Chromophore Attachment to Biliproteins: Specificity of PecE/PecF, a Lyase-Isomerase for the Photoactive 3^1-Cys-α84-phycoviolobilin Chromophore of Phycoerythrocyanin†

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ABSTRACT: PecE and PecF, the products of two phycoerythrocyanin lyase genes (*pecE* and *pecF*) of *Mastigocladus laminosus* (*Fischerella*), catalyze two reactions: (1) the regiospecific addition of phycocyanobilin (PCB) to Cys-α84 of the phycoerythrocyanin α-subunit (PecA), and (2) the Δ4→Δ2 isomerization of the PCB to the phycoviolobilin (PVB)-chromophore [Zhao et al. (2000) *FEBS Lett. 469*, 9−13]. The α-apoprotein (PecA) as well PecE and PecF were overexpressed from two strains of *M. laminosus*, with and without His-tags. The products of the spontaneous addition of PCB to PecA, and that of the reaction catalyzed by PecE/F, were characterized by their photochemistry and by absorption, fluorescence, circular dichroism of the four states obtained by irradiation with light (15-Z/E isomers of the chromophore) and/or modification of Cys-α98/99 with thiol-directed reagents. The spontaneous addition leads to a 3^1-Cys-PCB adduct, which is characteristic of allophycocyanins and phycocyanins, while the addition catalyzed by PecE and PecF leads to a 3^1-Cys-PVB adduct which after purification was identical to α-PEC. The specificity and kinetics of the chromophore additions were investigated with respect to the structure of the bilin substrate: The 3-ethylidene-bilins, viz., PCB, its 18-vinyl analogue phytochromobilin, phycoerythrobilin and its dimethylester, react spontaneously to yield the conventional addition products (3-H, 3^1-Cys), while the 3-vinyl-substituted bilins, viz., bilirubin and biliverdin, were inactive. Only phycocyanobilin and phytochromobilin are substrates to the addition-isomerization reaction catalyzed by PecE/F. The slow spontaneous addition of phycoerythrobilin is not influenced, and there is in particular no catalyzed isomerization to urobilin.

Phycobiliproteins are the major light-harvesting proteins from classical (class I) cyanobacteria, red, and cryptophyte algae (*3*). In the former two, they comprise up to 50% of the total protein and are organized in large and complex extra-membraneous antenna structures, the phycobilisomes (PBS, see ref *4* for leading citations). The PBSs are assembled from up to 30 different proteins in 2−100 copies each and carry hundreds of chromophores. They have intense absorptions in the "green gap" of the chlorophyll spectrum, which in most cyanobacteria are subject to regulation by (among other factors) light intensity and quality. The absorbed excitation energy is transferred with quantum efficiencies close to 100% to the core complexes in the photosynthetic membrane, mainly photosystem II (*5*). This efficient light-harvesting relies on an array of spectrally finely tuned and properly spaced bilin chromophores, which are covalently attached to the apoproteins. By a combination of biliproteins carrying different chromophores, phycobilisomes can span an absorption range from ∼460 to 670 nm. However, also the absorptions of the same type of chromophore can be tuned considerably: the PCB chromophores span an absorption range from 590 to 670 nm, with the particular absorption governed by very specific noncovalent pigment protein interactions at the respective binding sites (see ref *6*).

The assembly of phycobilisomes is a complex process. Besides several apoproteins which bind the bilin chromophores, it requires the coordinated expression of a series of additional proteins responsible for chromophore attachment to the apoproteins, for the organization of the phycobilins beyond the trimer stage, and for their attachment to the photosynthetic membrane. In some cases, the cyanobacterial building blocks of PBS are reflected in complex operons for each biliprotein: the two genes coding for the two structural biliprotein subunits (α and *β*), are followed by a series of

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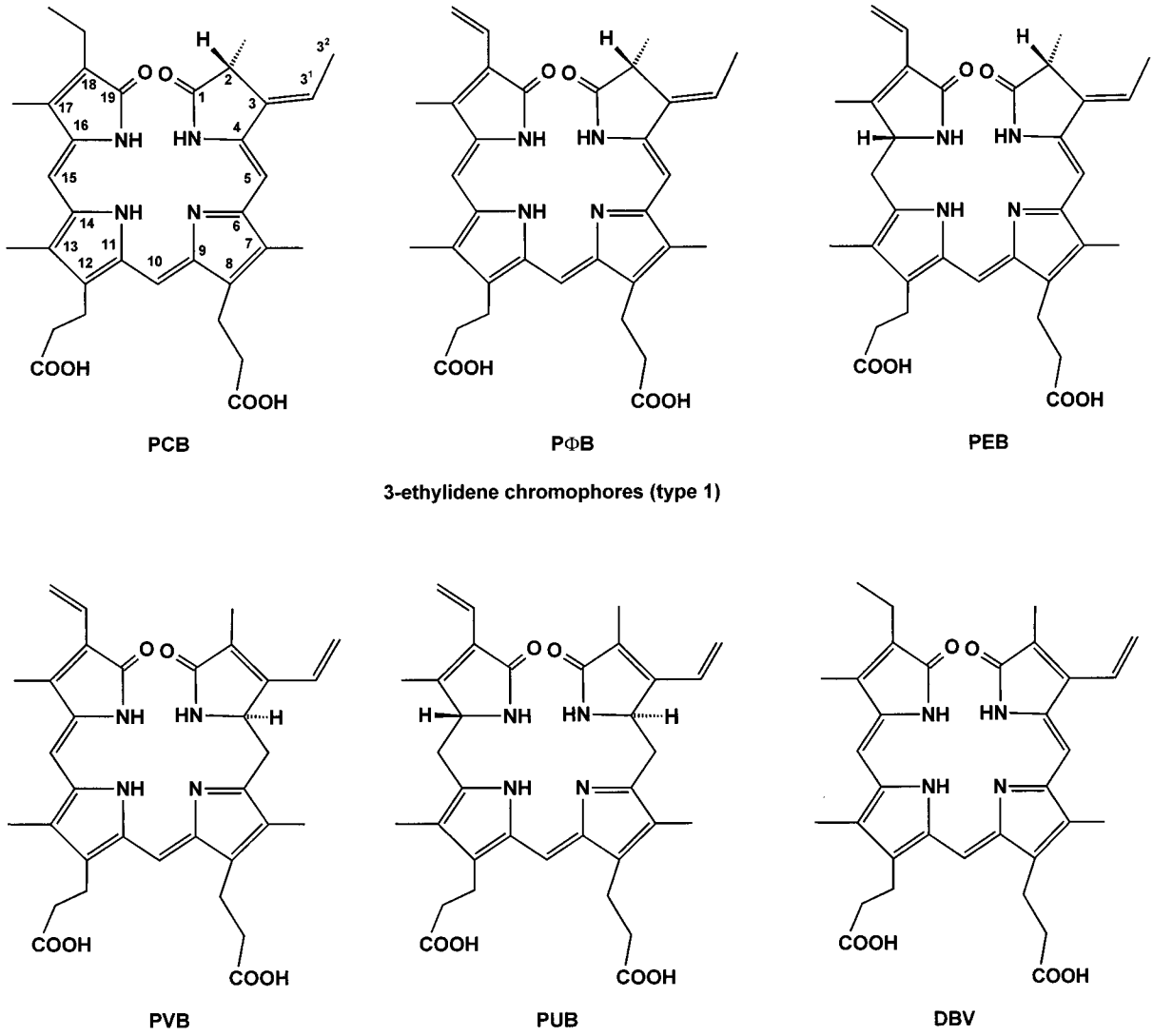

FIGURE 1: Structures of free bilins. (Top row) Type 1 chromophores carrying a Δ3,3¹-ethylidene group and a single bond between C-2 and C-3; bottom row: type 2 chromophores carrying a 3-vinyl group and a Δ2,3-double bond. The latter have neither been obtained from biliproteins, nor been found as biosynthetic intermediates.

additional reading frames coding for proteins involved in the assembly and integration of the biliproteins. An example is the phycocyanin operon of *Mastigocladus* (*M.*)[1] *laminosus*: *cpcB* and *cpcA* coding for the *β*- and α-subunit, respectively, are followed first by two genes (*cpcC*, *cpcD*) coding for linker proteins. They are components of the assembled phycobilisome and responsible for their macroscopic organization, by properly stacking trimers ($\alpha_3\beta_3$) to rods. Additional linkers are coded further downstream (*cpcG*$_1$, *cpcG*$_2$, *cpcG*$_3$), abd they are responsible for attaching the rods to the phycobilisome core (*7*−*11*). The intervening genes, *cpcE* and *cpcF*, code for two proteins which by their high homology with the respective genes from *Synechococcus* PCC 7002 (*12*) have been assigned to the two subunits of a bilin-lyase. These two proteins are not incorporated into the functional phycobilisomes, but rather involved in attaching the chromophores via thioether linkages to the apoproteins, as a prerequisite for further assembly under the control of the linker polypeptides.

