Catalytic Mechanism of S-type Phycobiliprotein Lyase

CHAPERONE-LIKE ACTION AND FUNCTIONAL AMINO ACID RESIDUES

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The phycobiliproteins are a homologous family of light-harvesting proteins present in cyanobacteria, red algae, and cryptophytes that absorb light in the spectral region between the chlorophyll absorption maxima at ~430 and ~680 nm: they contain linear tetrapyrroles (phycobilins) of which 1–4 are covalently attached to the subunits by thioether bonds to conserved cysteines (1–4). The chromophores are derived from heme by oxidative ring-opening and subsequent reduction (5) and then attached post-translationally. The correct attachment of most chromophores is catalyzed by lyases. Three phylogenetically unrelated types of lyases have been characterized in cyanobacteria (6). They are specific for certain binding sites and chromophores (7–16), but the reaction mechanisms are still unclear. A chaperone-like action has been proposed for E/F-type lyases that catalyze chromophore attachment to Cys-84 of β-subunits of phyco(erythro)cyansins (17, 18). A more highly evolved function is suggested, however, by concomitant isomerizing reactions catalyzed by certain lyases (12, 19, 20), and by the finding that most lyases can bind the chromophore and then transfer it to the acceptor protein (15, 21).

Chromophore binding is particularly pronounced with the S-type lyases. CpcS1 from Nostoc PCC71206 rapidly forms an adduct with phycocyanobilin (PCB),7 and the latter can be transferred in a much slower reaction to cysteine 84 of the β-subunits of phyco(erythro)cyansins (CpcB) or phycoerythrocyanin (PecB), suggesting that PCB-CpcS1 is an intermediate of the enzymatic reaction (21). Model reactions of PCB with nucleophiles resulted in the formation of addition products in which the chromophore is isomerized and which are also substrates for a CpcS1-catalyzed chromophore transfer to the apo-proteins (20). We now report chromophore binding kinetics obtained by stopped-flow techniques, and identification of functional amino acid residues for intermediate chromophore binding and catalysis by site-directed mutation and enzyme kinetics.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Iodoacetic acid, N-bromosuccinimide, and phenylglyoxal were from Sigma; diethylpyrocarbonate, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and phenylglyoxal were from Sigma; diethylpyrocarbonate, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and phenylglyoxal were from Sigma; diethylpyrocarbonate, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and phenylglyoxal were from Sigma; diethylpyrocarbonate, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and phenylglyoxal were from Sigma; diethylpyrocarbonate, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, and phenylglyoxal were from Sigma. All other biochemicals and separation materials were of the highest purity available and obtained from the sources described previously (19, 22).

Proteins—Cloning and expression followed generally the standard procedures of (23). The gene cpcB was PCR-ampli-
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fied from *Mastigocladus laminosus*, subsequently producing cpcB(C155I) via site-directed mutation (24), and *apcA* and *cpcS1* were obtained from *Nostoc PCC 7120* (14, 25). They were cloned first into pBlueScript (Stratagene) and then subcloned into pET-30 (Novagen, Munich). The native trimeric CPC was isolated from *Nostoc PCC 7120* via DEAE-ion-exchange chromatography (26) and then further purified via micro-crystallization with dialysis against 1 M potassium phosphate buffer (KPB), pH 7.0 (27).

Modifications of Amino Acids—Chemical modifications of arginine, carboxyl groups, cysteine, histidine, lysine, and tryptophan in CpcS1 were done as described before (15, 22). When a chemical modification affected enzymatic activity, the respective amino acids were screened by site-directed mutagenesis. Site-directed mutants CpcS(C51T), CpcS(C63T), CpcS(H22V), CpcS(R147Q), CpcS(R149L), CpcS(R169L), CpcS(W141), CpcS(W69M), CpcS(W75S), and CpcS(G119C/W140V) were generated by the PCR-based megaprimer method (28), using PCR primers P1 and P2 and mutation primers P3–P5 and P12–P18, respectively (supplemental Table S1). CpcS(H21T/H22V), CpcS(H80L), and CpcS(R18V) were generated with a mutation kit from Takara, Dalian, using the mutation primers P6–P11, respectively (supplemental Table S1). All mutations were verified by sequencing.

