.** Site-directed mutagenesis and CpcT lyase activity. a) Mutation sites are highlighted in colors corresponding to their roles in CpcT lyase activity: essential (red), critical (orange) and minimal effect (green). b) CpcT lyase activities of mutants relative to the wild type (WT = 100%), as measured by reconstitution of CpcB(C84S) with PCB in *E. coli*. A control in the absence of CpcT did not yield
chromophorylated proteins beyond the detectable limit (1%), i.e. spontaneous chromophore attachment did not occur (25,48).

FIGURE 6. Optical properties in solution and oligomeric state of PCB-CpcT complexes. Absorption (solid line) and fluorescence emission (dashed line, $\lambda_{ex} = 580$ nm) spectra of PCB complexes with wild-type CpcT (a), CpcT(Y65F) (b) and CpcT(D163A) (c). Excessive PCB was removed immediately before measurement from the CpcT-PCB complex purified by Ni$^{2+}$ affinity chromatography. d) Elution profile of CpcB(C84S) was obtained by gel chromatography on Superdex 75 eluted with KPB (20 mM, pH 7.2) containing NaCl (150 mM) (see Experimental Procedures). Two peaks correspond to CpcB oligomerized as dimer (40 kDa, calculated 47.4 kDa) and trimer (57 kDa, calculated 71.1 kDa), respectively. e) Elution profiles of CpcT at different protein concentrations (2.4mM and 36mM) under the same experimental condition as in (d). f) Elution profiles of CpcT in the absence (solid line) and presence (dashed line) of PCB (final concentration ~0.6 µM) in DMSO (final concentration ~0.016% (v/v)).

FIGURE 7. Structural comparisons among six bilin-binding members in the FABP protein family. All ribbon diagrams are shown in the structurally aligned orientation. Four of the six structures are bound with bilin chromophores (shown as CPK models with C in cyan, O in red, and N in blue). The PDB accession IDs are shown in parentheses and the chemical nature of the chromophore is indicated following a dash sign.

FIGURE 8. Conformations, accessibility and alignment of the bilin chromophores bound to three representative FABP proteins. In both CpcT and 1BBP, bilins are exposed to the molecular surface via their propionate side chains, although their chromophores open up at different methine bridges: PCB in CpcT (cyan) opens between rings A and D and biliverdin IX$\gamma$ (yellow) opens between rings B and C. The bilirubin chromophore in UnaG (green) adopts an extended conformation and is completely buried (visible only with semi-transparent surface). The chromophore alignment (right/bottom panel) is based on the least-squares fittings of their protein scaffolds. For clarity, rings A and D are labeled for three chromophores in corresponding colors; in BV IX$\gamma$ the central rings correspond to the outer rings in PCB and are, therefore, labeled in the same way.

FIGURE 9. Proposed docking mode of CpcT (green, this work) to the target site Cys-$\beta$155 of CpcB (gray, PDB ID: 1CPC). The locations of PCB are also shown at the docking interface between CpcT and CpcB before and after PCB is transferred from CpcT to CpcB for covalent attachment. Upon docking, a segment of CpcB containing Cys-$\beta$155 (in gold) may reach into the cleft of the CpcT monomer to bind the chromophore in the ZZZss geometry (cyan), and then undergoes structural transformation as PCB attached to CpcB adopts the ZZZasa geometry (gray).

FIGURE 10. Sequence alignment among $\beta$-subunits of CPC and PEC using pairwise HMM Logos (71). A signature motif E/D-R-D (residues 33-37-39) and a highly conservative D152 in CpcB/PecB are shown, both of which may play a role in guiding the docking of CpcT. The protein sequences of CPC and PEC were taken from Anabaena sp. 90 (NC_019427), Cyanothece sp. ATCC 51142 (NC_010546), Nostoc sp. PCC 7120 (NC_003272.1), Rivularia sp. PCC 7116 (NC_019678), Synechococcus elongatus PCC 6301 (NC_006576.1), Synechococcus sp. JA-2-3B'a(2-13) (NC_007776), Synechococcus sp. PCC 7002 (NC_010475.1), Synechocystis sp. PCC 6803
(NC_000911.1), *Thermosynechococcus elongatus* BP-1 (NC_004113.1), *Thermosynechococcus* sp. NK55a (NC_023033.1).

**FIGURE 11. Proposed reaction mechanism of CpcT.** CpcT carries and protects an unprotonated PCB chromophore in the **ZZZss** geometry. CpcB, the accepting apo-phycobiliprotein, presents an unstructured segment containing the attachment site Cys-β155 as a “fishing rod”. CpcB approaches the Δ3,3\(^1\) double bond of PCB from the sterically unhindered α-side and results in the 3\(^1\)S configuration. During or after its release from the CpcT pocket, the chromophore bound to CpcB assumes the **ZZZasa** geometry. The acylimmonium ion is formed only transiently after docking of CpcB. See lower-right structure for atom and ring labeling. In the mechanism shown, the critical Tyr65 and Asp163 play somewhat passive roles in polarizing the double bond and offering H-bond partners. However, more active roles are also possible, in particular transient protonation of a tyrosinate anion in the first steps of the reaction.
**TABLE 1. Statistics of data collection and structure refinement.**

| Data Collection         | Apo-CpcT | CpcT-PCB |
|-------------------------|----------|----------|
| Beamline                | 21-IDG   | 21-IDG   |
| Resolution (Å)          | 1.95     | 2.50     |
| $R_{\text{merge}}$ (%)  | 0.065    | 0.079    |
| Completeness (%)        | 97.9     | 80.2     |
| Number of unique reflections | 195467   | 76002    |
| Redundancy              | 12       | 4        |
| Space group             | P3,21    | P1       |
| Cell parameters (Å)     | $a=b=69.3, c=165.1$ | $a=69.7, b=69.6, c=162.6$ |
|                         | $\alpha=90^\circ, \beta=90^\circ, \gamma=120^\circ$ | $\alpha=90.2^\circ, \beta=90.3^\circ, \gamma=60.1^\circ$ |
| Structure Refinement    |          |          |
| R-factor                | 0.175    | 0.181    |
| Free R-factor           | 0.206    | 0.241    |
| # of molecules/ASU      | 2 monomers | 12 monomers |
| Waters and ligands      | 301 HOH  | 12 PCB   |
|                         | 74 HOH   |           |
| Solvent content (%)     | 51       | 51       |
| PDB ID                  | 4O4O     | 4O4S     |
Fig. 2
Fig. 3
Fig. 4
Fig. 6
CpcT-phycocyanobilin (4O4S)  
BBP-biliverdin IX\(\gamma\) (1BBP)  
BBP-biliverdin IX\(\gamma\) (1Z24)  
UnaG-bilirubin (4I3C)  
Apo-CpcS (3DBR)  
Apo-luciferase (1VPR)  

Fig. 7
Structure and Mechanism of the Phycobiliprotein Lyase CpcT
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J. Biol. Chem. published online July 29, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.586743

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