Phycobilin:cysteine-84 biliprotein lyase, a near-universal lyase for cysteine-84-binding sites in cyanobacterial phycobiliproteins

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Phycobilisomes, the light-harvesting complexes of cyanobacteria and red algae, contain two to four types of chromophores that are attached covalently to seven or more members of a family of homologous proteins, each carrying one to four binding sites. Chromophore binding to apoproteins is catalyzed by lyases, of which only few have been characterized in detail. The situation is complicated by nongenetic background binding to some apoproteins. Using a modular multiplasmidic expression-reconstitution assay in Escherichia coli with low background binding, phycobilin:cystein-84 biliprotein lyase (CpeS1) from Anabaena PCC7120, has been characterized as a nearly universal lyase for the cysteine-84-binding site that is conserved in all biliproteins. It catalyzes covalent attachment of phycocyanobilin to all allophycocyanin subunits and to cysteine-84 in the β-subunits of C-phycoerythrin and phycoerythrocyanin. Together with the known lyases, it can thereby account for chromophore binding to all binding sites of the phycobiliproteins of Anabaena PCC7120. Moreover, it catalyzes the attachment of phycoerythrobilin to cysteine-84 of both subunits of C-phycoerythrin. The only exceptions not served by CpeS1 among the cysteine-84 sites are the α-subunits from phycocyanin and phycoerythrocyanin, which, by sequence analyses, have been defined as members of a subclass that is served by the more specialized E/F type lyases.

bilibiliprotein biosynthesis | light-harvesting | photosynthesis | phycobilisome

Phycobilisomes, the extramembraneous light-harvesting antennae in cyanobacteria and red algae, use four different types of linear tetrapyrrrole chromophores to harvest light in the green gap of chlorophyll absorption (1–6). These phycobilins are covalently bound to seven or more proteins, each carrying one to four binding sites. The chromophores are biosynthesized from the cyclic iron-tetapyrrole, heme, by ring opening at C-5, followed by reduction and, sometimes, also by isomerization (7–9). In the last step, these phycobilins are covalently attached to cysteines of the apoprotein via a thioether bond to C-3 on ring A (Fig. 1) and in some cases by an additional thioether bond to C-18 on ring D (6, 10–12). This step, the binding to the apoprotein, is presently only poorly understood; it involves a considerable number of binding sites and chromophores, as well as the proper regulation and coordination of events.3

An increasing number of lyases has recently been identified that catalyze the chromophore addition and are specific not only for the chromophore but also for the apoprotein and the binding site (12–16). Based on the capacity of several of the respective apoproteins to also bind the chromophores autocatalytically (17–21), a chaperone-like function has been suggested (12). It enhances and guides the autocatalytic binding, which is generally of low fidelity, possibly by conformational control of the chromophore (18). At the same time, this autocatalytic binding interferes with the lyase analyses (22). The situation is somewhat similar to chromophore binding in cytochromes c, which is autocatalytic under well controlled conditions (23), but requires in situ a considerable number of proteins (24–26).

Of the biliprotein lyases, only the heterodimeric E/F-type has hitherto been characterized in detail: it is specific for the protein, namely α-subunits of cyanobacterial phycocyanin (CPC) and the related phycoerythrocyanin (PEC) and for the binding site (cysteine-α84) (12–14) and is often encoded by genes on the respective biliprotein operons (27, 28). The number of E/F-type lyases found in the genomes of sequenced strains of cyanobacteria, however, is insufficient to account for the multitude of binding sites in the phycobiliproteins present in the phycobilisomes (12). Moreover, the formation of functional chromophorylated phycobilisome cores in the absence of EF-type lyases indicated the presence of other lyases (27). The first direct evidence for other types of lyases was reported by Shen et al. (29), who identified a group of four genes (cpeS, cpeT, cpeU, and cpeV) in Synechococcus PCC7002 that encode lyases which attach phycocyanobilin (PCB; Fig. 1) to the β-subunits of CPC. Shen et al. further indicated first, that these lyases had a broader specificity, and second, that the substrate specificity was controlled by the amounts of the different lyases present in mixtures. Homologous genes are ubiquitous in cyanobacteria (15, 16). Phycobilin:cystein-84 biliprotein lyase (CpeS1) of Anabaena PCC7120 has subsequently been shown to catalyze the regiospecific attachment of PCB to cysteine-84 of the β-subunits of CPC and PEC (16), whereas CpeT from Synechococcus PCC7002 is regiospecific for the third binding site of CPC, namely cysteine-β155 (15). Together, the three lyases, CpeE/F (or PecE/F), CpeS, and CpeT in principle are sufficient for complete chromophore binding (chromophorylation) to the three binding sites of CPC and PEC.