Expressions—The pET-based plasmids were used to transform to *Escherichia coli* BL21 (DE3). Cells were grown at 20 °C in Luria-Bertani medium containing kanamycin (30 µg·mL⁻¹). When the cell density reached OD₆₀₀ = 0.5–0.7, isopropyl 1-thio-β-d-galactopyranoside (1 mM) was added. The 6 h after induction, cells were collected by centrifugation, washed twice with doubly distilled water, and stored at −20 °C until use. The recombinant proteins have the affinity tag of six consecutive histidines and could be purified with Ni²⁺-chelating chromatography as before (15). For CpcS1 to be used for the analyses of imidazole effects on kinetics, imidazole (500 mM) was replaced in the elution buffer of the Ni²⁺-chelating column by EDTA (50 mM) containing NaCl (500 mM), and the protein was purified further by fast-protein liquid chromatography (Amersham Biosciences) over a DEAE-FF column developed with a gradient of 0.1–0.6 M NaCl in KPB (20 mM, pH 6.5).

SDS-PAGE—SDS-PAGE gels (29) were stained with Coomassie Brilliant Blue for the protein, and with zinc acetate for bilin chromophores (30). The UV-induced fluorescence of protein-bound bilins was recorded digitally by camera, and the bilin bound to CpcS1 was quantified by comparing their scanned fluorescence intensities to that of a standard, namely Cpc, on the same SDS-PAGE, using Photoshop 6.0 (Adobe) (15).

Spectroscopy—Enzyme reactions and amino acid modifications were followed by UV-visible spectrophotometry (model Lambda 25, PerkinElmer Life Sciences) and fluorometry (LS45, PerkinElmer Life Sciences). The formation of chromophorylated phycobiliprotein was detected by the emission at 645 nm for PCB-containing chromoproteins (15), and at 575 nm for PEB-containing chromoproteins (13). Complete spectra in the 350 to 700 nm range were recorded to verify correct attachment and the absence of spontaneous addition products (6). Far-UV CD spectra were recorded at 20 °C with a CD spectrometer (J-810, JASCO).

PCB, PEB, and Protein Concentration Determinations—PCB and PEB were prepared as described before (19, 31). Their concentrations were determined spectrophotometrically using the extinction coefficients ε₆₉₀ = 37,900 M⁻¹·cm⁻¹ in methanol/2% HCl (19) and ε₅₉₃ = 49,000 M⁻¹·cm⁻¹ in methanol/5% HCl (32), respectively. Protein concentrations were determined by the Bradford assay (33), using bovine serum albumin as the standard.

Lyase Activity Assay—Chromophore reconstitution with apo-phycobiliprotein was assayed as described before (13, 15, 25) using the following standard reaction conditions: KPB (500 mM, pH 7.5) containing NaCl (150–200 mM), CpcS1 (or mutants, 10–30 µM), and apo-phycobiliprotein (5–30 µM). PCB (final concentration = 2–15 µM) was added as a concentrated DMSO solution where the final concentration of DMSO in the reaction mixture was 1% (v/v). The mixture was incubated at 35 °C for 1 h in the dark. For kinetic tests, purified CpcS1 (or mutants, 10–30 µM), apo-phycobiliprotein (5–30 µM), and different concentrations of the chromophore substrate, PCB, were mixed in the reconstitution system (see above) and incubated at 35 °C. Chromophore attachment to the apo-protein was followed by recording the fluorescence of the product (22). Kₘ (for PCB), Vₘₐₓ, and kₘₐₓ were calculated from Lineweaver-Burk plots, using Origin V7 (Origin Lab Corp., Munich).

Some CpcS1 mutants were insoluble: their enzymic capacity was evaluated by reconstitution of PCB-CpcB(C155I) in *E. coli*. Appropriately modified cpcS1 in plasmid pET30 were transformed into BL21(DE3) together with cpcB(C155I) in plasmid pCDF, and PCB-generating genes *hlo* (encoding heme oxygenase 1) and *pcaY* (encoding PCB: ferredoxin oxidoreductase) were transformed in plasmid pACYC (15). For PCB binding assay to CpcS1 in *E. coli*, cpcB(C155I) was omitted. After purification of CpcS1 (mutants) by Ni²⁺ affinity chromatography, the protein was denatured in acidic urea (8 M, pH 1.5). Non-covalently bound PCB was assayed by its absorption at 690 nm using an extinction coefficient of 37,900 M⁻¹·cm⁻¹, covalently bound PCB was assayed by its absorption at 660 nm using an extinction coefficient 35,500 M⁻¹·cm⁻¹ (34), and protein was assayed by using the Bradford method (33).