There are currently eight different chromophores known for the biliproteins (*13*), four of them occurring in the cyanobacteria, viz., the 3¹-Cys adducts of phycocyanobilin (PCB), phycoerythrobilin (PEB), phycoviolobilin (PVB), and phycourobilin (PUB) (Figure 2). All of them are attached to the apoproteins via a thioether bond linking C-3¹ of the chromophore to specific cysteins on the apoproteins, in some of them a second bond is formed to C-18¹. There is one common bilin binding site in all biliprotein subunits (Cys-

[1] Abbreviations: BR, bilirubin; BV, biliverdin; CD, circular dichroism; $\Delta\Delta A_{xxx/yyy}$, amplitude of photochemical signal with difference maxima at *xxx* and *yyy* nm; normalized to maximum absorption (see ref *1* for details); DBV, 18¹,18²-dihydrobiliverdin; DME, dimethylester; *M.*, *Mastigocladus*; PCB, phycocyanobilin; PEB, phycoerythrobilin; PΦB, phytochromobilin; PUB, phycourobilin; PVB, phycoviolobilin [there are two terms for this chromophore in the literature: phycobiliviolin (*61*) and phycoviolobilin (*67*); the latter is used because it is analogous to the names of the major phycobilins, viz., phycocyano- and phycoerythrobilin], the names of all chromophores refer to the free chromophores shown in Figure 1, while the chromophores attached to the apoproteins are characterized as addition products (see Figure 2 and ref *2*); PC, C-phycocyanin; PEC, phycoerythrocyanin; α-PEC, chromophorylated phycoerythrocyanin α-subunit; PecA, apoprotein of phycoerythrocyanin α-subunit; PecE and PecF, components of α-PEC-phycoviolobilin lyase; TX-100, Triton X-100.

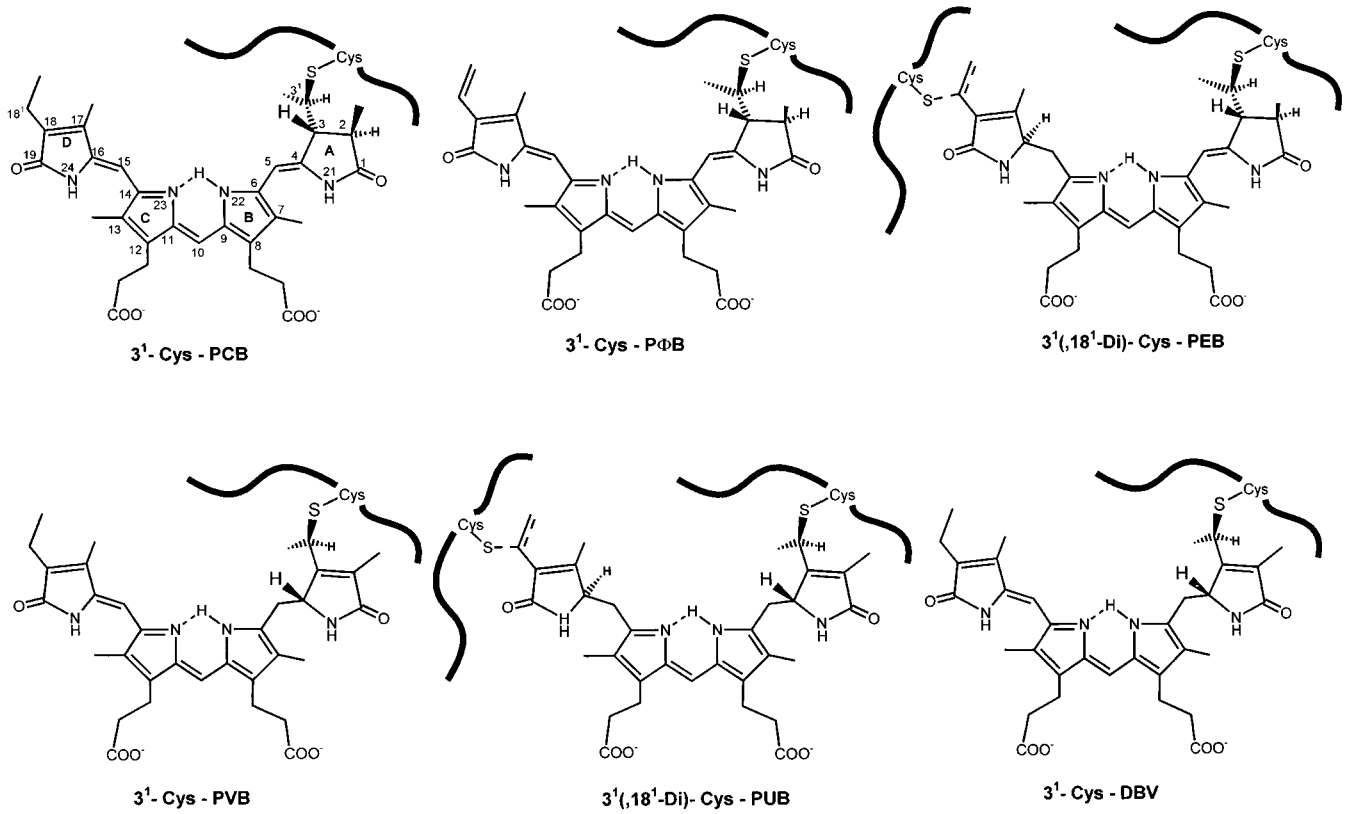

FIGURE 2: Structures of bound bilins. (Top row) Type 1 chromophores with a single bond between C-2 and C-3; bottom row: type 2 chromophores with a Δ2,3-double bond. There is always a 3^1-linkage present, for some chromophores an optional second linkage (18^1) is indicated.

84 in the consensus sequence), but up to two additional binding sites are present in the subunits of the rod-pigments, phycocyanin (PC), phycoerythrin (PE) and phycoerythrocyanin (PEC). Bilin binding sites have furthermore been located on the γ-subunit of rhodophytan PE (which shows some homology to linker proteins, ref *14*), and on the core-membrane linker L_{CM}, which is responsible for attaching the phycobilisome to the photosynthetic membrane (*15*, *16*). The chromophores on a biliprotein may be of the same or of different types. Allophycocyanin (APC) and PC contain for example PCB chromophores at all binding sites, while PEC contains two PCB chromophores on the β-subunit and one PVB-chromophore on the α-subunit.

The details of PBS assembly are currently only partly understood. In a likely scenario, the first step is the attachment of the chromophores to the apoproteins. It is followed by the aggregation of biliproteins to trimers ($\alpha_3\beta_3$), whose combination with linkers triggers the buildup of the core and the attachment of rods to the entire phycobilisome. Among the many problems, the first one in this scenario is the attachment of chromophores to their apoproteins. In phycocyanin from *Synechococcus* PCC7002, which has been studied in some detail, two of the binding sites (α-84, β-84) show autocatalytic binding upon addition of PCB to the α- and β-subunit, respectively. The third binding site (β-155) is inactive and does not bind PCB spontaneously (*17*). However, the situation is even more complicated since of the two sites which bind PCB, only β-84 is truly autocatalytic in terms of largely attaching the chromophore without further modification and with the correct stereochemistry. This site is therefore similar to the binding sites of the sensory biliproteins, the plant, and bacterial phytochromes, where the selectivity and recent mutagenesis experiments (*18*, *19*) support an autocatalytic ligation. By contrast, the "autocatalytic" ligation to α-84 is less specific, and the substrate, PCB, becomes largely oxidized to a $18^1,18^2$-dihydrobiliverdin in the process (*17*, *20*).

For this site, the proper binding can be brought about in vitro by the joint action of two lyase proteins encoded by the genes *cpcE* and *cpcF* of the C-phycocyanin (*cpc*)-operon (*21*−*25*), the same proteins also seem to catalyze the cleavage of the thioether bond as evidenced by chromophore exchange experiments (*21*). Homologous bilin-lyase genes have been found, too, in other biliprotein operons (*10*, *26*−*31*), but also in noncontiguous regions (*32*). In all cases tested, they are (at least in vitro) specific for the chromophore attachment to Cys-α84. Disruption of *cpcE* or *cpcF*, or of their equivalents, has been shown in several species to result (i) in production of PC lacking the α-84 chromophore and (ii) in a downregulation of PC synthesis to 10−30% of the wild-type content. Few of the expressed proteins (lyases) have, however, been studied enzymatically for their activities and specificities (*21*, *25*, *33*). Another problem has been pointed out by Glazer and co-workers (*13*): the numbers of pairs of lyase genes found by homology searches, is insufficient to code for lyases of each specific bilin binding site. Even if the attachment to β-84 were autocatalytic in vivo, too, no candidates are currently known for β-155 lyases, or the lyases for the up to four additional bilin binding sites in PEs.

There is yet a further complication in chromophore ligation. The bilin chromophores can be divided into two

types, depending on their structures and reactivities. Type 1, contains a Δ3,3¹-ethylidene group. The respective chromophores, PCB and PEB, have been studied in considerable detail. They can add reversibly thiols (*34*−*36*), as can the chromophore PΦB of the phytochromes (*35*−*40*). These chromophores can also be cleaved chemically from the holoproteins by treatment with HBr/TFA (*36*) or by heating in alcohols (*41*−*44*). The second type of chromophores comprising phycoviolobilin and phycourobilin, can not be added by the same mechanism, because their Δ3,4 double bond precludes a Δ3,3¹ double bond. The bound chromophores could principally arise from addition to a 3-vinyl group in the hypothetical free chromophores, similar to the addition of heme to c-type cytochromes (*45*, *46*), or the addition of thiols to vinyl-substituted bilins (*47*, *48*). However, no 3-vinyl chromophore has yet been isolated from the respective biliproteins. They can not be cleaved off the protein by the methods used both for PCB and PEB and for c-type cytochromes. We have, for example, been unable to cleave the PVB chromophore at all from PEC, even by methods used to cleave heme from the c-type cytochromes (*49*), which show a similar binding situation, and we are not aware of a report on such cleavage in any of the other biliproteins containing the putative 3-vinyl chromophores.