Much less is known about the chromophorylation of other cyanobacterial phycobiliproteins (12, 29–32). All phycobiliproteins

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Abbreviations: APC, allophycocyanin; APB, APC B; CPC, cyanobacterial phycocyanin; CPE, cyanobacterial phycocerythrin; CpeS1, phycobilin:cystein-84 biliprotein lyase; KPB, potassium phosphate buffer; PCB, phycocyanobilin; PEB, phycoerythrobilin; PEC, phycoerythrocyanin.

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†APC, apoproteins of α- (ApcA) and β-subunits (ApcB) are encoded by aprA1 and aprB; β-APC*, homologue of β-APC encoded by aprF; APF, encoded by aprD; ApcA, homologue of ApcA in some cyanobacteria; CPC, apoproteins of α- (CpcA) and β-subunits (CpcB) are encoded by cpcA and cpcB; CPE, apoproteins of – (CpeA) and β-subunits (CpeB) are encoded by cpeA and cpeB; CpeS1, bilin:Cys84-biliprotein lyase encoded by aprL0617; CpeS2, CpeS1-like protein encoded by alls529; CpeS3, CpeT1, CpcS; β-chlorophyll-biliprotein lyase encoded by all5339; CpeT2, CpeT1-like protein encoded by allr6647; PEC, apoproteins of α- (PecA) and β-subunits (PecB) are encoded by pecA and pecB. If not stated otherwise, the amino acid positions of biliproteins refer to the consensus sequences shown in SI Fig. 9. The numbering of the chromophore is shown in Fig. 1. This article contains supporting information online at www.pnas.org/cgi/content/full/0706209104DC1.

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contain a complex group of allophycocyanins (APC) that constitute
the core, and many phycobilisomes contain phycocerythrins that are
located in the distal parts of the rods and have a complex chromophore complement (6). Intrigued by the proposal of Shen et al.
(29) that the new group of lyases may singly, or in combination, have
almost no discrimination to the protein substrate. A single protein,
the four putative lyases, CpeS1, showed any catalytic activity
C-phycoerythrin (CPE) subunits (CpeA and CpeB). Only one of
APC subunits and also to attach phycoerythrobilin (PEB; Fig. 1) to
catalytic background, for their ability to attach PCB to the various
studied, in a multiplasmidic
Anabaena
PCC7120, which contains two
cpeS-like genes (cpeS1 = alr0617, CpeS2 = all5292) and two
cpeT-like genes (cpeT1 = all5339 and cpeT2 = alr0647) (33). The
enzymatic functions of the respective gene products have been studied,
in a plasmid-encoded
Escherichia coli
system with low auto-
catalytic background, for their ability to attach PCB to the various
APC subunits and also to attach phycocerythrin (PEB; Fig. 1) to
C-phycoerythrin (CPE) subunits (CpeA and CpeB). Only one of
the four putative lyases, CpeS1, showed any catalytic activity
specific for the cysteine-84 site (consensus sequence) but it showed
almost no discrimination to the protein substrate. A single protein,
CpeS1, therefore, is capable of catalyzing chromophore binding to
a surprisingly large number of apoprotein subunits.

