Novel activity of a phycobiliprotein lyase: both the attachment of phycocyanobilin and the isomerization to phycoviolobilin are catalyzed by the proteins PecE and PecF encoded by the phycoerythrocyanin operon

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Abstract The structure of phycoviolobilin, the photoactive chromophore of α-phycoerythrocyanin, is incompatible with a chromophore ligation to the apoprotein via SH-addition (cysteine) to a Δ3,3[1]-double bond of the phycobilin. The two putative phycoerythrocyanin lyase genes of *Mastigocladus laminosus*, *pecE* and *pecF*, were overexpressed in *Escherichia coli*. Their action has been studied on the addition reaction of phycocyanobilin to apo-α-phycoerythrocyanin (PecA). In the absence of the components of α-PEC-phycoviolobilin lyase PecE and PecF, or in the presence of only one of them, phycocyanobilin binds covalently to PecA forming a fluorescent chromoprotein with a red-shifted absorption (λ_{max} = 641 nm) and low photoactivity (< 10%). In the presence of both PecE and PecF, a chromoprotein forms which by its absorption (λ_{max} = 565 nm) and high photoreversible photochromism (100% type I) has been identified as integral α-phycoerythrocyanin. We conclude that PecE and PecF jointly catalyze not only the addition of phycocyanobilin to PecA, but also its isomerization to the native phycoviolobilin chromophore.

Key words: Phycobiliprotein; Photosynthesis; Phytochrome; Chromophore; Thiol addition; Double bond shift; Isomerization; Cyanobacterium; Phycobilin lyase

1. Introduction

Phycobiliproteins, the light-harvesting proteins from cyanobacteria and certain algae, share several structural features with the plant sensory pigments, the phytochromes, and with the functionally yet unexplored cyanochromes. The spectral changes of the chromophores induced by the native apoprotein are similar, and the chromophores are generally covalently bound to cysteines of the apoproteins via thioether-linkages (structures **1**-**4**). In the phyto- and cyanochromes, the chromophore addition is an autocatalytic process[2], which has been used to introduce non-natural chromophores [1–5]. The situation is more complex in the phycobiliproteins. In phycocyanin, which has been studied in some detail, two binding sites (α-84, β-84) show autocatalytic binding, while the third does not bind the chromophore [6]. However, even for the former two sites is the attachment unspecific, and the phycocyanobilin (PCB, **5**) becomes oxidized to a mesobiliverdin (MBV)(4) in the process [7]. Proper binding to at least one of the sites, viz. α-84, has however been shown for the PCB chromophore in the presence of a lyase encoded by *cpcE* and *cpcF*, on which on the *cpc*-operon two genes are located 3′ of the phycocyanin genes [8–12]. Homologous genes have been found, too, in other biliprotein operons including that for phycoerythrocyanin (PEC) [13–16] as well as in non-contiguous regions [17]. Their mode of action is unclear, and their numbers are insufficient if a specific pair of lyase proteins were needed for each bilin binding site.

The situation is even more complicated when considering the different types of biliprotein chromophores. In those cases where spontaneous or lyase-catalyzed binding has been studied, PCB can be replaced by PEB (**3**) [18], the chromophore typical for phycoerythrins, and by PΦB, the chromophore of higher-plant phytochromes, or vice versa. In their unbound state, these three chromophores are all characterized by having a Δ3,3[1] ethylidene double bond, which is conjugated to the chromophore system. The addition reaction of a cystein–SH to this terminal double bond (Scheme 1) is then a common mechanism, even if details like e.g. the absolute stereochemistry at C-3′ may differ [19]. Methods have also been established to cleave these chromophores from the apoprotein [20,21] and at least in one case a chromophore exchange has been reported under the action of a lyase [8].

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Abbreviations: MBV, mesobiliverdin; PCB, phycocyanobilin; PEB, phycoerythrobilin; PΦB, phytochromobilin; α-PEC, phycoerythrocyanin α-subunit; PecA, apoprotein of phycoerythrocyanin α-subunit; PecE and PecF, components of α-PEC-phycoviolobilin lyase; PVB, phycoviolobilin (there are two terms for this chromophore in the literature: phycobiliviolin and phycoviolobilin. The latter is used because it is analogous to the names of the major phycobilins, phycocyanobilin and phycoerythrobilin)

[2] Thiols can be added to PCB, phycoerythrobilin (PEB) and phytochromobilin (PΦB) in a simple chemical reaction. While this can presently not be excluded for the addition of the apoprotein, the selectivity and recent mutagenesis experiments with bacterial 'phyto'chromes [41] support an autocatalytic process in this system.