Recent studies with phycoerythrocyanin (PEC) indicated a possible way out of this problem. PEC carries a chromophore of the second type on the α-subunit, viz., 3¹-Cys-α84-PVB (Figure 2, see footnote 1 for nomenclature). PEC is a light-harvesting pigment and a component of the phycobilisome in certain filamentous cyanobacteria, in addition it shows a photoreversibly photochromic behavior which is reminiscent of the sensory biliproteins, the phytochromes (see refs *19* and *50*−*52* for review). This intermediate situation as well as the unusual chromophore made it an interesting object to investigate pigment−protein interactions in biliproteins, including dynamic interactions which govern the photophysical properties of the chromophores. When the respective two lyase genes, *pecE* and *pecF*, were overexpressed in *Escherichia coli*, the crude extracts showed a remarkable reactivity. They did not only catalyze the chromophore attachment to Cys-84 of PecA, but surprisingly their substrate was PCB which in the attachment process was isomerized to yield 3¹-Cys-α84-PVB (*33*). This result might suggest, that other addition products of type 2-chromophores (e.g. 3¹-Cys phycourobilin) may also result from the addition of type 1 chromophores (PEB in the given case) and a concomitant isomerization. To further characterize this new enzyme, we now report the purification of the overexpressed lyase subunits, their interactions with PCB, details of the products of the PCB addition/isomerization, and the pigment specificity of the PEC-Cys-α84 PCB lyase/isomerase.

MATERIALS AND METHODS

Clones. Work has been carried out in parallel with two strains of *Mastigocladus* (*M.*) *laminosus*, which internally have been termed "Munich" and "Wuhan". The genes *pecA*, *pecE*, and *pecF* were PCR-amplified from both strains. The three sequences of the "Munich" strain were identical to those reported for *Fischerella* PCC7601 (= *M. laminosus*) (*53*, *10*, *11*), those for the "Wuhan" strain showed several differences in each gene. A detailed characterization of the two strains, which also show some physiological and morphological differences, is in progress and will be published separately. *Sam*I−*Xho*I fragments of *pecA*, *pecE, and pecF* from the "Wuhan" strain were cloned first into pBluescript pUC19, and then transferred into pGEMEX (Promega). *E. coli* BL21(DE3) was transformed with the resulting plasmids pGEMEX-*pecA*, pGEMEX-*pecE* and pGEMEX-*pecF*, to overexpress separately PecA (= apo-α-PEC), PecE, and PecF, respectively. On account of the plasmids used, all resulting proteins carry an N-terminal extension of 8 amino acids (MEQNPQSQ). All fragments (*pecA*, *pecE*, and *pecF*) from the "Munich" strain, as well as *pecA* of the "Wuhan" strain, were cloned into a pET30 plasmid (Novagen), resulting after expression in *E. coli* BL21(DE3) in the respective proteins with N-terminal extensions containing a His- and a S-tag, plus two protease sites for thrombin and enterokinase. All constructions were verified by sequencing.

Proteins. *(i) E. coli Cells.* One-liter cultures of *E. coli* were grown at 37 °C in rich medium (1% tryptone/0.5% yeast extract/0.5% NaCl/0.2% glucose, pH 7.5) containing kanamycin (30 μg/mL for pET30 containing strains), or ampicillin (100 μg/mL) for the pGEMEX containing strains. At an OD_{600} of 0.45−0.5, isopropyl-β-D-thiogalactopyranoside was added (1 mM). Cells were collected by centrifugation 3 h later, washed twice with distilled water, and kept frozen at −20 °C.

(ii) Crude PecA (apo-α-PEC), PecE, PecF. Frozen cells were resuspended in Tris-HCl buffer (50 mM, pH 6.5) sonified, and centrifuged (20.000*g* for 30 min). The supernatant was dialyzed against the same buffer and stored at −20 °C until use.

(iii) His-tag-PecA (Apo-α-PEC), His-tag-PecE, and His-tag-PecF with N-Terminal Extensions Containing a $(His)_6$-tag and a S-tag, Plus Two Protease Sites for Thrombin and Enterokinase. For activity tests, frozen cells were resuspended in Tris-HCl buffer (50 mM, pH 6.5), sonified, and then centrifuged at 20.000*g* for 30 min to yield clear supernatant which was used directly in the reconstitution experiments. For preparative experiments, His-tagged proteins were purified via Ni-affinity chromatography on chelating Sepharose (Amersham-Pharmacia) according to the suppliers protocol, using columns and elution with imidazol (1 M), and then dialyzed against Tris-HCl buffer (50 mM, pH 6.5). The same procedure was also used to purify His-tagged PecA after ligation with chromophores. The separation of PecA carrying different chromophores was done by chromatography on Biogel P10 (Bio-Rad, Richmond, CA).

Pigments. Phycocyanobilin (PCB) was obtained by refluxing methanol-washed *Arthrospira* (= *Spirulina*) *platensis* (lyophilised, W.Behr, Bonn) in methanol for 16 h (*54*). PCB was purified by flash chromatography on silica RP8 (ICN) (50 mM potassium phosphate buffer, pH 2.1 60%, 2-propanol 40%) (modified from ref *17*). Forty grams of lyophilized bacteria yielded ∼60 mg of PCB (55% yield with respect to the phycocyanin plus allophycocyanin contained), and ∼27 mg (25%) after purification.

Phycocyanobilin-dimethylester (PCB-DME) was prepared by refluxing PCB in boron trifluoride in methanol (5% w/v) for 20 min. The solvent was evaporated by a stream of nitrogen, and the PCB-DME was purified on silica gel ($CHCl_3$/2-propanol = 98:2) (*40*, *55*).

Phycoerythrobilin (PEB) was isolated from partially purified phycoerythrin from *Porphyra yezoensis* by refluxing in methanol for 16 h (*54*) and purified by flash chromatography on silica RP8 (ICN) (50 mM potassium phosphate buffer, pH 2.1 70%, 2-propanol 30%) (modified from ref *17*). Phycoerythrobilin-dimethylester (PEB-DME) was a gift of Prof. A. Gossauer, Fribourg.

Phytochromobilin (PΦB) was prepared from PEB by oxidation with $FeCl_3$ (M. Maisch and W. Rüdiger, private communication) and purified like PCB.

Biliverdin (BV) was prepared from commercial bilirubin (BR) (Serva) by oxidation with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone in dimethyl sulfoxide for 30 min. BV was purified on silica plates with the upper phase of toluene−acetic acid−water (5:5:1) (*44*, *56*, *57*).

Reconstitution/Ligation. To PecA [alone or in combination with the supernatant(s) containing PecE and/or PecF] in Tris-HCl buffer (50 mM, pH 6.5) containing mercaptoethanol (5 mM) and sometimes (depending on the isolation procedure, see Results) Triton X-100 (0.2%), was added a solution of the phycobilins in DMSO (1 mM), such that the final DMSO concentration was 1%, and the final phycobilin concentration in the reconstitution mixture was 10 μM. After incubation in the dark at ambient temperature for the period detailed in the results, the mixture was centrifuged for 15 min at 15000*g* to remove any particulate matter, and the supernatant investigated by UV−vis absorption (Perkin-Elmer, Lambda2), fluorescence (Hitachi, F-2000) and light-induced absorption changes. For the latter, an actinic cold-light source (Volpi, 150 W) was used, equipped with interference filters (10 nm fwhm) of the suitable wavelength. Photochemistry is expressed in $\Delta\Delta A_{xxx/yyy}$-units as defined in ref *1*. Briefly, it is defined as the amplitude of the S-shaped difference signal ($\lambda_{max} = xxx$ and *yyy* nm) relative to the maximum absorption. In cases where the His-tagged PecA was used, the resulting chromoprotein was purified via Ni-affinity chromatography (see above). If necessary (e.g., for fluorescence or activity tests), the sample was dialyzed against phosphate buffer (50 mM, pH 7). PCB- and PVB-containing reconstitution products were separated by chromatography on Biogel P10 (Bio-Rad, Richmond, CA) under the conditions used for subunit preparation by Füglistaller et al. (*49*). The PVB-containing proteins are not retained on the column and elute in a spectrally nearly pure form, while PCB-containing proteins are retained and elute only with 30% acetic acid.

SDS−polyacrylamide gel electrophoresis was performed with the buffer system of Laemmli (*58*) at an acrylamide: bis-acrylamide ratio of 37.5:1, and concentrations of 5 and 15% for the stacking and resolving gels, respectively. The gels were stained with zinc acetate for fluorescence detection of bilin chromophores (*59*) and/or with Coomassie brilliant blue R for the protein.

Extinction Coefficients. Three methods were used. In each case, the absorption spectrum of a solution of the reconstitution product was recorded at the beginning. Method 1: after treatment with TPCK-treated trypsin (3 × 1 mg over a period of 16 h, ambient temperature, pH 6.5), and subsequent acidification to pH 1.5 with HCl, the spectrum was again recorded. Method 2: the chromoprotein was acidified with HCOOH (1%) and treated with pepsin (3 × 5 mg over a period of 16 h, ambient temperature). Method 3: the chromoprotein was denatured by addition of urea (8 M) and HCl (pH 1.5). From the known extinction coefficients of the cations of 3^1-Cys-PCB- (*60*), -PVB (*61*), -PEB (*62*, *63*), and -PΦB-peptides (*35*) (in 8 M urea/HCl, that of the 3^1-Cys-PVB peptide in TFA), and the dilution during proteolysis/denaturation and acidification procedure, those of the reconstitution products were determined to 61×10^3 $M^{-1}cm^{-1}$ (PCB adduct), 77×10^3 M^{-1} cm^{-1} (PVB adduct), 66×10^3 M^{-1} cm^{-1} (PEB adduct) and 56×10^3 M^{-1} cm^{-1} (PΦB adduct).