Results
Biosyntheses of APC and Homologous Subunits. Screening of putative
lyases. The APC lyase functions of CpeS1, phycobilin:cytochrome-
β155 biliprotein lyase (CpeT1), and of the homologous CpeS2 and
CpeT2 were screened in an
E. coli system extended from that of Tooley et al. (16, 34) to contain compatible plasmids confer-
ing synthesis of the chromophore PCB (i.e., heme oxygenase
and biliverdin reductase), one of the His-tagged APC subunits
as acceptor and one or more of the putative lyases. Free PCB is
nonfluorescent under UV excitation but becomes fluorescent
upon binding to native apoproteins (35–38). After induction,
when chromophorolyminated subunits were formed, the
E. coli cultures became brightly fluorescent [supporting information
(SI) Fig. 4]. To assay the chromoproteins formed, the cells were
broken and the supernatant analyzed by fluorescence spectroscopy
(Table 1); the products were then further analyzed after purification by Ni²⁺ chromatography. This
E. coli system was superior to in vitro
studies for poorly soluble proteins like CpeA, CpeB, ApcA2, and ApcB, and, furthermore, autocalytic chromophore addition (17–20) was suppressed; it is generally <10% (Table 1). Possibly the most striking example was ApcA1, which
is known to attach PCB autocalytically in good yield (19), but,
in the
E. coli system, this background autocalytic binding, in the absence of the lyase, was strongly reduced (Table 1). Moreover, the fluorescence emission of the autocalytically bound product was red-shifted compared with that of the product of lyase-catalyzed binding, which indicates some chromophore oxidation to mesobiliverdin during autocalytic binding.

The results obtained with the five APC subunits and the four
putative lyases are summarized in Table 1. Because the fluo-
rescence yields of the chromoproteins differ (see below), quantitative
comparison is possible only within a series of experiments with the
same acceptor protein, e.g., within the columns of Table 1. Therefore,
each column has been normalized to the amount of chro-

Table 1. Chromoprotein yield in
E. coli in both the absence and presence of CpeS1, CpeS2, CpeT1, or CpeT2

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<tbody>
<tr>
<td>None</td>
<td>30 (±2)</td>
<td>16 (±3)</td>
<td>7 (±1)</td>
<td>7 (±1)</td>
<td>9 (±0)</td>
<td>2 (±0)</td>
<td>1 (±0)</td>
</tr>
<tr>
<td>CpeS2</td>
<td>10 (±0)</td>
<td>7 (±0)</td>
<td>3 (±0)</td>
<td>6 (±1)</td>
<td>2 (±0)</td>
<td>8 (±0)</td>
<td>0 (±0)</td>
</tr>
<tr>
<td>CpeT1</td>
<td>5 (±0)</td>
<td>11 (±0)</td>
<td>4 (±0)</td>
<td>2 (±0)</td>
<td>6 (±3)</td>
<td>6 (±0)</td>
<td>0 (±0)</td>
</tr>
<tr>
<td>CpeS1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CpeT2</td>
<td>11 (±1)</td>
<td>8 (±1)</td>
<td>6 (±0)</td>
<td>3 (±0)</td>
<td>5 (±0)</td>
<td>7 (±0)</td>
<td>0 (±0)</td>
</tr>
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</table>

Relative fluorescence yields were averaged from two independent measurements and normalized in each column to the yield obtained with CpeS1. With APC subunits, PCB was generated as chromophore; with phycocerythrin subunits, PEB was generated.
were purified via Ni2+ affinity column in potassium phosphate buffer (20 mM, pH 7.0) containing 0.5 M NaCl [last sample plus 10% glycerin (vol/vol) to improve solubility]. Reconstitutions in E. coli (44); the somewhat lower (solubility) conditions. The spectroscopic data of the reconstituted chromoproteins, over the spectral range from 300 to 700 nm, agree both qualitatively and quantitatively with those of the respective isolated native biliproteins. Fig. 2 (and SI Fig. 7) show that the products from ApcA1 and ApcB exhibited the characteristic absorption, fluorescence emission, and CD spectra of the α- and β-subunits of APC (41–43) and showed complementary chromopptide maps (SI Fig. 6). Likewise the absorption maxima (622 nm), CD [617 (+)/343 (−) nm], and fluorescence maxima (645 nm) of PCB-ApcF are very similar to those reported for the native 16.2-kDa subunit of Mastigocladus (PCC7603) (44); the somewhat lower (−19%) extinction coefficient and increased (+15%) fluorescence yields of the former may reflect the different origin and measurement conditions.

