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# Toward a Mechanism for Biliprotein Lyases: Revisiting Nucleophilic Addition to Phycocyanobilin

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Biliproteins are a remarkable example of molecular adaptation.[1] The photophysics and photochemistry of the linear-tetrapyrrolic bilin chromophores are profoundly changed by interactions with the apoprotein, thereby optimizing them for their light-sensing[2] and light-harvesting functions.[3] The chromophores are covalently bound to the apoproteins via thioether bonds to conserved cysteine sites of the apoproteins. This binding is autocatalytic in the phytochromes[4] but enzymatic in the phycobiliproteins of cyanobacteria.[5,6] Up to four chromophores are attached in these light-harvesting pigments by a set of lyases, of which three phylogenetically unrelated types (E/F, S/U, and T) have recently been characterized; they are distinguished by their specificities for the acceptor proteins, the binding sites, and the chromophores.[6] All three lyase types directly attach phycocyanobilin (PCB, **1**) (or phycoerythrobilin, which differs by the length of the conjugated $\pi$ system) by nucleophilic addition to the common $\Delta 3,3^1$ double bond of the two chromophores.[7] Two additional chromophores, phycoviolobilin (PVB, **5**) and phycourobilin, are produced from **1** and phycoerythrobilin, respectively, during the attachment by an isomerizing subtype of E/F lyases.[8]

The reaction mechanism of these lyases is poorly understood.[6d] A chaperone-like function had been suggested on the basis of the spontaneous but low-fidelity addition of chromophores to the apoproteins.[5] Such a function has been supported experimentally for the EF type,[9] but other evidence indicates a more involved action of the lyases. This includes the $\Delta 4/\Delta 2$ isomerization catalyzed by some E/F-type lyases[8] and, in particular, the discovery of transient chromophore binding that is most pronounced in the S-type lyases[6d,10] and triggered the work reported here.

When attempting to identify the binding site in the complex of **1** and the lyase CpcS by trypsin digestion, we failed to obtain chromopeptides, and free **1** was released instead, indicating noncovalent (or extremely weak covalent) binding. Additional low-molecular-weight products were formed, however, when a nucleophile such as mercaptoethanol (ME, added as an antioxidant) or imidazole (Im, used in $Ni^{2+}$ affinity chromatography) was present in the reaction mixture. Two products each were identified with ME (**3a** and **3b**) and Im (**3c** and **3d**). Their mass spectra corresponded to adducts of **1**, and ME or Im signals, respectively, were verified by NMR spectroscopy (Tables S1−S3 in the Supporting Information). Their optical absorption spectra were reminiscent of those of chromopeptides, namely, thiol adducts to C-$3^1$ of **1**, analogous to **4**. A different structure was indicated, however, by a split near-UV band and a considerably higher intensity ratio (1.5 vs 0.5) of the vis/near-UV bands. This was confirmed by NMR identification of isophycocyanobilin chromophores (**3**) for all four products: in every case, the 3-H signal is missing, and the protons at C-2 and C-$3^1$ couple only to 2-H and $3^2-CH_3$, respectively (Tables S2 and S3 in the Supporting Information). All of the other expected signals are present, but some (in particular 5-H) show significant shifts compared with those of chromopeptides of type **4**.[11]

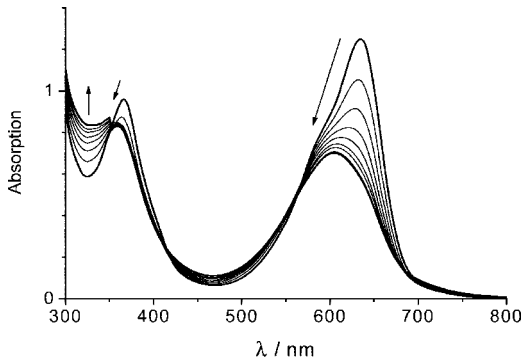


***Figure 1.*** Chromophore transfer from the complex of the lyase CpcS with phycocyanobilin **1** to imidazole. Absorption changes were recorded after mixing of the CpcS−**1** complex [obtained from **1** (5 $\mu$M) and CpcS (15 $\mu$M)] with Im (500 mM) in potassium phosphate buffer (500 mM, pH 7.5) containing NaCl (100 mM). The first spectrum was obtained 1 min after mixing, and the subsequent spectra were recorded at 30 min intervals thereafter ($T = 35$ °C). The directions of change are indicated by the arrows.

The same adducts were also formed when the CpcS−PCB intermediate[10] was treated with the respective nucleophile. The reactions of Im with **3c** and **3d** were accompanied by a diminished and blue-shifted visible absorption band (Figure 1). The resulting adducts were retained with $His_6$-tagged CpcS during $Ni^{2+}$ affinity chromatography but lost during proteolysis with trypsin, again indicating a strong noncovalent (or weak covalent) interaction with the lyase. **1** does not bind Im in the absence of CpcS (Figure S1 in the Supporting Information). The time course of the reaction is slow: it is comparable to that of the transfer of **1** from CpcS to the acceptor protein CpcB[10] or from **3c** or **3d** to the acceptor protein catalyzed by CpcS (see below). This indicates that the slow kinetics of the lyase[6d] is not related to protein−protein interactions but rather to chromophore transfer, internal secondary transfer, and/or related pigment−protein rearrangements. The isolated adduct **3c** is stable toward ME, while the chromophore is transferred to CpcS in the absence of excess Im. Obviously, binding of the chromophore **3** to CpcS is of comparable strength as that to Im.