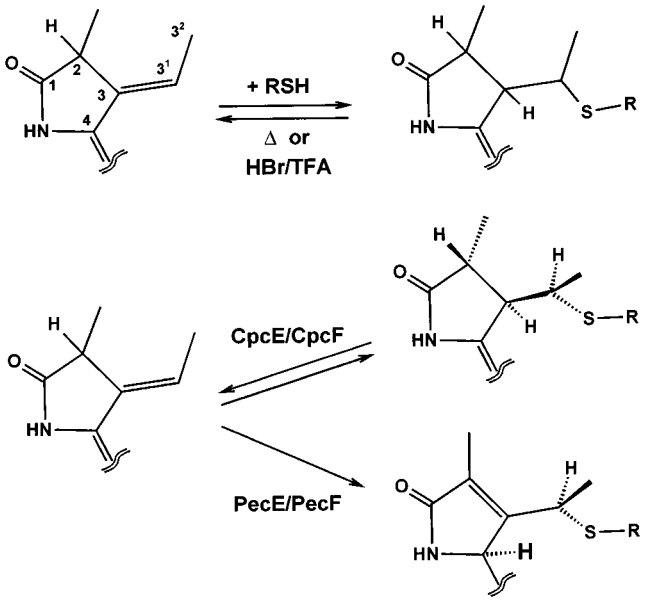

FIGURE 3: Addition reactions of PCB to biliprotein α-subunits. (Top) Spontaneous ("autocatalytic") reaction and its chemical reversion; center: reversible enzymatic ligation catalyzed by PCB-PC-Cys-84α lyase (CpcE/CpcF); (bottom) irreversible enzymatic ligation-isomerization catalyzed by PCB-PEC-Cys-84α lyase-isomerase (PecE/PecF).

RESULTS

The apoproteins of the PEC α-subunit (PecA), and of the two subunits of the PCB−PEC lyase/isomerase (PecE and PecF) were overexpressed in two forms: (1) with a short N-terminal extension (MEQNPQSQ) introduced by the vector, and (2) with an N-terminally positioned extension containing a His-Tag, an S-tag, and two specific protease sites, one between the two tags (thrombin) and the other between the tags and the enzyme proper (enterokinase). The His-tag has been introduced to simplify purification via metal affinity chromatography. The S-tag was attached as an alternative binding site for affinity chromatography with the viral S-protein. Free bilins are suceptible to rapid oxidation by heavy metals (*6*), traces of which might interfere with the ligation of proteins purified over chelating columns. However, no such loss of chromophore was observed, and therefore only the His-tag has been used in this work for affinity chromatography. The cleavage sites have been introduced in case the tags might interfere with the ligation reaction, but neither of these have been used. There was no evidence for any interference of the N-terminal extensions, and the solubility and reactivity of the tagged proteins was generally better than that of the untagged ones (see Figure 5, panels c and d). The overexpressed proteins are deposited mainly in inclusion bodies, but can be well solubilized by extended sonication. This method results in superior activities

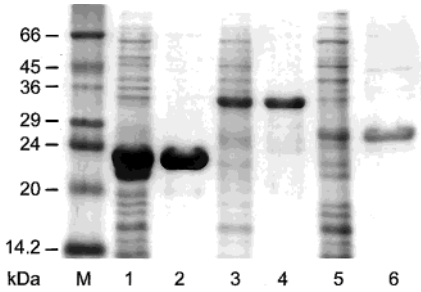

FIGURE 4: SDS−polyacrylamide gels of His-tag-PecA, His-tag-PecE and His-tag-PecF overexpressed in *E. coli* and purified by metal-affinity chromatography. Sample loads: Lane M, marker proteins (Dalton Markers VII-L, Sigma, Taufkirchen); lane 1, supernatant from lysis of *E. coli* expressing His-tag-PecA; lane 2, purified His-tag-PecA; lane 3, supernatant from lysis of *E. coli* expressing His-tag-PecE; lane 4, purified His-tag-PecE; lane 5, supernatant from lysis of *E. coli* expressing His-tag-PecF; lane 6, purified His-tag-PecF.

and is therefore preferable over the treatment with urea and/or detergents (see however the positive effect of TX-100).

Many reconstitution experiments have been carried out with crude extracts, obtained as supernatants of centrifuged, sonicated cell lysates. They gave qualitatively the same results irrespective of the presence or the absence of the tags (see Figure 5, panels c and d). Since the tagged proteins were much more readily purified by metal affinity chromatography (Figure 4), these were used for most preparative procedures and for experiments with purified proteins. Typical yields were 100−150 mg of purified proteins from 1 L of *E. coli* culture. To optimize the reconstitution, the influence of several additives and cofactors was tested on the spontaneous addition of PCB to PecA, and on the ligation catalyzed by PecE and PecF in a 1:1 ratio, which are detailed below.

Spontaneous Addition of PCB to PecA. Incubation of the cell extract containing PecA with PCB ($\lambda_{max} \approx 618$ nm in the reconstitution buffer system) resulted in an absorption increase at 641 nm (Figure 5a) and the induction of a red fluorescence ($\lambda_{max,emission} = 660$ nm, see Figure 6a) which is already visible with the naked eye. The addition reaction is independent of the presence or absence of EDTA or the addition of divalent metals, and inhibited by mercaptoethanol (5 mM). No dialyzable factors were needed of the *E. coli* supernatant, because the addition reaction was not affected by dialysis prior to the incubation with PCB. The optimum temperature is in the range of 20−30 °C, the optimum pH at 6.5−7.0. The same product is formed from untagged and

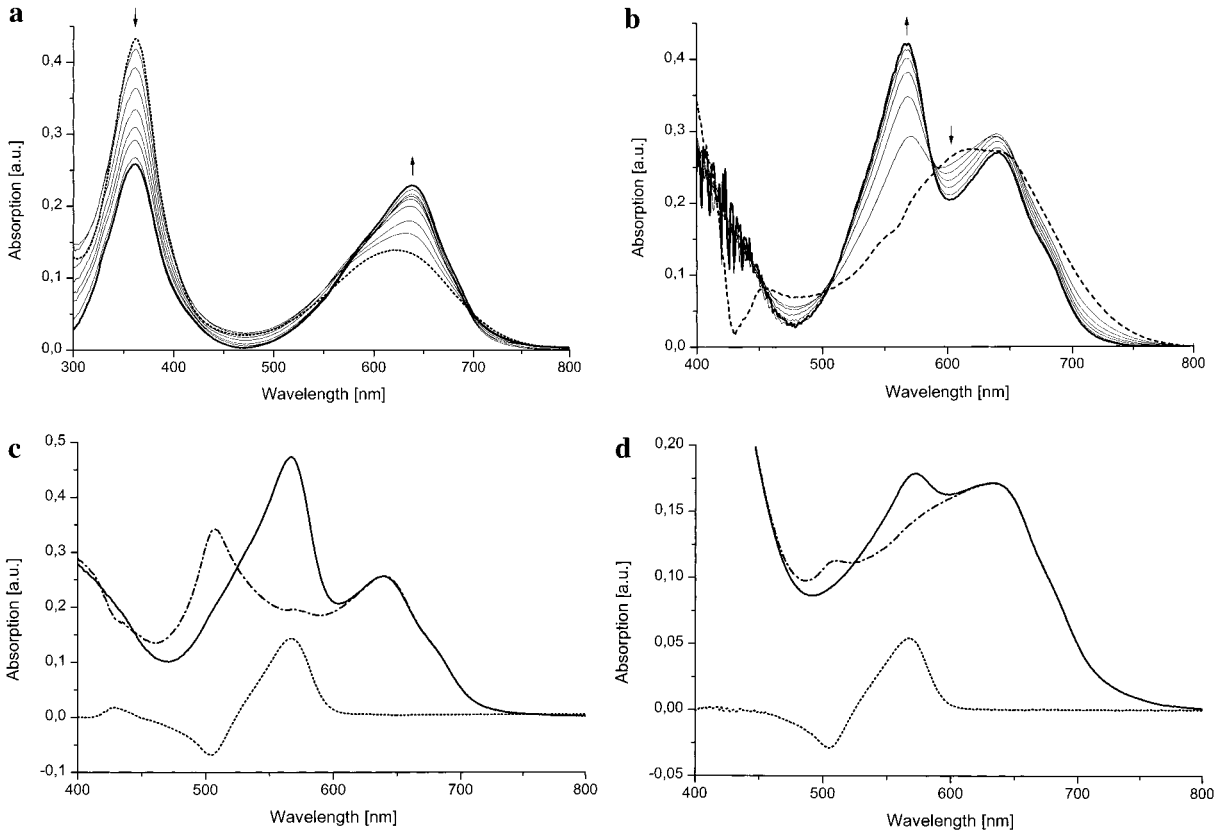

FIGURE 5: (a) Spontaneous addition reaction of PCB to PecA (apo-α-phycoerythrocyanin, crude extract of overexpressing *E. coli*) followed by absorption spectroscopy. The original spectrum after preparation of the reaction mixture (- - -) and the final spectrum after 24 h incubation (—) are traced in bold, all intervening spectra (taken at 3 h intervals) are traced in thin lines. The direction of the absorption changes are indicated by the arrows. The reference cell contained the same cell lysate of *E. coli* overexpressing PecA, but with PCB omitted. (b) PecE/PecF-catalyzed addition reaction of PCB to PecA (apo-α-phycoerythrocyanin) followed by absorption spectroscopy. The original spectrum after preparation of the reaction mixture (- - -) and the final spectrum after 16 h incubation (—) are traced in bold, all intervening spectra (taken at 2.5 h intervals) are traced in thin lines. The reaction was carried out with the crude extracts of the respective overexpressing *E. coli* strains. The reference cell contained the same cell lysates of *E. coli* overexpressing PecA, PecE, and PecF, but with PCB omitted; the noise <450 nm is due to strong scattering in both the sample and reference cells. (c) Reversible photochemistry (type I) of a crude extract of *E. coli* overexpressing His-tag PecA after reconstitution with PCB in the presence of PecE and PecF (crude extracts). Absorption spectra of the reaction mixture after irradiation with 500 nm light (—) and 570 nm light (— · — · —), and difference spectrum (···). (d) Reversible photochemistry of a crude extract of *E. coli* overexpressing untagged PecA after reconstitution with PCB in the presence of PecE and PecF (crude extracts). Labels as in panel c.