The strong absorption at 650 nm and relatively weak fluorescence emission at 663 nm of the reconstitution product of ApcF (Fig. 2, Table 2) agree well with those of the native α-subunit of APC B (APB) (45). The hitherto unreported CD spectrum is typical for a biliprotein. In this case, unlike the others, the absorption and fluorescence maxima of whole cells (SI Fig. 4) differ from those of the purified samples (Fig. 2): the absorption maximum of the E. coli cells was at 605 nm (PCB-ApcD605), with a long-wavelength shoulder ~650 nm, whereas the purified product absorbed at 650 nm (PCB-ApcD650) (Fig. 2). The origin of this shift is probably related to aggregation (unpublished work).

In addition to the four ubiquitous apc genes, Anabaena PCC7120 contains a fifth gene, apcA2, that is highly homologous (70% amino acid identity) to that encoding the APC α-subunit, apcA1. To our knowledge, the respective chromoprotein has never been isolated; it may be an APC-like protein (46). The heterologous reconstitution product PCB-ApcA2 had absorption and fluorescence emission maxima at 622 and 641 nm, respectively, that are very similar to those of PCB-ApcF. The strong fluorescence and the large intensity ratio of the Vis and UV absorptions are characteristic of a native biliprotein; the CD signals, however, are of opposite sign (SI Fig. 7), indicating a different chromophore conformation.

The enzyme kinetics for PCB attachment to two substrates, ApcA1 and ApcF were studied in vitro, because these proteins are readily soluble after expression. From the linear Lineweaver–Burk plots, kinetic constants $K_m = 2.7 \pm 0.4 \mu M$, $k_{cat} = 9.5 \times 10^{-5} s^{-1}$, $K_m = 2.4 \pm 0.1 \mu M$, $k_{cat} = 3.8 \times 10^{-5} s^{-1}$ were obtained with ApcA1 and ApcF, respectively. These values agree well with the range obtained for PCB attachment to cyanine-84 of CPC and PEC by the E/F-type lyases (47, 48) or CpeS1 (16), thus further supporting both the rather broad substrate specificity and the functional role as an APC lyase.

**Chromophore Binding to CPE Subunits.** The surprisingly wide substrate spectrum of CpeS1 prompted a study of its activity (and that of the other three putative lyases) for phycoerythrin, which, with respect to the lyases, is the least-studied cyanobacterial biliprotein (12, 31). The multiplasmidic E. coli system was modified for this study. The gene encoding the PCB oxidoreductase (pcyA) was replaced by two genes (peaB), which encode the reductases converting biliverdin to PEB (49). Because Anabaena PCC7120 does not produce PEB, the respective genes, as well as cpeA and cpeB, which encode the two CPE subunits, were taken from *Calothrix* PCC7601.

The situation with CPE is more complex than with singly

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**Table 2. Quantitative absorption and fluorescence data of biliproteins obtained by CpeS1-catalyzed reconstitution**