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There exists, however, a series of biliprotein chromophores, in which binding cannot arise directly by this mechanism. The common structural feature of this second type of chromophores (e.g. **2** and **4** in Scheme 1) is the presence of a Δ2,3 double bond, which is incompatible with the presence of a Δ3,3′ double bond in the precursor. The phycoviolobilin (PVB)-chromophore (**2**) is of this type [22], the urobilin chromophores which are abundant in marine phototrophs [23] and several chromophores of cryptophyte biliproteins [24,25]. Several alternative mechanisms can be envisioned on how these chromophores might be formed, of which two are depicted in Scheme 2. One is the addition to a hypothetical precursor carrying a conjugated Δ3,3′ double bond followed by, or coupled to, an isomerization reaction (paths A/C or D), respectively. The other is, similar to the addition of heme to *c*-type cytochromes, the addition to a vinyl-group, which however is not conjugated to the extended π-system but only to the isolated Δ2,3 double bond (path B).

A distinct phycobiliprotein carrying a type II-chromophore is the α-subunit of phycoerythrocyanin (PEC). Integral PEC is a functional component of the light-harvesting phycobilisome in some cyanobacteria [26]. However, isolated PEC and particularly its α-subunit (α-PEC) also show a remarkable photoreversible photochromism resembling that of the phytochromes [27,28]. In an attempt to explore the origin of this reactivity and to engineer photochromic protein materials, we are currently engaged in modifying both the chromophores and the apoprotein of α-PEC. Here, we wish to report evidence that the putative α-PEC lyase, encoded by the *pecE* and *pecF* genes of the pec-operon [13–16], catalyzes not only the addition of PCB to the apoprotein of phycoerythrocyanin α-subunit (PecA), but also its isomerization to the PVB chromophore yielding native and fully photoactive α-PEC (D in Scheme 2).

2. Materials and methods

2.1. Proteins

The genes *pecA*, *pecE* and *pecF* were PCR-amplified from *Mastigocladus laminosus* DNA and then cloned into pUC19 (work has been carried out in parallel with two strains, e.g. *Fischerella PCC 7603* and a yet unclassified species). By inserting the *pecA*, *pecE* and *pecF* into pGEMEX (Promega), the plasmids pGEMEX-*pecA*, pGEMEX-*pecE* and pGEMEX-*pecF*, respectively, were obtained. *Escherichia coli* BL21(DE3) was transformed with these plasmids to overexpress (separately) PecA, the components of α-PEC-phycoviolobilin lyase (PecE and PecF), respectively. On account of the plasmids used, all resulting proteins carry a N-terminal extension of eight amino acids (MEQNPQSQ). Alternatively, *pecA* was cloned into a pET30 plasmid (Novagen), resulting after expression in *E. coli* BL21(DE3) in a C-terminal extension containing a His- and a S-tag, plus two protease sites for thrombin and enterokinase. Details of the constructions, which were all verified by sequencing, will be published separately. The *E. coli* cells were harvested by centrifugation, washed twice with distilled water, and then suspended in Tris–HCl buffer (50 mM, pH 6.5). The suspensions were sonified, and then centrifuged to yield clean supernatants which were used in the reconstitution experiments. His-tagged PecA was purified after ligation via Ni-affinity chromatography (Pharmacia).

2.2. Phycobilins

PEB is a gift from Prof. A. Gossauer, Fribourg. PCB was obtained by refluxing methanol-washed *Spirulina platensis* (lyophilized, W. Behr, Bonn, Germany) in methanol [20] and purification by chromatography on silica RP8 (ICN) modified from [6]. 40 g of lyophilized bacteria yielded 60 mg PCB (55% yield with respect to the phycocyanin plus allophycocyanin contained), and 20 mg (18%) after purification.

2.3. Reconstitution/ligation

PecA (alone or in combination with the supernatant(s) containing PecE and/or PecF) in Tris-buffer (50 mM, pH 6.5) was treated with 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 Section 3, the mixture was centrifuged to remove any particulate matter, and the supernatant investigated by UV–vis absorption spectrophotometry (Perkin Elmer model Lambda2), fluorescence (Hitachi F-2000) and light-induced spectral changes. For the latter, a 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 [29]. Briefly, it is defined as the amplitude of the S-shaped difference signal (λ_{max} = xxx and yyy nm) relative to the maximum absorption. In those cases where the His-tagged PecA was used, the resulting chromoprotein was purified via Ni-affinity chromatography.