Stopped-flow Kinetics of PCB Binding to Lyase—Stopped-flow measurements were done with a model SFM-300 machine equipped with two 10-ml syringes and a third one of 1.9 ml, controlled by Bio-Kine software (V4.21a) using a TC-100/10T cuvette with a 10-mm light path (BioLogic France), and a diode array detector (TIDAS MMS 100-1, controlled by TIDASAQ software V2.15, J&M, Germany). The reaction was followed at 20 °C with a time resolution of 10 ms. Data were collected in the spectral range of 350–800 nm with data points every 1 nm. Solutions were adjusted to result, after mixing, in equimolar amounts of CpcS1 and the apo-protein, and the desired amounts of pigment. The resulting data were analyzed using SPECFIT/32 (Spectrum Software Associates, Boston, MA) as before (18).

PCB Binding Assay of Lyase—CpcS1 (or mutants, 5–30 µM) were incubated at 35 °C for periods of 5 min to 1 h in the recon-
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RESULTS

CpcS1 Binds PCB Rapidly and PEB Even More Rapidly—The binding of PCB by the lyase, CpcS1, was studied, after rapid mixing, by time-resolved absorption spectroscopy. Three species could be resolved using a sequential fitting model (Fig. 1, a and b): At the right in Fig 1, A represents free PCB, and C is the final product, PCB-CpcS1 (21). The absorption spectrum of the intermediate, B, was similar to that of PCB-CpcS1, and, therefore, indicative of a similar conformation, but it had a lower extinction coefficient. This intermediate was formed with a time constant of \(\tau_1 = 38 \text{ ms} \) and transformed to the final adduct with \(\tau_2 = 119 \text{ ms} \).

The binding kinetics of PEB (13) were even faster. Using again a biexponential model (Fig. 1, c and d), the intermediate formed in the first phase (\(\tau_1 = 12 \text{ ms} \)) was near the time resolution of the apparatus (death time, 3.2 ms). This is also indicated by a contribution of absorption at 596 nm to the main absorption of A at 547 nm that was similar to that of free PEB. Both the intermediate, B, and the final product, C (PEB-CpcS1), absorbed at 596 nm; obviously the major conformational change took place during the first phase, indicating that binding with PEB was even faster than with PCB. Transformation to the final product, PEB-CpcS1, was again accompanied by a small absorption increase. This second step was much slower with PEB (\(\tau_2 = 8.3 \text{ s} \)) than with PCB (\(\tau_2 = 119 \text{ ms} \)), which may relate to the more rigid structure of the former (36).

Chromophore Exchange on CpcS1—When PEB was added to PCB-CpcS1 solution, it replaced most of the bound PCB (Fig. 2). Vice versa, PCB could also replace the chromophore of PEB-CpcS1, but only incompletely, using the same concentrations of the respective components indicating, therefore, a tighter binding of PEB. This reaction was also considerably slower than replacing PCB in PCB-CpcS1 with PEB. Fitting the exchange reaction by a sequential model required in both cases three kinetic components, but all were faster for PEB replacing PCB in PCB-CpcS1 (Fig. 2, a and b) than for PCB replacing PEB in PEB-CpcS1 (Fig. 2, c and d). Together, with the more rapid binding of PEB (Fig. 1), this indicates that PEB is a competent substrate for CpcS1.

PCB or PEB Bound to CpcS1 Is Only Slowly Transferred to Apro-proteins—When PCB-CpcS1 was mixed with an apo-protein, PCB was transferred from CpcS1 to the cysteine 84 of the apo-protein. The kinetics of this transfer reaction were studied with CpcB(C155I), in which the second binding site, Cys-155, was blocked and, therefore, the spontaneous background binding to the latter site was inhibited. The reaction could be fitted by a biexponential model (Fig. 3, a and b). The intermediate, B, was formed rapidly (\(\tau_1 = 16.5 \text{ s} \)). Its spectrum was similar to that of the product, PCB-CpcS1, and this intermediate proba-
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bly results from the interaction between PCB-CpcS1 and CpcB(C155I). Its transformation to the final product PCB-CpcB(C155I), was much slower and not yet complete within the measuring period; the time constant ($\tau_2 \sim 29.6$ min) obtained by the fit is, therefore, only an estimate. The reaction was accompanied by a 7 nm blue-shift, and the spectrum of the final product, C, was similar to that of PCB-CpcB(C155I); therefore, we assigned the slow phase to the transfer of PCB from the lyase, CpcS1, to cysteine 84 of the acceptor protein, CpcB(C155I).