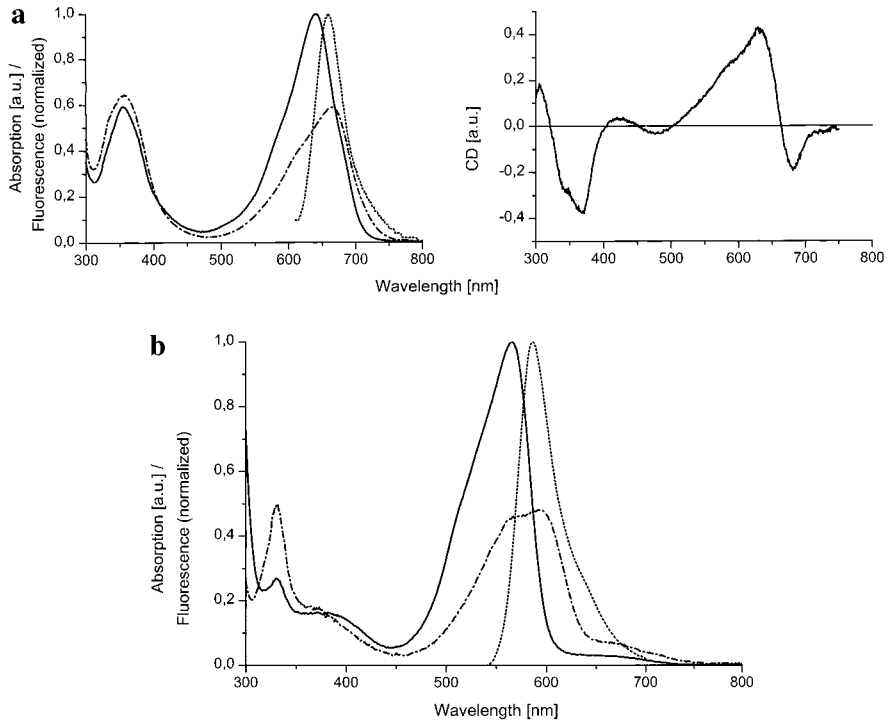

FIGURE 6: (a) Purified spontaneous addition product of PCB to PecA (apo-α-phycoerythrocyanin). (Left) Absorption (—) and fluorescence spectra (···) of the native chromoprotein, and absorption of the denatured chromoprotein (8 M urea) at pH 1.5 (— · — · —), (right) CD spectrum, (b) purified PecE/PecF-catalyzed addition product PCB to PecA (apo-α-phycoerythrocyanin). Absorption (—) and fluorescence spectra (···) of the native chromoprotein, and absorption of the denatured chromoprotein (8 M urea) at pH 1.5 (— · — · —) see Figure 7 for the CD spectrum.

His-tagged PecA, if judged from absorption and fluorescence spectroscopy and photochemical activity of the chromoprotein and of the derived chromopeptides. The adduct with His-tagged PecA was purified by two different methods, viz., Ni-affinity chromatography or chromatography on Biogel P10 (see below), with identical results. The absorption spectrum of the purified protein fraction has the characteristics of a native biliprotein (Figure 6a, ref *64*): there is an intense absorption in the visible spectral range ($\lambda_{max} = 641$ nm) and a smaller absorption band in the near UV ($\lambda_{max} = 354$ nm). Compared to free bilins, the relative intensities of these two bands are inverted in the adduct (*6*, *35*). The absorption ratio (1.64) is however, less than that of native α-PC, which has a ($Q^{A}_{vis/UV} \approx 5$), with the exact value depending on the aggregation state, pH, etc. (*6*). This low value probably reflects the fact that PCB is not the native chromophore of α-PEC, and therefore the interactions with the apoprotein are less evolved. Covalent binding of the chromophore was verified by SDS−PAGE and subsequent fluorescence detection of the bilin-chromophore as the Zn-complex (*59*). The colored protein migrated with an apparent MW of 23K (calcd 23 324), and an orange fluorescence was induced by treatment with zinc-acetate (Figure 7).

Arciero et al. (*20*) have identified the major spontaneous addition product of CpcA (= apo-α-PC) from *Synechococcus* PCC 7002 as a $18^1,18^2$-dihydrobiliverdin (3^1-Cys-DBV in Figure 2). In the native protein, it absorbs at 650 nm, while native α-PC absorbs around 620 nm. The red-shift of the CpcA-adduct is due to the additional conjugated Δ2,3 double bond (*17*, *20*). The absorption of the spontaneous addition product of PCB with PecA absorbs between these two positions. Since the absorptions of 3^1-Cys-PCB can be considerably modified by noncovalent interactions with the apoprotein (*64*), the product was investigated under conditions where the chromophore is uncoupled from the protein by denaturation in acidic urea. Under these conditions, the absorption maximum at 663−664 nm is nearly identical to that of denatured PC ($\lambda_{max} = 665$ nm), and ∼26 nm blue-shifted as compared to 3^1-Cys-DBV (*17*). The absorption bands of phycobilins are generally rather broad and even for the somewhat narrower and more intense cations formed with acid, the maxima can only be given at best to ±1 nm. However, within this uncertainty, the positions of these maxima are independent of the acid used (HCl, TFA) at pH

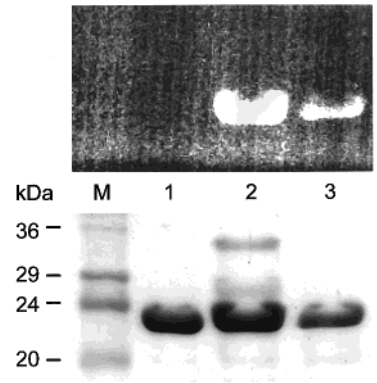

FIGURE 7: SDS−PAGE of Apo-α-PEC (PecA) and reconstituted α-PEC stained for the chromophore with zinc acetate (top) (59) and for protein with Coomassie blue (bottom). The gel was first photographed under ultraviolet light (fluorescence, top), and subsequently stained with Coomassie blue (bottom). Sample loads: Lane M: marker proteins (Dalton Markers VII-L, Sigma); lane 1, purified His-tag-PecA; lane 2, purified reconstitution product of His-tag-PecA with PCB *plus* His-tag-PecE/His-tag-PecF; lane 3, purified reconstitution product of His-tag-PecA with PCB.

Table 1: Comparison of Spontaneous Addition Products of PCB to PecA with C-Phycocyanin: Absorption Maxima of Denatured Chromoproteins and of Peptic peptides

λ_{max} (nm)	conditions
664	purified chromoprotein (His-tagged), in 8 M urea, 1% HCOOH
664	purified chromoprotein (His-tagged), in 8 M urea/HCl (pH 1.5)
657	peptic digest of purified chromoprotein (His-tagged) in HCl (pH 1.5)
657	peptic digest of purified chromoprotein (His-tagged) in 1% HCOOH
662	purified peptide from digestion of chromoprotein (His-tagged) with TPCK-treated-trypsin at pH 6.5, measured in HCl (pH 1.5)
662	purified peptide from digestion of chromoprotein (His-tagged) with TPCK-treated-trypsin at pH 6.5, measured in 1% HCOOH
665	PC in 8 M urea/HCl (pH 1.5)
662.5	PC in 8 M urea/HCl (pH 1.5 and 3.0) (*60*)
665	PC in 8 M urea/HCl (pH 2) (*35*)
655	PC in 30% acetic acid (*87*)
660	PC-peptide (30% acetic acid) (*87*)
665	PC-peptide (HCl, pH 2) (*35*)
648	PC-peptide (10 mM TFA) (*17*)

<2 and also independent of whether the chromoprotein has been denatured by 8 M urea or proteolytically degraded (Table 1). The absorption data therefore clearly indicate that 3^1-Cys-PCB rather than 3^1-Cys-DBV is formed by the spontaneous addition of PCB to PecA. This conclusion is supported by peptic degradation of the addition products, whereby the chromophore is also largely (*65*) uncoupled from the protein. The chromopeptides absorb again close to those obtained from PC and at much shorter wavelengths than DBV-derived chromopeptides (Table 1). Finally, the chromoprotein was treated with HBr/TFA (*36*). It yielded a single free bilin which by absorption and HPTLC (silica-RP8) was identical to PCB and in a yield (65%) which is only little less than that obtained with PC trimer (75%). We therefore conclude that the main product of the spontaneous addition of PCB to PecA carries a 3^1-Cys-PCB chromophore which is characteristic of PC and APC, and not the 3^1-Cys-DBV chromophore.