<table>
<thead>
<tr>
<th>Biliprotein</th>
<th>$\lambda_{max} [nm]$</th>
<th>$Q_{vis/uv}$</th>
<th>$\varepsilon_{vis} \left( M^{-1} cm^{-1} \right)$</th>
<th>$\lambda_{max} [nm]$</th>
<th>$\Phi_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB-ApcA1</td>
<td>338/618 (4.3)</td>
<td>111,000 (±600)</td>
<td>641</td>
<td>0.37 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>PCB-ApcB</td>
<td>344/613 (2.4)</td>
<td>86,700 (±200)</td>
<td>640</td>
<td>0.20 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>PCB-ApcC</td>
<td>365/622 (3.2)</td>
<td>71,800 (±800)</td>
<td>641</td>
<td>0.18 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>PCB-ApcD650</td>
<td>410/602 (2.5)</td>
<td>69,000 (±500)</td>
<td>635</td>
<td>0.080 (±0.001)</td>
<td></td>
</tr>
<tr>
<td>PCB-ApcE650</td>
<td>346/650 (2.3)</td>
<td>62,000 (±4,000)</td>
<td>663</td>
<td>0.074 (±0.002)</td>
<td></td>
</tr>
<tr>
<td>PCB-ApcF</td>
<td>355/622 (2.4)</td>
<td>96,400 (±300)</td>
<td>645</td>
<td>0.19 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>PEB-CpeA(C139S)</td>
<td>361/564 (5.8)</td>
<td>104,400 (±700)</td>
<td>573</td>
<td>0.51 (±0.03)</td>
<td></td>
</tr>
<tr>
<td>PEB-CpeB(C48A/C59S/C165S)</td>
<td>390/560 (5.2)</td>
<td>120,000 (±6,000)</td>
<td>574</td>
<td>0.63 (±0.01)</td>
<td></td>
</tr>
</tbody>
</table>

Data are given for the biosynthesized and purified products, PCB-ApcA1, -ApcB, -ApcC, -ApcD, and -ApcF; PEB-CpeA(C139S) and -CpeB(C48A/C59S/C165S). Data were averaged from two independent experiments. Reconstitutions in E. coli were done with genes from Anabaena PCC7120, except cpeAB and peaAB, which are from Calothrix PCC7601; $Q_{vis/uv}$ denotes the absorbance ratio of the visible and near-UV bands.
strongly fluoresce in the presence of Zn\(^{2+}\) (SI Fig. 5(50), and they also had typical CD spectra (51) (SI Fig. 7). The report of Shen et al.

Discussion

The activity with all other mutants, as well as the background activities, was particularly low with the CpeB; the absorption maxima of these products were also red-shifted to \(\approx 640\) nm. The biosynthesized and purified PEB proteins had absorption and fluorescence maxima \(\approx 560\) and 575 nm, respectively (Fig. 2B), typical of native phycoerythrin subunits (50), and they also had typical CD spectra (51) (SI Fig. 7). SDS/PAGE (SI Fig. 5B) showed proteins of the expected size that strongly fluoresce in the presence of Zn\(^{2+}\), as is expected for bilins covalently bound to their proteins (39). The attachment to the correct site was further confirmed by mass spectrometry (Table 3).

We therefore conclude that CpeS1 also possesses CPE lyase activity; that it uses both subunits as substrates; and that the activity, in both cases, is restricted to a single cysteine, cysteine-84.

Table 3. Molecular weights (m/z) of chromopeptides from tryptic digestion

<table>
<thead>
<tr>
<th>Biliprotein</th>
<th>Chromopeptide</th>
<th>Ion</th>
<th>Peptide peak (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCB-ApcA1</td>
<td>RPDVVSPGGNAYG QEMTATC(PEB)LR</td>
<td>[M + 4H](^{4+})</td>
<td>727.47 727.86</td>
</tr>
<tr>
<td>PCB-ApcA2</td>
<td>RPDIVSPGGNAYG QEMTATC(PEB)LR</td>
<td>[M + 3H](^{2+})</td>
<td>968.96 970.09</td>
</tr>
<tr>
<td>PCB-ApcB</td>
<td>YAA(PCB)IR</td>
<td>[M + 2H](^{2+})</td>
<td>641.56 641.78</td>
</tr>
<tr>
<td>PCB-ApcD</td>
<td>ALCP(PEB)IR</td>
<td>[M + 2H](^{2+})</td>
<td>581.02 581.28</td>
</tr>
<tr>
<td>PCB-ApcF</td>
<td>LAACP(PEB)LR</td>
<td>[M + 2H](^{2+})</td>
<td>616.56 616.78</td>
</tr>
<tr>
<td>PEB-CpeA(C139S)</td>
<td>CPEA(PEB)LR</td>
<td>[M + 2H](^{2+})</td>
<td>467.86 468.21</td>
</tr>
<tr>
<td>PEB-CpeB(C48A/C59S/C165S)</td>
<td>CPEB(PEB)AR</td>
<td>[M + 2H](^{2+})</td>
<td>625.57 625.79</td>
</tr>
</tbody>
</table>

Calc., calculated; Expt., experimentally determined.