Studies of bilin lyases are complicated by a background of spontaneous, but faulty, attachment of chromophores to the acceptor protein, CpcB(C155I). Its transformation to the final product PCB-CpcB(C155I), was much slower and not yet complete within the measuring period; the time constant ($\tau_2 \sim 29.6$ min) obtained by the fit is, therefore, only an estimate. The reaction was accompanied by a 7 nm blue-shift, and the spectrum of the final product, C, was similar to that of PCB-CpcB(C155I); therefore, we assigned the slow phase to the transfer of PCB from the lyase, CpcS1, to cysteine 84 of the acceptor protein, CpcB(C155I).

A third reaction catalyzed by CpcS1 is the attachment of PEB to apo-proteins of cyanobacterial phycoerythrin, CpeA and CpeB (13). This reaction, however, could not be studied in the same way as the PCB additions described above, because both apo-proteins could not be solubilized from the E. coli cells used for overexpression (13). Consequently, we studied the reaction of PEB-CpcS1 with the (non-natural) apo-protein for this chromophore, CpcB(C155I), which has been previously shown to bind PEB under catalysis of CpcS1 (13). When PEB-CpcS1 was mixed with CpcB(C155I), the reaction could be fitted with a three-step model (Fig. 3, e and f). The first step ($\tau_1 = 3.4$ s) is faster than with PCB-CpcS1 as the chromophore donor, the last step is comparable ($\tau_3 = 24$ min). Surprisingly, there is at early times an absorption at $\sim 620$ nm, which decreases at later times. Such absorption is characteristic for a PCB chromophore, but it is unlikely that the latter is formed from PEB. The more likely explanation is protonation: free PEBH$^+$ absorbs in methanol at 594 nm (32), and, interestingly, protonation is also discussed as an early step during the spontaneous attachment of bilin chromophores to phytochrome (40).

PEB also binds spontaneously (i.e. in the absence of lyase) to Cys-84 of CpcB but, as with PCB, the reaction is faulty and the chromophore is in part oxidized (41). This spontaneous reaction could be fitted with a sequential three-step model (supplemental Fig. S2, a and b); in this case the absorption at $\sim 605$ nm keeps growing with time, which agrees with the (irreversible) formation of oxidation products. If CpcS1 was added to the
that the first reaction, probably protonation of PEB, had already occurred during the dead-time of the apparatus (3.2 ms). As shown above, CpcS1 bound PEB very rapidly in the absence of CpcB thus supporting this interpretation. If this is correct, then binding of PEB to CpcS1 would, again, out-compete binding to the apo-protein, thereby preventing the faulty side reactions of the free chromophore with CpcB.

Identification of Critical Amino Acid Residues in CpcS1—PCB bound to CpcS1 is only very weakly fluorescent, whereas the final catalytic product PCB-CpcB(C155I) is strongly fluorescent. This increase in fluorescence was used to quantify the catalytic activity of modified CpcS1 and to identify amino acid residues that are critical for the activity. As a first test, arginine, carboxyl groups, cysteine, histidine, lysine, or tryptophan residues in CpcS1 were modified chemically as in previous studies with E/F-type lyases (15, 22). Modification of Asp, Glu, or Lys did not change the enzymatic activity of CpcS1, but it was decreased after modification of Arg, Cys, His, or Trp (supplemental Table S2), indicating a functional significance of the latter group of amino acids.

Conserved sites with these amino acids were identified in the CpcS sequences of various cyanobacteria (supplemental Fig. S4). The conserved residues Cys-51, Cys-63, His-21, His-22, His-80, Arg-18, Arg-147, Arg-149, Arg-169, Trp-14, Trp-69, Trp-75, and Trp-140 (sequence numbering for CpcS1 from Nostoc PCC7210) were selected for site-directed mutations. The enzyme activities of all mutants were also assayed in multiplasmidic E. coli capable of biosynthesis of the chromophore, PCB. Enzyme activities of all mutants were then assayed in E. coli extraction. The double mutant G119C/W140V was moderately active in the E. coli assay, indicating that Trp-140 is not required for the enzymatic activity of CpcS1; it decreases, however, the solubility of CpcS1 sufficiently to prevent an in vitro analysis. We have also observed other mutants that were inactive in vitro but active in E. coli, which is an advantage of the E. coli system for such studies.