3. Results and discussion

3.1. Autocatalyzed 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 increased absorption at 640 nm and the induction of a red fluorescence ($\lambda_{max,\ emission} = 660$ nm). The absorption spectrum of the protein fraction obtained with His-tagged PecA, purified by Ni-affinity chromatography, has the characteristics of a native biliprotein (in Fig. 1): there is an intense absorption in the visible spectral range ($\lambda_{max} = 641$ nm) and a smaller absorption band in the near UV, while the relative intensities of these two bands are inverted in the free bilins [30]. The absorption maxima are characteristic of a covalently bound MBV-adduct (**4** in Scheme 1), the red-shift as compared to that of an α-subunit containing a PCB-chromophore (**1**,

Table 1
Addition of PCB to PecA and type I photochemical activity (expressed as $\Delta\Delta A_{505/565}$, [29]) of the resulting chromoprotein containing the PVB chromophore

Components on reconstitution[a]	Chromophore in product[b]	Photoactivity ($\Delta\Delta A_{505/565}$)
PCB+PecA[a]	MBV-α-PEC	< 1%
PCB+PecE	–	–
PCB+PecF	–	–
PCB+PecE+PecF	–	–
PCB+PecA[a]+PecE	MBV-α-PEC	< 1%
PCB+PecA[a]+PecF	MBV-α-PEC	< 1%
PCB+PecA[a]+PecE+PecF	α-PEC (plus MBV-α-PEC)	102%

See text for abbreviations and for other photochemical reactions of the MBV-chromophore adduct
[a]Two PecA were tested which were derived from two strains of *M. laminosus* and expressed in *E. coli*, the results are very similar.
[b]MBV-chromophore identified by absorption spectroscopy, the structure of this chromophore has been identified by Arciero et al. [6,7].

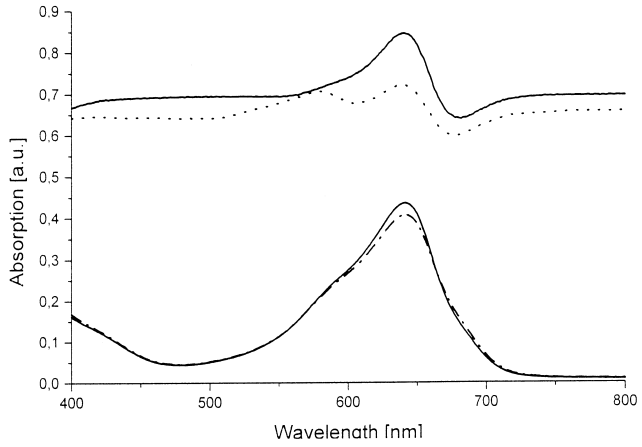


Fig. 1. Absorption spectra of affinity-purified chromoprotein obtained by addition of PCB to His-tagged PecA in the absence of ligase (PecE/F) before (solid line) and after (dot-dashed line) irradiation with 639 nm light (lower curves), and difference absorption spectra (before minus after irradiation) of an irradiation at 639 nm (solid line) and at 572 nm (dotted) (top curves).

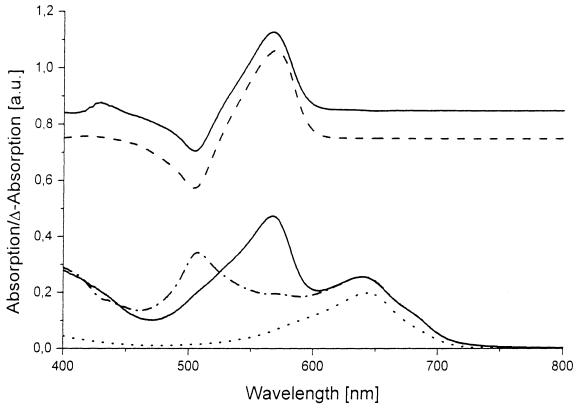
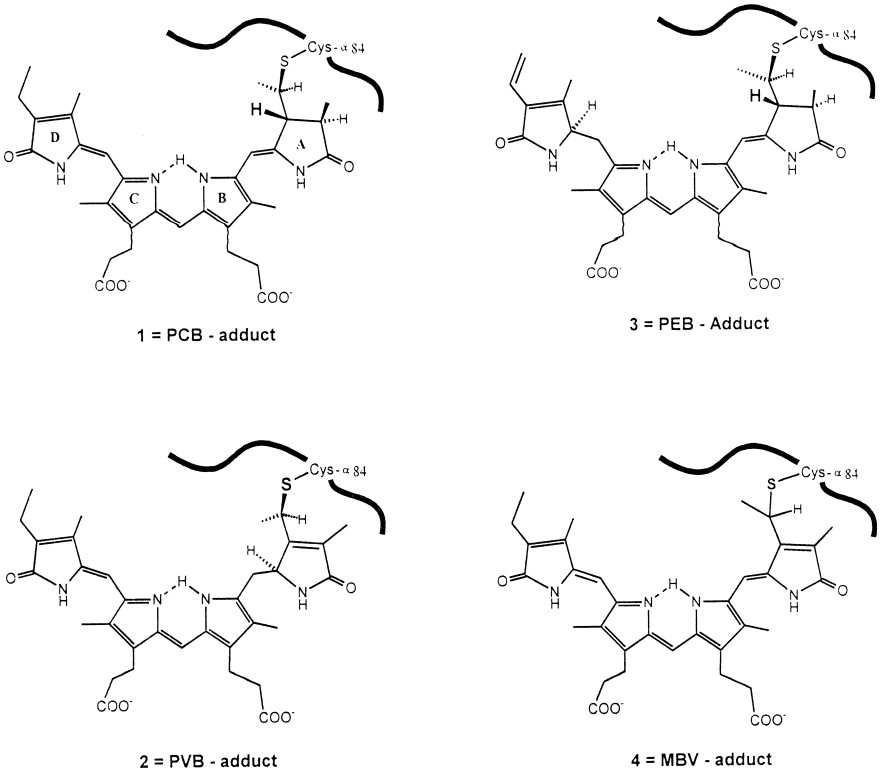