Minor, but distinct photochemical activities were observed in the spontaneous reconstitution product. Irradiation with red light (639 nm) induced a bleaching at 641 nm and an increased absorption at the red-wing (Figure 8). Similar to the assay of PEC activity (*1*), the amplitude of the difference signal was normalized to the absorption at the 641 nm maximum: it amounts to $\Delta\Delta A_{641/682} = 9.4\%$. The same difference bands were observed upon irradiation of the chromoprotein at 572 nm, but the signal was smaller ($\Delta\Delta A_{641/682} = 5.5\%$), and they were accompanied by an additional difference peak at 572−579 nm ($\Delta A_{578}/A_{641} = 3\%$). The latter is fully reversible upon subsequent irradiation at 639 nm, while the changes at $\lambda > 600$ nm are only partly reversible (appx. 60%). This indicates the presence of two photochemical signals, an irreversible bleaching which is superimposed on a small but distinct red (641 nm)/green (576 nm) photoreversibly photochromic signal. It is known that most phycobilins exhibit small photoreversible photochemistry if the protein is partly unfolded, but the amplitudes are generally only small (*66*, *6*). The comparably large signals under environmental conditions which favor native biliproteins (pH 6.5−7, ambient temperatures, no urea) may reflect the fact that the binding pocket of α-PEC is not optimized for 3^1-Cys-PCB, but rather for 3^1-Cys-PVB. This is supported by "curing" experiments: The product was first unfolded with 8 M urea, and then allowed to refold slowly by dialysis against a buffer free of urea. Under these conditions, any photochemistry of PC or its subunits is abolished, while the photochemistry of the spontaneous addition product persisted.

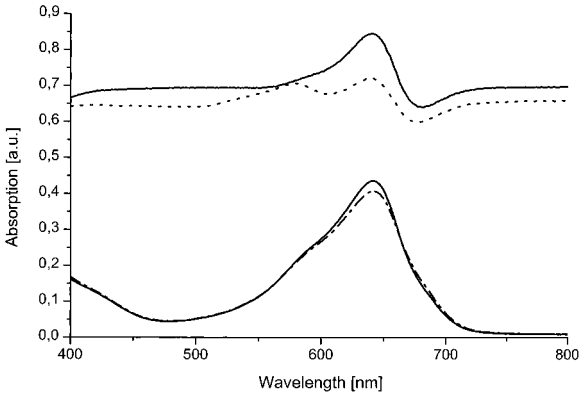

FIGURE 8: Reversible photochemistry of the spontaneous addition product of PCB to PecA. Absorption spectra of affinity-purified chromoprotein obtained by addition of PCB to His-tagged PecA in the absence of ligase (PecE/F) before (—) and after (— · — · —) irradiation with 639 nm light (bottom), and difference absorption spectra (before *minus* after irradiation) of an irradiation at 639 nm (—) and at 572 nm (- - -) (top).

Ligation-Isomerization of PCB to Cys-α84, Catalyzed by PecE and PecF. When PCB and PecA are incubated with crude lysates of *E. coli* overexpressing PecE and PecF, there is only a minor increase of absorption at 641 nm. Instead, there is a gradual increase of absorption at 565 nm at the expense of the 618 nm absorption of PCB, which continued over 16 h (Figure 5b). Native α-PEC absorbs at 565 nm and shows a pronounced photoreversible photochromism [$\Delta\Delta A = 100-110\%$ (*67*)]. On the basis of these criteria, we have previously suggested that the product of the reaction catalyzed by PecE/F carries the native phycoviolobilin chromophore (*33*). While previous work involved crude extracts only, the chromoprotein has now been purified from excess chromophore and from the contaminating spontaneous addition product, by chromatography on Biogel P10. This polyacrylamide material is normally used for gel filtration, but acts here as a selective adsorbent which retains the free chromophores as well as the spontaneous addition product carrying the 3^1-cys adduct of PCB, which may be incompletely folded (see above). The major fraction of the reaction mixture is not adsorbed, however, and elutes as a purple chromoprotein which by the following criteria is identical to α-PEC. (1) Its mobility on SDS−PAGE is identical to that of authentic α-PEC (not shown). (2) Its absorption and fluorescence spectra are within the limits of error identical to the spectra of an authentic sample of native α-PEC (Figure 6b). (3) Native α-PEC shows a characteristic photochromic signal, which relates to a 15Z-15E isomerization of the 3^1-Cys-PVB chromophore. The isolated chromoprotein shows qualitatively and quantitatively the same signal (Figure 9a), proving that the chromophore of the reconstituted α-subunit has full type I photoactivity, and showing at the same time that the N-terminal His-tag does not interfere with the native folding (see also Figure 5, panels c and d). (4) The

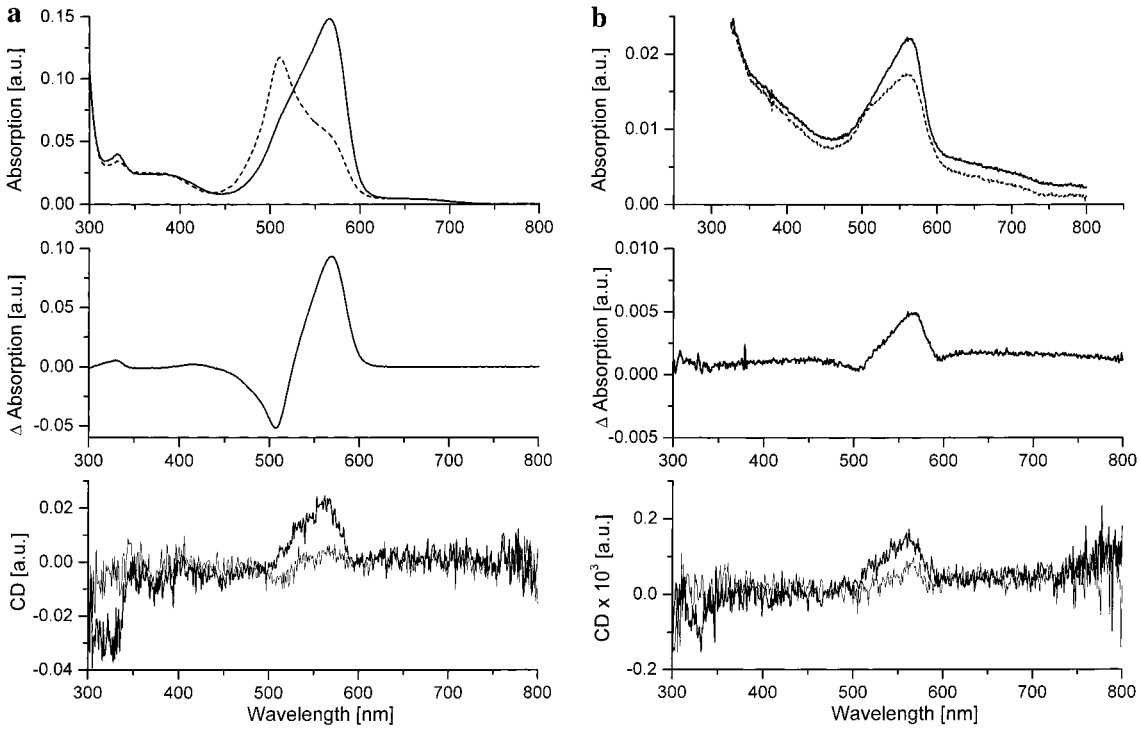

FIGURE 9: Reversibe photochemistry of the PecE/PecF catalyzed addition product of PCB to PecA. (a) Type I photochemistry of the purified chromoprotein obtained by addition of PCB to His-tagged PecA in the absence of ligase (PecE/F). (Top) Absorption spectra after preirradiation with 500 nm light (—) and after subsequent irradiation with 570 nm light (- - -), (center) difference absorption 500 nm irradiation *minus* 570 nm irradiation, (bottom) CD spectra after preirradiation with 500 nm light (bold line) and after subsequent irradiation with 570 nm light (thin line), (b) Type II photochemistry of the purified chromoprotein in the presence of PCMS (0.06 mM). (Top) Absorption spectra after preirradiation with 500 nm light (—) and after subsequent irradiation with 570 nm light (- - -), (center) difference absorption 500 nm irradiation *minus* 570 nm irradiation, (bottom) CD spectra after preirradiation with 500 nm light (bold line) and after subsequent irradiation with 570 nm light (thin line).

photochemistry of PEC depends on the status of Cys-98/99: upon treatment with PCMS, another photoreversible photochromism is observed (type II). The same behavior is seen for the adduct: The two forms connected by the type II photochemistry show absorptions with $\lambda_{max} = 563$ and 560 nm, and an amplitude $\Delta\Delta A_{564/597} = 20\%$ (Figure 9b), which qualitatively and quantitatively agrees with the respective signals of an authentic sample. (5) The CD spectra of both the 566/507 nm forms (Figure 9a) and the 563/560 nm forms (Figure 9b) were identical to those of an authentic sample (*68*). Accordingly, we conclude that under the action of PecE and PecF, the PCB chromophore is ligated to Cys-84 of PecA in a regio- and stereoselective fashion to yield integral α-PEC.