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**TABLE 1**

| CpcS1 mutants | Relative activity by assay | \( K_m \) | \( k_{cat} \times 10^{2} \) | \( k_{cat}/K_m \times 10^{3} \) | PCB retention of CpcS1 relative to wild type | Activity/binding index* | UV-CD, \( \lambda_{max} \) |
|---------------|---------------------------|---------|-----------------|-----------------|---------------------------------|----------------------|----------|
|               | in E. coli | in vitro | \( \mu \text{M} \) | \( s^{-1} \) | \( s^{-1}\mu \text{M}^{-1} \) |                        |          |
| Wild type     | 100       | 100      | 1.14 (±0.06) | 2.59 (±0.16) | 2.19 (±0.05) | 100/100 | 1  | 213.5 |
| W14H         | 3 (±0.4)  | b        | 1.2          |               |               |         |    |        |
| R18V         | 51 (±1)   | a        | 2.15 (±0.70) | 0.73 (±0.06) | 0.29 (±0.02) | 54 (±8) | 1/8 | 213   |
| H21T/H22V    | 48 (±3)   | 13 (±1)  | 1.34 (±0.11) | 0.95 (±0.07) | 0.71 (±0.01) | 23 (±6) | 1/2 | 209   |
| H22V         | 64 (±3)   | 34 (±1)  | 1.38 (±0.01) | 1.19 (±0.07) | 0.86 (±0.005)| 38 (±4) | 1/2 | 213   |
| C51T         | 2 (±1)    | 2 (±1)   | 1.22 (±0.1)  |               |               |         |    |        |
| C63T         | 82 (±6)   | 108 (±0) | 1.55 (±0.20) | 2.81 (±0.45) | 1.81 (±0.04) | 98 (±14) | 1/2 | 214.5 |
| W69M         | 56 (±4)   | 46 (±5)  | 1.39 (±0.11a) | 3.43 (±0.01) | 2.47 (±0.22) | 35 (±51) | 1/2 | 212.5 |
| W75S         | 69 (±7)   | 21 (±2)  | 1.44 (±0.25) | 1.06 (±0.18) | 0.74 (±0.04) | 87 (±7) | 1/2 | 212.5 |
| H80L         | 81 (±11)  | 54 (±1)  | 1.70 (±0.28) | 2.46 (±0.16) | 1.47 (±0.16) | 90 (±2) | 1/2 | 212.5 |
| G119C/W140V  | 43 (±2)   | a        | 1.37 (±0.02) |               |               |         |    |        |
| R147Q        | 42 (±3)   | 15 (±1)  | 1.54 (±0.08) | 1.69 (±0.30) | 0.74 (±0.03) | 25 (±4) | 3/3 | 1/2   |
| R149L        | 64 (±1)   | 4 (±0)   | 1.22 (±0.02) |               |               |         |    |        |
| R169L        | 64 (±7)   | 72 (±3)  | 1.39 (±0.02) | 1.80 (±0.02) | 1.29 (±0.10) | 54 (±7) | 1/2 | 213   |

*The activity/binding index is the ratio of the activity in *E. coli* and the PCB retention determined by acid denaturation, both normalized to the wild-type lyase.

*Insoluble mutants, no data in *vitro*.

*Insoluble protein, no binding in *vitro*.

*K* kinetic constants of spontaneous adduct formation of PCB with CpcB; the mutant allowed simultaneous determination with the lyase-catalyzed reaction (see text).

*2* Mutant is catalytically almost inactive; rapid formation of spontaneous adduct of PCB with CpcB(C155I) (\( \lambda_{max, emission} \sim 660 \text{ nm} \)) inhibited determination of the kinetics of the lyase-catalyzed reaction.

*Broad line.

The results were therefore inconclusive: the conserved residues Cys-51 and Trp-14 may be essential for the enzymatic activity, but this could also be an indirect effect of improper folding of CpcS1.