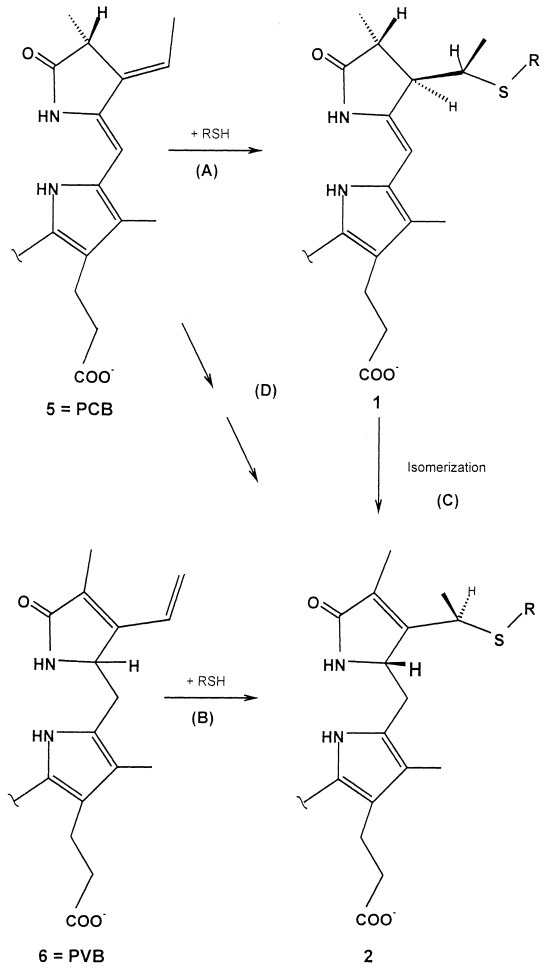
Fig. 2. Absorption spectrum of chromoprotein obtained by addition of PCB to His-tagged PecA in the presence of lyase (PecE plus PecF) before (solid line) and after (dot-dashed line) irradiation with 570 nm light (bottom curves), and difference absorption spectrum (before minus after irradiation) of an irradiation at 570 nm (solid line) (top curve). The spectrum of the non-enzymically formed and affinity-purified non-enzymatic addition-product of PCB to PecA (dotted line) and the light-induced difference spectrum of α-PEC isolated from *M. laminosus* (dashed line), are shown for comparison.

$\lambda_{max} \approx 620$ nm) due to an additional conjugated Δ2,3 double bond [6,7]. Covalent binding of the chromophore was verified by SDS–PAGE and subsequent fluorescence detection of the bilin-chromophore as the Zn-complex [31]. The colored protein migrated with an apparent molecular weight of 22.8 kDa (calc. 22738 Da), and an orange fluorescence was induced by treatment with Zn–acetate.

Minor, but distinct spectral changes were observed when this solution was irradiated with red light (639 nm). There was a bleaching at 641 nm and an increased absorption at the red-wing (Fig. 1), which was only partly reversible upon irradiation at 682 nm (not shown). The difference spectrum gave a $\Delta\Delta A_{641/682}$ of 9.4%. Irradiation of the original sample at 572 nm resulted in the same difference bands $\lambda > 600$ nm, albeit with reduced amplitude (5.5%) and an additional difference peak at 572–579 nm ($\Delta A_{578}/A_{641} = 3\%$). The change at 578 nm is fully reversible upon subsequent irradiation at 639 nm, and also that at 641 nm if the contribution of the first photoreaction is subtracted, indicating a small but dis-



Scheme 1.