To optimize the reaction conditions, the addition/isomerization reaction was assayed using the following criteria: (i) the ratio $Q^{A}_{565/641}$ of absorption at 565 (PVB-adduct) over that at 641 nm (PCB-adduct), (ii) the kinetics of the rise of the 565 nm absorption, and (iii) the amplitude of the reversible photochemistry [type I, (*67*)]. According to all three criteria, reconstitution requires mercaptoethanol (5 mM), and either of the divalent metals, Mg^{2+} or Mn^{2+}, which can only partly be substituted by Ca^{2+}. The most characteristic effects on the amplitude of the reversible photochemistry are summarized in Table 2, a full enzymological characterization is in progress. Despite the requirement of divalent ions, the reaction is not influenced by EDTA <50 mM, indicating a high affinity of the lyase for the metals. Possibly due to the requirement of Mg^{2+} or Mn^{2+}, Tris-HCl buffer was favorable over phosphate buffer, although a mixture containing Tris (2/3) and potassium phosphate (1/3) was tolerated. Since the solubility and stability of PecA and PecF were better in phosphate buffer, most reconstitutions were carried out in this mixture. The reconstitution can be improved by the addition of TX-100 at concentrations of 0.2−1%. This is not due to speeding up of the ligation-isomerization, but to inhibition of the spontaneous addition reaction (not shown). The presence of O_2 is of no influence on the reconstitution kinetics. However, PCB is rather sensitive to oxygen, in particular at pH >7 in the presence of metal ions (*69*), which leads to partial loss of the free chromophore during the relatively long incubation. The optimum pH is in the same range as for the spontaneous addition (6.5−7.5), the temperature optimum is increased to 37 °C. Temperatures of 37 °C and a pH 7 were therefore generally used for the subsequent tests. They have not been

Table 2: Relative Lyase-isomerase Activity of the $(His)_6$-tagged-PecE and $(His)_6$-tagged-PecF with Different Cofactors[a]

conditions	relative activity (%)
0 mM of ME	0
5 mM ME	21
5 mM ME, 3 mM Mn^{2+}	100
5 mM ME, 10 mM Mn^{2+}	13
50 mM ME, 5 mM Mn^{2+}	42
5 mM ME, 2.5 mM Mg^{2+}	58
5 mM ME, 10 mM Mg^{2+}	74

[a] The activities have been determined from the amplitude photoreversible signal after 15 min of the reaction time, that of the current optimum conditions (5 mM ME, 3 mM Mn^{2+}) has been set to 100%.

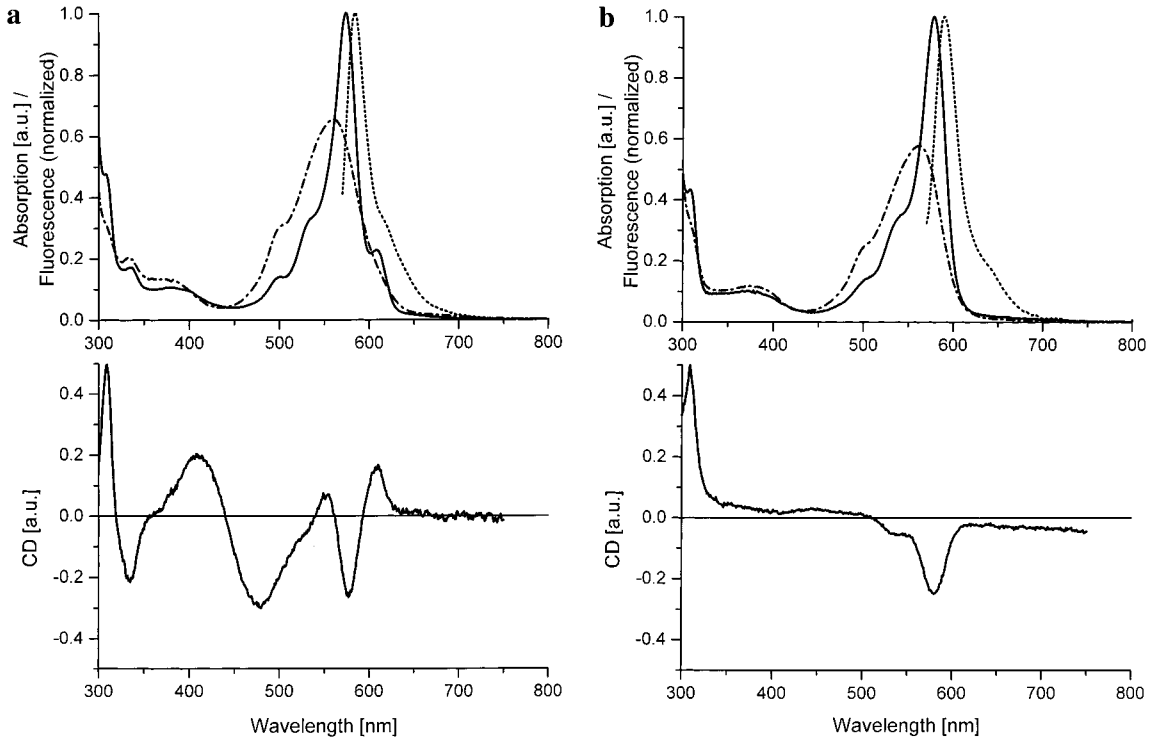

FIGURE 10: Products of spontaneous addition of PEB (a) and PEB-DME (b) to PecA (apo-α-phycoerythrocyanin) after purification via Ni-affinity chromatography. (Top) Absorption (—) and fluorescence spectra (•••) of the native chromoprotein, and absorption of the denatured chromoprotein (8 M urea) at pH 1.5 (— • — • —), (bottom) CD spectrum.

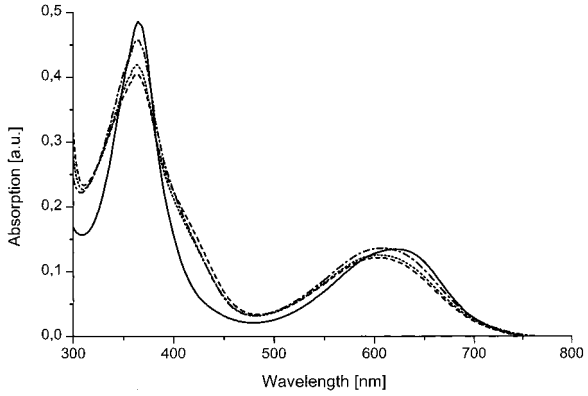

FIGURE 11: Interaction PCB and PecE/PecF. Absorption spectra of free PCB in the reconstitution buffer (—), and of the same solution after addition of PecE (— • — • —), PecF (•••) and PecE *plus* PecF (- - -).

optimized further, and optimum conditions also seem to vary with the proteins used and other details.

The next question studied was if there is any interaction of PecE or PecF with PCB. Incubation of free PCB with either lyase protein alone, or with both of them, resulted only in small changes of the spectrum of the incubation mixture (Figure 11). The long-wavelength band is shifted by 10−20 nm to the blue, and the near-UV maximum is broadened. However, there is always an increase in the 420 nm region, leading to a distinct shoulder. It is well-known (*48*, *70*) that thiols can add to the central methine-bridge of bilins, resulting in bilirubin-type pigments absorbing in the 420 nm region. The shoulder at 420 nm may then indicate the formation of such a bilirubin-type pigment, e.g., by addition of a thiol group from the lyase subunit(s) to C-10 of PCB. These data indicate that PCB is transiently bound to either or both of the lyase subunits, via addition to the central methine bridge. Both lyase subunits (PecE, PecF) carry more than one cystein, but there is only a single consensus cystein (PecE Cys-91) with other homologous biliprotein α-84 lyases, which shall be tested by mutagenesis experiments.

Substrate Specificity. Several chromophores were tested as substrates for the reconstitution (Figure 1). These are two vinyl bilins, viz., phytochromobilin (PΦB, which differs from PCB only by the C-18 substituent, and PEB, which also lacks the Δ15,16 double bond), two pigments, biliverdin (BV) and bilirubin (BR), carrying a Δ2,3 double bond and a 3-vinyl group, and two pigments doubly esterified at C-13^3 and C-17^3, viz., PCB-DME and PEB-DME. The latter are insoluble in water, as is BR, but were solubilized by DMSO. The spectroscopic analyses of the incubations of these pigments with PecA, in the presence and absence of PecE and PecF, are summarized in Table 3.

While PCB-DME, BR, and BV do not bind covalently to the apoprotein, spontaneous ligation takes place with PCB, PEB, PEB-DME, and PΦB. The products are heterogeneous in each case if judged from the appearance of shoulders (Figure 10, Table 3). The structure assignments in Table 3 are tentative in most cases, however, and based only on the absorption spectra, but it is clear that despite the binding of these chromophores, the reaction is quite heterogeneous.