The CpcS1 mutants C63T, H22V, H21T/H22V, H80L, R18V, R147Q, R149L, R169L, W69M, and W75S were soluble and shared similar secondary structures with wild-type CpcS1, as judged by their far-UV CD spectra (Table 1 and supplemental Fig. S5). The CD spectra indicate a high content of \( \beta \)-sheet, which (in agreement with the lipocalin-type structure (42) (Protein Data Bank code 3bdr; see “Discussion”) and in contrast to the mainly \( \alpha \)-helical E/F-type lyases (22). The maxima are consistently at 213 ± 1.5 nm, but three mutants, H21T/H22V, R147Q, and R149L, were characterized by significantly broadened bands that correlate with impeded PCB binding (see below). Mutants, C63T, H80L, and R169L, had comparable activities to the wild-type CpcS1, which was further verified by kinetic studies (Table 1). Amino acids Cys-63, His-80, and Arg-169, therefore, were not required for CpcS1 activity. Mutant R149L was inactive both in *vitro* and in *E. coli*, indicating a critical function. Activities of mutants H22V, R147Q, W75S, and, particularly, R18V were strongly reduced in *vitro* (5–34% compared with that of the wild type) but were only moderately reduced in *E. coli* (42–69%), indicating that one or more factors in *E. coli* facilitate the proper function. The \( K_m \) of mutants H22V, R147Q, and W75S did not change markedly, and their \( k_{cat}/K_m \) ratio decreased only ~50%, thus indicating that these residues are only indirectly involved in the enzymatic activity of CpcS1. Mutant R18V had only low activity in *vitro* and a reduced affinity for PCB, and the \( k_{cat}/K_m \) ratio was only 13% of the wild type. The W69M mutant was unique, because its enzymatic activity was only moderately reduced, but the product was heterogeneous as judged from the double-peaked fluorescence emission: the fluorescence maximum at 645 nm is characteristic for the correctly attached chromophore, whereas the second peak at 660 nm is characteristic of partial chromophore oxidation. Possibly, Trp-69 is important for controlling the correct conformation of PCB during attachment to the apo-protein. There may be cumulative effects of mutations, as indicated by the double mutant H21T/H22V (\( k_{cat}/K_m \sim 30\% \) of wild type).

The second property tested with the mutants was chromophore binding by the lyase. PCB bound to wild-type CpcS1 was largely retained during Ni\(^{2+}\)-affinity chromatography but mostly lost during SDS-PAGE, tryptic digestion or mass spectrometric analysis (20). Chromophore loss during Ni\(^{2+}\) affinity chromatography (Table 1) and the intensity of the Zn\(^{2+}\)-induced fluorescence on SDS-PAGE (supplemental Fig. S6) were used as a measure of chromophore binding by the mutants. The latter criterion seems of limited value because small amounts of bound PCB (~10%) were seen on SDS-PAGE, irrespective of the mutation employed, which may even constitute a by-product. Extensive washing of the His\(_{6}\)-labeled protein was more telling: the chromophore content was determined by quantifying the loss during Ni\(^{2+}\) affinity chromatography and by acid denaturation of the purified CpcS1-bilin. The effects of mutations on chromatophore loss during affinity chromatography were partly different from those observed for catalytic activity. In Table 1, this is indicated by the activity/binding index (defined in the legend). For several mutants, for example W69M, it is >1.5; thus, their catalytic activity is less impaired than chromophore binding. There is, in particular, no correlation between the affinity for PCB (\( K_m \)) in the catalytic attachment reaction (i.e., chromophore attachment to the apo-protein) and chromophore binding by the lyase. The W69M mutant, however, allowed spontaneous binding with kinetics...
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Imidazole Inhibits CpcS1 Catalysis Non-competitively—PCB binds imidazole and mercaptoethanol covalently in vitro in a reaction that is catalyzed by CpcS1 (20, 21). The reduced enzymatic activities of the H22V and H21T/H22V mutants of CpcS1 may therefore relate to the lack of (transient) binding to one, or more, of these residues, which are chemically equivalent to imidazole. Consequently, we tested whether imidazole inhibits the enzymatic reaction (supplemental Fig. S3). Imidazole was a non-competitive inhibitor, indicating that the imidazole and PCB binding sites are not the same. It is probably that the two sites are close, because imidazole is attached catalytically to PCB on catalysis (20, 21) and may substitute for a histidine residue of the acceptor protein.

Exchange of PEB and PCB in Phycobiliproteins—The stopped-flow experiments unexpectedly revealed indications for a chromophore exchange not only in the lyase (Fig. 2) but also on the chromophorylated acceptor protein, PCB-CpcB(C155I). This was indicated first using crude extracts of PEB-producing E. coli (supplemental Fig. S8, a and b). Because binding increases the extinction coefficient of the chromophores, the exchange is indicated by the absorption increase at 546 nm due to PEB binding, and a decrease at 602 nm and 618 nm (detachment of PCB), which is obvious from the difference spectrum. This reaction was then verified using purified chromoproteins and isolated PEB. If His147Q had reduced lyase activity and was catalytically inactive. Both the R147Q and R149L mutants bound less PCB than the wt (Table 1), but in R149L binding was much less reduced than the catalytic activity: the activity/binding index was reduced to 0.14. The basic residues, Arg-147 and Arg-149, might interact with the two propionic acid side chains of PCB. There is an interesting correlation in three mutants, H21T/H22V, R147Q, and R149L, between a strongly reduced chromophore binding, low activity in E. coli, and a distinctly broadened UV-CD band of the lyase (Table 1): if the broadened band indicates conformational heterogeneity and, possibly, increased flexibility, then lyase activity may rely on a well defined, rigid structure.