There is, however, one notable exception to this broad protein specificity: the \(\alpha\)-subunits of phycoerythrin (CpeA) and PEC (PecA) both have a cysteine-84-binding site, but here CpeS1 is inactive (16), and the sites are rather served by the site- and protein-specific EF-type lyases (12, 52). This exception and the presence of specialized enzymes indicate some special status of these subunits, which may relate to the variety of chromophores attached in different phyco(erythro)cyanins, namely PCB, phycoviolobilin, or phycourobilin (6). One could argue that with PecA [and possibly R-phycoerythrin (53)], the EF-type lyase has a second function as a chromophore isomerase (14). However, this additional isomerase function is not required for CpaC, where CPC is attached in the conventional fashion by addition to the \(\Delta_{3,3}\) ethylenediene double bond and with stereochemical and conformational properties very similar to those of PCB bound to cysteine-84 of the CPC and PEC \(\beta\)-subunits (54–56). This indicates a structural difference that, among the cysteine-84-binding sites, distinguishes CpaC and PecA from other biliproteins. Such a difference is supported by a sequence analysis of the apoproteins (SI Fig. 9). In addition to the well known phylogenetic characteristics of the different types of biliproteins (6) and the N-methylation of asparagine found only in \(\beta\)-subunits (57), there are several sequence traits that set CpaC and PecA apart from all other subunits. Most of the respective amino acids map to the surface of the protein and cluster largely, but not exclusively (see below), near the cysteine-84-binding site (Fig. 3). They include an insertion after amino acid 77 between helices b and c of M. laminosus PCC 7603 (54).

Fig. 3. Distinction of cysteine-84-binding sites served by EF-types lyases and by CpeS1. Characteristic amino acids are shown, including the insertion in the b-e loop, that distinguish \(\alpha\)-subunits of C-phycoerythrin and PECs from all other biliprotein subunits (in purple), projected on the structure of C-phycoerythrin from M. laminosus PCC7603 (54). Analysis by grouped alignment is shown in SI Fig. 9. The molecule (CPK style; no hydrogens shown) is viewed from the “chromophore face” of the cysteine-84-binding site; the chromophore is shown in blue, the sulfur of the binding cysteine-84 in yellow. This figure was prepared with DSViewer Pro V. 6 (Accelrys, San Diego, CA).
complexity of CpeS1 and the other three proteins; however, in view of the function, a notion supported by evidence of interaction among X.W., and M.Z., unpublished work). This may indicate a regulatory chromophore addition (Table 1) and enzymatic catalysis by CpeS1 lyase activity (Table 1); further, they inhibited both autocatalytic PCC7120 (33), only one encodes the product, CpeS1, which has the a shifting specificity depending on the ratios of the STUV-type, 9; see in particular T22, Q25, R30, and S37), of which only R30 is seen at the bottom of the model shown in Fig. 3. The inactivity of the K53A mutant of NblA suggests electrostatic interactions with the biliprotein subunits, which may be unfavorable with most of the biliproteins, because of the presence of arginine-37, but more favorable with CpeA and PecA that lack this residue.

The second part of the suggestion of Shen et al. (29), concerning a shifting specificity depending on the ratios of the STUV-type lyases, may have to be modified, at least for the system studied in this paper. Of the four genes of this lyase family present in Anabaena PCC7120 (33), only one encodes the product, CpeS1, which has the catalytic capacity for chromophore attachment to cyanine-84. CpeS2 and the two members of the CpeT family did not show any lyase activity (Table 1); further, they inhibited both autotrophic and photosynthetic addition (Table 1) and enzymatic catalysis by CpeS1 (K-H.Z., J. Zhang, J.-M.T. S. Böhm, M.P., L.E. C. Buberer, H.S., X.W., and M.Z., unpublished work). This may indicate a regulatory function, a notion supported by evidence of interaction among CpeS1 and the other three proteins; however, in view of the complexity of the E. coli system, this notion needs thorough investigation, especially in cyanobacteria.