Only two of the chromophores which bind spontaneously are recognized by the lyase. These are PCB and its 18-vinyl analogue, PΦB. In all other cases, the reaction is basically unchanged by the presence of PecE and PecF. As compared to the absorption maximum of the PVB adduct arising from ligation-isomerization of PCB, the corresponding product of PΦB absorbs 10 nm to the red at 651 nm. This shift difference corresponds to the one observed in plant (*71*−*74*) and cyanobacterial phytochromes (*19*, *51*, *78*), it also corresponds roughly to the spectral differences among the

Table 3: Reaction of Free Bilins with PecA (= apo-α-PEC) in the Presence (+) and Absence (−) of the Lyase, PecE/PecF[a]

components in reconstitution			reconstitution	chromophores bound to reconstituted system	
Apo-α-PEC (PecA)	lyase (PecE/PecF)	chromophore	duration of reconstitution (h)	structure	absorption λ_{max} (nm) ($\epsilon \times 10^{-3}$ (M^{-1} cm^{-1})) {$\Delta\Delta A$}
+	−	PCB	18	3^1-Cys-PCB (+ 2 byproducts)	642 (61) {<10%, Figure 8}
+	+	PCB	12	3^1-Cys-PVB (+ 3^1-Cys-PCB)	567 (77) {102%}
+	±	PCB-DME	− −	− −	− −
+	−	PΦB	18	3^1-Cys-PΦB (+ 2 byproducts)	651 (56) {n.d.}
+	+	PΦB	12	3^1-Cys-18-vinyl-PVB (+ 3^1-Cys-PΦB)	577 {94%}
+	±	PEB	1	3^1-Cys-PEB (+ 3 byproducts)	574 (66) {< 1%}
+	±	PEB-DME	4	3^1-Cys-PEB-DME (+ 2 byproducts)	579 (75) {< 1%}
+	±	BV	− −	− −	− −
+	±	BR	− −	− −	− −

[a] All reactions were carried out with the crude extracts of the respective over expressing *E. coli* strains. Reactions are considerably faster with the isolated lyases (see text). See text for abbreviations. the reversible photochemistry has been characterized by the amplitude difference $\Delta\Delta A$ of the sigmoid feature in the difference spectrum (*1*). − − Indicates no reaction.

free bilins, PCB, and PΦB (*35*). Since the addition product of PΦB absorbs at much longer wavelengths, it is clear that this chromophore must be isomerized, too, during the ligation catalyzed by PecE/PecF.

DISCUSSION

Biosynthesis of the phycobiliprotein chromophores (*2*, *13*) has been studied in particular in the eucaryotic alga, *Cyanidium caldarium* (*Galdieria sulfuraria*) (*79*, *80*). Here, cleavage of heme and subsequent reduction produce a dihydrobiliverdin, which is first isomerized to 3,3^1Z-phycoerythrobilin, then to 3,3^1Z-PCB and finally to 3,3^1E-PCB. Some of these intermediates have also been identified in cyanobacteria, while alternative pathway(s) are discussed in higher plants yielding PΦB (*38*, *81*, *82*). None of the unusual chromophores carrying a Δ2,3 double bond, which is not conjugated to the main chromophoric π-system, has been accounted for by any of these schemes, and to our knowledge none of them has hitherto been isolated in free form from a natural source. Our results substantiate, by contrast, earlier data obtained with crude extracts of the overexpressing *E. coli*: at least one of these type II-chromophores, viz., 3^1-Cys-PVB, is not formed prior to, but rather during the addition to the apoprotein. Although in our reconstitution system, PecE and PecF cannot transform PCB quantitatively to 3^1-Cys-PVB, this can be ascribed to the competing nonenzymatic side reaction yielding mainly 3^1-Cys-PCB. Obviously, this is no intermediate in the catalyzed reaction, because it cannot be converted further to yield holo α-PEC. It is likely that this reaction is circumvented in vivo by proper regulation of PCB synthesis or the presence of other factor(s).

Isomerizations of bilin chromophores are frequently observed during their biosynthesis (see refs *2*, *13*, and *38* for leading references). The mechanism of any of these reactions is to our knowledge presently not known. The formation of the shoulder at 420 nm upon incubation of PCB with the lyase subunits, individually or together, indicates a possible way for such isomerizations: addition of, e.g., a SH-group of the enzyme to the central methine bridge would uncouple the two dipyrrolic halves of the molecule, rendering changes on the two independent halves more selective. However, more work is needed to verify the formation of such a product, and its reactivities.

In contrast to crude extracts from *E. coli* overexpressions, the current study has concentrated on the isolated chromophore binding protein, PecA, and both lyase subunits. The reconstituted chromoproteins were also purified by chromatography. The study confirms the previous results obtained with crude extracts, but the speed of the reaction is accelerated by an order of magnitude: There is an autocatalytic binding of the PCB chromophore to PecA, which in the presence of PecE/F is competed for by an additional isomerization-reaction leading to a chromoprotein which by its spectroscopic (absorption, CD), photochemical, and electrophoretic properties is indistinguishable from native α-PEC isolated from *M. laminosus*. The reactions have also narrowed the spectrum of potential cofactors: with the exception of a divalent metal, in particular Mg^{2+} or Mn^{2+}, phosphate ions, and a thiol (mercaptoethanol), no other cofactors were required for the reaction. The metal ions required, are those involved in reactions depending on nucleotides. However, if there was any effect of ATP or GTP on the reaction, it was a slight retardation, possibly by competition of the ATP for the metal (not shown).

The reaction of the purified lyase with isolated PecA is considerable faster than with the crude cell lysates of the overexpressing *E. coli*. However, the fastest reaction times (∼1 h) are still slow compared to the times required by phytochromes for the autocatalytic ligation, which is completed within few minutes. This may be due to the fact that the reaction involves a protein as substrate, and that the binding site is at least partly buried (*83*). The size of the substrate (18 kDa) reduces the diffusion time compared to small molecules, and the buried reaction site may indicate that the protein has to undergo in addition a transient conformational change which further reduces the reaction time.

The substrate spectrum for ligation is rather narrow: of the several chromophores which add non- or auto-catalyti-

cally to PecA, only PCB and PΦB are recognized by the lyase (PecE/F). Both pigments are ligated and isomerized in a similar way if judged from the absorption spectra. Replacement of the 18-ethyl- by a vinyl group therefore does not inhibit the enzyme. By contrast, PEB, which also has a 18-vinyl group but lacks the Δ15,16 double bond, is not recognized by the enzyme. The putative product of such a reaction was 3^1-Cys-PUB, a chromophore which is abundant in PE from marine cyanobacteria and from rhodophytes (*2*, *14*, *84*): PEB has the same structure in the ring A,B,C-region as PCB, and application of the ligation/isomerization sequence to PEB would yield 3^1-Cys-PUB. A short wavelength shoulder around 500 nm, the absorption for urobilin, can be seen in the spectrum of the spontaneous addition product. However, there was no indication for an increased formation of this chromophore when PecA was incubated with PEB in the presence of the lyase. If indeed a homologous reaction were catalyzed by a PEB lyase-isomerase, its specificity is expected to differ from the PCB lyase-isomerase studied here.

The high specificity of the latter enzyme underlines the question for the "missing lyases" in cyanobacteria. Since it is possible that there is more than one lyase family which are only distantly related, we have carried out an extended search for homologous proteins. A search for proteins which are homologous to the α-84 lyases of established function yields a considerable number of hits. However, even in the case where the full genome is known (*Synechocystis* PCC6803), it yields only five currently recognized (by BLAST, ref *85*) lyase genes, while there are at least eight chromophore binding sites requiring by the simplest calculation 16 lyase subunits (*13*). Some of the PEB- and PUB-chromophores are also doubly ligated (3^1 and 18^1) and may therefore require additional and possibly rather different lyases. One possibility to rationalize the attachment to the other sites is that ligation of a chromophore to one site may render the addition of all other chromophores autocatalytic. However, this does not appear to be the case, if judged from experiments in which lyases were mutated and/or inactivated (reviewed in ref *13*). Such mutants generally form only small amounts of PC, which points to the lyases being involved in some regulatory cycles (*32*). Several cases have been reported where the small amount of PC formed lacks the α84 chromophore, while bearing the properly attached two *β*-chromophores. However, this is not universally true. Inhibition of CpcE in *Synechocystis* PCC6803 leads to reduced levels of PC, but both subunits carry the regular chromophore complement (*13*). And inactivation of *pecE* and/or *pecF* in *Anabeaena* PCC7120 leads to an impaired PEC in which the α-subunit is chromophorylated but carries a chromophore assigned as PCB on the basis of its absorption (*27*). In view of the spontaneous addition of PCB to PecA found here for *M. laminosus*, it is not clear if this addition is the result of an action of a α-PC lyase on α-PEC (*27*) or the product of a spontaneous addition in which the PCB chromophore is not further modified.

The lyases active in the assembly process of chromophores other than α-84 may be only distantly related and therefore not readily found by sequence comparison with known Cys-α84 bilin lyases.[2] One such class of proteins are the phytochromes, which on account of their autocatalytic chromophore addition have to be classified as lyases. Phytochromes have also been found in cyanobacteria, two of them have been characterized in some detail from *Synechocystis* PCC6803 (*19*, *85*, *86*), and the autocatalyzed chromophorylation has been shown for six additional ones (*40*). While the meaning of this functional homology, and that of distant homologies with other proteins[2] remains unclear, it should also be considered that the catalytic activity resides at least partly on the apo-biliproteins, and the lyases have mainly a chaperone-type function.

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[2] The lack of homologous sequences has prompted a search for more distant relatives of the "classical" lyases using Ψ-BLAST with *pecE* and *pecF* (H. Scheer and J. Houmard, unpublished results). It only yielded an unexpected homology to nuclear import factors, the karyopherins or importins (*88*). This convergence was supported by an inverse search starting from yeast karyopherin-α, which after several rounds yielded genes coding for known lyases including *pecE* and *pecF*.

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