Irrespective of the exact nature of the binding, the amounts of bound chromophore and the spectroscopic changes were sufficient to analyze, for the first time, the kinetics of chromophore binding to a biliprotein lyase. It is multiphasic with both PCB and PEB chromophores, and the first phase, showing the largest spectral change, is always very rapid (τ < 50 ms).
There are two biliproteins that do not require lyases for correction chromophore binding. One class comprises the phytochromes and related cyanobacteriochromes, which have the lyase function integrated into the apo-protein (24, 45–49). The other autocatalytically assembling chromoprotein is the core-membrane linker of phycobilisomes, ApcE (55). The difference in attachment modes may well reflect the functions and abundance of the chromoproteins: phytochromes and ApcE are large proteins, but only minor constituents of the cell, whereas phycobiliproteins, by contrast, are major products that can constitute 50% of cellular protein in cyanobacteria (56). Biosynthetic expenditures for their synthesis are, therefore, a major factor in cellular energetics. This would favor the evolution of smaller but more abundant proteins for the function of light-harvesting role, which would also benefit from a high pigment-protein ratio (57). Such a situation would, in turn, favor the evolution of specialized lyases that ensure correct chromophore binding, but do not have to be produced in comparable amounts to their substrates.

**Chromophore Transfer to Apo-proteins**—Transfer of PCB or PEB from CpcS1 to the apo-proteins is a very slow reaction ($\tau > 10$ min, Figs. 3 and 5 and supplemental Figs. S1 and S2). Rapid binding to CpcS1, even in the presence of the apo-protein (see above), and the slow subsequent transfer to the latter strongly suggests that PCB–CpcS1 is an intermediate in the enzymatic reaction. This is also supported by the finding that interaction of the apo-protein with the lyase is stimulated when the latter carries a chromophore.

Transfer to the apo-protein, and thereby the overall binding kinetics, is also much slower than the spontaneous binding. This is a clear indication that the function of CpcS1 is not catalytic in the classic enzymological sense of speeding up the reaction, but rather chaperone-like to control the correct attachment. A chaperone-like action had already been demonstrated for an E/F-type bilin lyase (18), suggesting similar functions for these phylogenetically unrelated lyases, which also have very different secondary structures. Further, a chaperone-like action has also been demonstrated for another tetrapyrrole-attaching enzyme, the cytochrome $c$ lyase (58). Redox control is considered critical for the cytochrome $c$ lyase, whereas conformational control of the chromophore, and possibly also the apo-protein (supplemental Fig. S2), seems critical for the bilin lyases (see Scheer and Zhao (6)). Indirectly, conformational control as a determinant for chromophore binding is also supported by the effect of Triton X-100 on the spontaneous chromophore attachment (39). Although phycobilins are prone to oxidation (59), direct redox control seems unlikely, because CpcS1, in vitro, does not require reducing agents and is even inhibited by mercaptoethanol (14). E/F-type lyases require thiols, but there is no indication for the presence of disulfides that have to be reduced (15).

Judged from the reconstitutions in vitro, residues His-21/His-22, Arg-18, Arg-147, Arg-149, and Trp-75 are involved in the enzymatic function of CpcS1 (Table 1). Residue Trp-140 is probably only indirectly involved by inhibiting folding; the data with mutants of Cys-51 and Trp-14 were inconclusive, because they were non-functional in vitro and in E. coli. The protein encoded by gene til1699 of *Thermosynechococcus elongatus* has recently been assigned to a CpcS-III lyase (42) and is homolo-
gous to CpcS1 from Nostoc PCC7120, with 70% identity, including all critical amino acids identified by us (Fig. 4). The x-ray structure of Tll1699 (Protein Data Bank code 3bdr) shows that it belongs to the calyx-shaped lipocalin family (InterPro IPR002345). Their central cavities can adapt to a wide variety of small molecules (52), including biliverdin IXα (IPR002345). Their central cavities can adapt to a wide variety of small molecules (52), including biliverdin IXα (IPR002345).