What about the other members of the family? Does a similarly broad activity holds for CpeT/CpeT, which chromophorolates CpeB (and PecB; unpublished work) at cyanine-155 (15)? Although several CPC-producing cyanobacteria have two pairs each of CpeS and CpeT homologues, those producing the PEB chromophore have even more members; for example, there are six cpeS and four cpeT homologues in Gloeobacter violaceus PCC7421 (64). Possibly, lyases with similarly broad specificity as CpeS1 can be found for the other binding sites and for the occasional secondary attachment to ring D in phycocerythins. Currently, very little is known about the mode of action of any of these lyases; it also has to be separated from the autotrophic catalyses, albeit of low fidelity, of many of the apoproteins (17–19). Consequently, we emphasize the advantages of the multiplastic lactic E. coli system for these studies. Besides biotechnological applications, it offers rapid and flexible screening, including that of multiple protein attachment. Particularly advantageous is the low background caused by spontaneous chromophore addition, which is generally <10%.

In summary, CpeS1 fills a crucial gap; the attachment of all chromophores present in a cyanobacterial phycoobilin can be accounted for in combination with the two E/F-type lyases [cyanine-84 of CpaA and PecA (13, 14), one T-type lyase [cyanine-155 of CpeB and PecB (15)], and the autotrophic activity of the core-membrane linker, ApcD (65). The situation, however, is still less clear for those organisms producing phycocerythins, but the broad specificity of CpeS1 indicates that the respective sites may be served by other members of the S- and T-type lyases. Finally, an important and unresolved question is the sequence of events in the multiple chromophore attachment to phycocerythins and the beta-subunits of phycocyanins and PEC, which can also be a point of regulation and contribute to mutual interference of lyases. We have evidence that the E. coli system can be helpful, too, for investigating these questions, when combined with studies in vitro and in the parent cyanobacteria.

Materials and Methods

Cloning. Cloning and expression generally followed standard procedures (66). Full-length cpeA, pecA, cpeS1, ho1, and pcyA were PCR-amplified from Anabaena PCC7120 or Mastigocladus laminosus (Fischerella PCC7603), as described (14, 18, 61). For the construction of dual plasmids, ho1 and pcyA were cloned together in pACYCDuet (Novagen, Munich, Germany) to produce pHO1-PcY.A. CpeS1, without His-tag, was obtained by expressing pGE-MEX (Promega, Beijing, China) containing cpeS1 (16). The plasmids containing apcA1, apcB, apcD, apcF, cpeS1, cpeT1, or cpeT2 from Anabaena PCC7120 and cpeA, cpeB, peA, or peB from Calothrix PCC7601 were constructed by using the primers P1–P24, shown in SI Table 4. They were cloned first into pBluescript (Stratagene, Beijing, China) and then subcloned into pET30a or pETDuet (Novagen) and ho1 plus pebB constructed in pCDFDuet (Novagen) to produce pHO1-PcY.A, CpeS1, and ho1 plus pebB constructed in pCDFDuet (Novagen) to produce pHO1-PcY.A, CpeS1, and ho1 plus pebB constructed in pCDFDuet (Novagen) to produce pHO1-PcY.A, CpeS1, PcyA, and pebB from Calothrix PCC7601 with a mutation kit from Takara Bio, Dalian, China (see SI Table 4 for the construction primers P25–P38). All molecular constructions were verified by sequencing.