Scheme 2.

tinct red (641 nm)/green (576 nm) photoreversible photochromism. It is known that most phycobilins exhibit small photoreversible photochemistry if the protein is partly unfolded, but the amplitudes are generally only small [27,30]. The photochemistry of MBV-adducts (**4**) has not been studied to our knowledge, but a similar behavior may be expected. However, the red/green photochromism persisted after urea unfolding and subsequent refolding, which is not the case with e.g. α-PC, which indicates a specific influence of the environment of the α-84 binding site of PecA.

A very different spectral evolution was obtained when PCB and PecA were incubated in the presence of PecE and PecF, under otherwise identical conditions. There was only a minor increase of absorption at 641 nm, while over a period of 4 h there appeared gradually an absorption at 565 nm, which is the absorption maximum of integral α-PEC containing the PVB-chromophore (**2**) [27,29]. This absorption continued to increase over 16 h, at the expense of the 618 nm absorption of PCB, while the absorption at 640 nm remained constant, yielding the final spectrum shown in Fig. 2 (bottom, solid line). The conversion of the added PCB (**5**) to protein-bound PVB (**2**) was verified by the characteristic photoreversible photochromism of the product (Fig. 2). After irradiation at 570 nm, the band at 565 nm decreased, and a new absorption band arose at 505 nm, corresponding to the characteristic *Z-E* isomerization of the native phycoviolobilin (PVB) chromophore of α-PEC. Shape and maxima (505/565 nm) of the resulting difference spectrum were identical to those of type I-photochemistry of isolated α-PEC (505/565 nm, [29]), and the photoreaction was fully reversible after irradiation with 500 nm light. The amplitude of this difference spectrum (defined as $\Delta\Delta A_{505/565\,nm}$, [29]) was 102%. Within the limits of error, this is identical to that of isolated α-PEC ($\Delta\Delta A_{505/565\,nm} = 105\%$), and proves that the chromophore of the reconstituted α-subunit has maximum photoactivity. This behavior identifies the resulting chromoprotein unequivocally as α-PEC.

Control experiments showed, that neither PecE nor PecF alone were capable of generating the proper product (Table 1), the spectra of the resulting products were like the one obtained with PecA and PCB only (not shown). These results show that both lyase proteins are required for the formation of α-PEC from PecA and PCB, and that their joint action catalyzes a remarkable reaction which involves not only binding of the PCB-chromophore by addition of the SH-group of Cys-α-84, but also an isomerization involving (formally) the migration of the Δ4,5 double bond to the Δ2,3-position. This dual activity of α-PEC-PVB lyase (i.e. PecE and PecF) is intriguing if compared to that of the homologous α-phycocyanin-PCB lyase (CpcE/F, [8]). The latter acts as a lyase only, i.e. it is responsible only for the correct addition of PCB to the apoprotein of α-PC. Clearly, the details of these enzyme activities and their relations to that of other ligases need to be studied in more detail.

Biosynthesis of the biliprotein chromophores has been studied in particular in the eukaryotic alga, *Cyanidium caldarium* [32–39]. Here, cleavage of heme and subsequent reduction produce a dihydrobiliverdin, which is first isomerized to 3,3^{1}Z-PEB, then to 3,3^{1}Z-PCB and finally to 3,3^{1}E-PCB (**5**). Some of these intermediates have also been identified in cyanobacteria [40], while alternative pathway(s) are discussed in higher plants yielding PΦB [1,2]. 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 indicate, that at least one of them, e.g. 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 PVB, this can be ascribed to the non-enzymatic side reaction yielding probably bound MBV (**4**), which cannot be converted further to α-PEC. It is likely that this reaction is circumvented in vivo by proper regulation of synthesis or the presence of other factor(s). It should be emphasized that all reactions reported here were carried out under aerobic conditions. The formation of MBV (**4**) from PCB (**1**) is an oxidation, however, the products are the same under an Ar atmosphere.

In summary, our results rationalize the unsuccessful attempts to isolate free PVB from PEC-containing organisms, as well as unsuccessful attempts to cleave the chromophore from the apoprotein by methods designed for PCB, PEB or PΦB. We therefore suggest that in cyanobacteria, PVB results in vivo from the PecE/F-catalyzed addition/isomerization reaction of PCB to bound PVB. Work is in progress to investigate if similar pathways can account for the formation of other chromophores of this type.

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