One exception is Trp-75: this amino acid is part of a missing loop in the x-ray structure. The other exceptions are His-21 and Trp-140, which face to the outside. Because we failed to obtain a single site-mutant His-21, its function cannot be assessed reliably; the strongly decreased in vitro activity of the His-21/22 double mutant compared with the His-22 single-site mutant indicates that it is prone to misfolding. This is more obvious for Trp-140: the mutant is insoluble in vitro, but moderately functional in E. coli, indicating that the mutant does not fold properly in vitro. Based on this model structure, we suggest that CpcS lyses of type III bind PCB or PEB transiently within the central cavity; the two arginines are probably candidates for interacting with the propionic acid side chains of the chromophores.

Variations of the chromophore absorptions in the CpcS1-PCB complex indicate furthermore that the conformation of the chromophore and/or its state of protonation are affected by these mutations. X-ray structures of bilin complexes with Tll1699 are desirable to test this model.

Critical amino acid residues have also been determined for two EF-type lyses, CpcE/F (15) and PecE/F (22), whose structures are as yet unsolved. In both E-subunits, one arginine and one tryptophan are essential, as well as an additional histidine in that of the isomerizing lyase. One acidic residue is involved in CpcF, and one tryptophan plus Cys-121 and His-122 are involved in PecF; the latter two are critical for the isomerization (12). Arginine, tryptophan, and sometimes cysteine and histidine, therefore, seem essential amino acid residues for the phycobiliprotein lyase activity. The combination of cysteine and histidine is also important in the autocatalytic chromophore binding of phytochromes (46): both are nucleophilic amino acids that can add reversibly to the chromophore during catalysis by CpcS1 and then permit the chromophore to be transferred to the apo-proteins (20). The non-competitive inhibition of CpcS1 by imidazole (supplemental Fig. S3) also supports the relevance of histidine(s).

These data, furthermore, suggest that binding to the lyase is not strictly related to the lyase function (Table 1). Mutations of His-21/22, Trp-69, and Arg-147 reduce the lyase activity much less than the capacity of PCB binding by the lyase, resulting in an activity(binding index of 1.6. In the case of the W69M mutant, however, the reaction results in a product mixture: besides the native product fluorescing at \( \lambda_{\text{max}} = 645 \) nm, a second product is formed that fluoresces at longer wavelengths (\( \lambda_{\text{max}} = 660 \) nm, Table 1). Obviously, loss of chromophore binding to the lyase correlates, in this case, with a loss of fidelity of the attachment to the apo-protein. The Arg-149 mutant is an opposite example: in this case the chromophore binds well, yet the lyase activity is strongly inhibited.

CpcS1 Function—Identification of chaperone-like mechanistic traits in a second biliprotein lyase indicates that all biliprotein lyses may function in such a way. Genes that are homologous to cpcS1 exist in all cyanobacteria, red algae, and glaucocystophytes (9). The frequent finding of several copies, including in Nostoc PCC7120 (61), is surprising because of the near-universal substrate specificity with regard to both the chromophore and the apo-protein (9, 13). This suggests a higher degree of complexity of their actions and interactions, which is also supported by the “beneficial” effects of E. coli crude extracts on CpcS1 function (see above). Rapid binding by the lyase, which out-competes the apo-protein, may thus be crucial in protecting the chromophore (Fig. 5) while fine-tuning of the subsequent transfer is subject to thermodynamic control. An indication for this is the possibility of chromophore exchange not only on the lyase, CpcS1 (20) but also on the chromoproteins under catalysis by CpcS1 (supplemental Fig. S8). The latter was previously unnoticed and is less pronounced than with E/F-type lyses (6) but is still significant under our assay conditions. In combination, these exchange reactions would permit chromophore reshuffling and equilibration. Because none of them is energy requiring, this would avoid the problem of futile cycles. It remains to be seen if exchanges also occur in vivo, in particular in species containing PE besides PC, and how this activity is controlled.

One level of regulation is indicated by the gene arrangement in the phycoerythrin-containing cyanobacterium, Gloeobacter violaceus PCC7421. The gene coding for the lyase, cpeS, and those coding for chromophore biosynthesis, pebAB, are arranged in an operon (cpeS/pebA/pebB), and transcribed in the same direction (62). This may reduce the chance of degration of the rather labile chromophore by binding to the lyase. Substrate channeling has been suggested for the reductases, PcbA and PcbB (5), and it may extend further to the lyase.

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FIGURE 5. Catalytic scheme of CpcS1. PCB-CpcB is the reconstituted chromoprotein with PCB correctly attached to cysteine 84, PCB-CpcB* is the spontaneous addition product with incorrectly attached and partially modified PCB.