Expressions. The pET-based plasmids were expressed in E. coli BL21(DE3) as before (67). The dual plasmids were transformed together into BL21(DE3) cells under the appropriate antibiotic selections (chloromycin for pHO1-PcY.A, streptomycin for pCDEF derivative, kanamycin for pCDEF-derivative or pETDuet-derivative: see SI Table 5). To produce the dual plasmids, pET-ApcA1, -B, -A2, -D, -F was used together with pHO1-PcY.A, or pET-ApcA1, -B, -A2, -D, -F was used together with pHO1-PcY.A, or pCDEF derivative, or pETDuet derivative, containing cpeS1, cpeT1, cpeT2, or cpeT3. In the control experiments, plasmids containing cpeS1, cpeT1, cpeT2, or cpeT3 were omitted from the transformations. For reconstitution in E. coli, cells were grown at 20°C (APC) or 18°C (CEP) in LB medium containing kanamycin (20 μg/ml), chloramphenicol (17 μg/ml), streptomycin (25 μg/ml), and ampicillin (40 μg/ml). Twelve (APC) or 18 (CEP) h after induction with isopropyl β-D-thiogalactoside (1 mM), cells were collected by centrifugation, washed twice with doubly distilled water, and stored at −20°C until use (16).

Cells were lysed and tagged proteins isolated by Ni²⁺-chromatography, as described (16). If necessary, the affinity-enriched proteins were further purified by FPLC (Amersham–Amersham Pharmacia, Shangai, China) over a Superdex 75 column developed with buffer [50 mM potassium phosphate buffer (KPB)/150 mM NaCl, pH 7.0]; or with a DEAE FF column developed with a gradient of 0–1 M NaCl in KPB (20 mM, pH 7.0). UV-VIS absorption spectra of the proteins were performed with the buffer system of Laemmli (68). The gels were stained with Coo massie brilliant blue R for the protein and with ZnCl₂ for bilin chromophores (39).

Spectroscopy and Enzyme Kinetic Assay. UV-VIS absorption spectra were recorded with a Lambda 25 spectrometer (Perkin–Elmer, Shangai, China). Fluorescence spectra were recorded with a LS 45 spectrofluorimeter (Perkin–Elmer) and are not corrected. CD was...
measured with a J-810 CD spectrometer (Jasco, Munich, Germany).

**Extinction Coefficients.** Concentrations of the reconstituted and biosynthesized biliproteins were determined by using the extinction coefficient of PCB in CPC in 8 M acidic urea \((\varepsilon_{560} = 35,500 \ M^{-1}cm^{-1})\) (69) and of PEB in R-phycocyanin in 8 M acidic urea \((\varepsilon_{560} = 42,800 M^{-1}cm^{-1})\) (70). Fluorescence quantum yields, \(\Phi_F\), were determined in KPB (50 mM, pH 7.2), using the known \(\Phi_F\) (0.27) of CPC from *Anabaena PCC7120* (71) as standard.

**Enzyme kinetic assays** were carried out as described (48), \(K_{m}\), \(v_{max}\), and \(k_{cat}\) were all calculated from Lineweaver–Burk plots, using Origin V7 (Origin Lab Corporation, Northampton, MA) using a gradient of KPB (100 mM, pH 7.2). For HPLC analyses, the desired chromoprotein solution was acidified with HCl to pH 1.5 digested with pepsin (1:1, wt/wt) for 3 h at 37°C and then fractionated on Bio-Gel P-60 (Bio-Rad, Hercules, CA), equilibrated with dilute HCl (pH 2.5). Colorless peptides and salts were eluted with the same solvent (Bio-Rad, Hercules, CA), equilibrated with dilute HCl (pH 2.5) for 3 h at 37°C and then fractionated on Bio-Gel P-60 using Origin V7 (Origin Lab Corporation, Munich, Germany).

Reconstituted chromoproteins were diazayed against KPB (20 mM, pH 7.2). For HPLC analyses, the desired chromoprotein solution was acidified with HCl to pH 1.5 digested with pepsin (1:1, wt/wt) for 3 h at 37°C and then fractionated on Bio-Gel P-60 (Bio-Rad, Hercules, CA), equilibrated with dilute HCl (pH 2.5). Colorless peptides and salts were eluted with the same solvent (Bio-Rad, Hercules, CA), equilibrated with dilute HCl (pH 2.5) for 3 h at 37°C and then fractionated on Bio-Gel P-60 using Origin V7 (Origin Lab Corporation, Munich, Germany).