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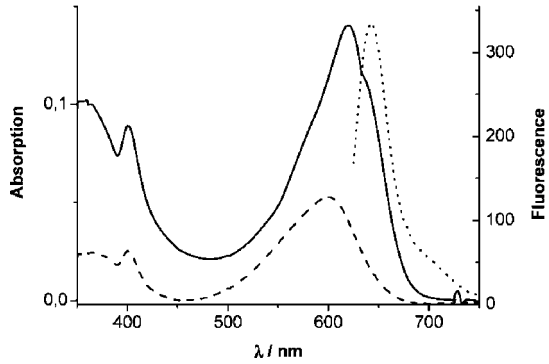


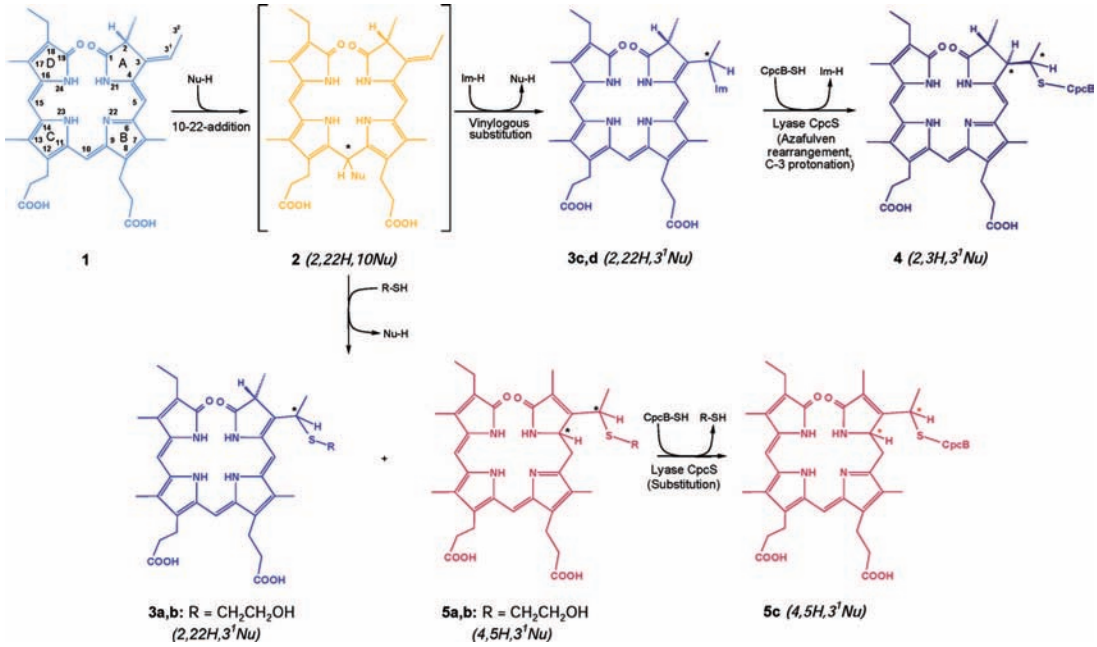

***Figure 2.*** Chromophore transfer from **3c** to CpcB(C155I). Absorption spectra of **3c** (2 $\mu$M), CpcS (10 $\mu$M), and CpcB(C155I) (10 $\mu$M) 1 min after mixing (dashed curve) and after incubation for 2 h at 35 °C (solid curve) are shown, along with the fluorescence emission spectrum after incubation for 2 h (dotted curve). The fluorescence of the starting solution was zero on this scale.

An isophycocyanobilin chromophore **3** had been proposed earlier by Stumpe et al.[12] as an intermediate during attachment of **1** to apoproteins but had never been isolated. Supposedly, it was generated from a rubinoid precursor **2** that was initially formed by nucleophilic attack at the central methine bridge[13] (see below). This prompted us to investigate whether the adducts are substrates for the lyases. With **3c** as the donor, the apo-$\beta$-subunit of C-phycocyanin (CpcB) as the acceptor protein,[6e,f] and the lyase CpcS as the catalyast, the chromophore was transferred to cysteine-$\beta$84 (Figure 2); the product was identical to a control sample in which free **1** was supplied as the substrate. Obviously, the isophycocyanobilin **3** (*2,22H,3$^1$Nu*) was reisomerized during this reaction to the well-known **4** (*2,3H,3$^1$Nu*) (Scheme 1).

The reaction of ME with **1** had previously been studied by Köst-Reyes and Köst.[17] On the basis of the similarity of the optical spectra with those of PCB chromopeptides, the products had been assigned as *2,3H,3$^1$Nu* adducts of type **4** but not further characterized. A reinvestigation of this reaction now showed the formation of four products, none of which had the suggested structure. Two of them were identical to the adducts **3a** and **3b** obtained with CpcS; that is, isophycocyanobilins (*2,22H,3$^1$Nu*) are also formed during the spontaneous addition of ME to **1**. The two other products contained the PVB chromophore **5** (*4,5H,3$^1$Nu*; $\lambda_{max}$ = 570 nm), which is characteristic for the α-subunit of phycoerythrocyanin[11b] and possibly also present in certain cyanobacteriochromes.[18] The mass spectra of **5a** and **5b** showed molecular ions of addition products, and the NMR spectra lacked the low-field 5-H singlet and 2-H quartet but showed instead the 4H-5H$_2$ pattern. Chromophore **5** has until now been known only as a protein adduct (**5c**) generated from **1** during attachment to the α-subunit of phycoerythrocyanin (PecA) in a reaction that is catalyzed by specialized E/F-type lyases.[8a] No intermediate had been identified for this reaction, and neither a spontaneous nor a catalytic addition product to PecA of type **4** could be transformed by the lyase to a PVB product **5**.[4b] We therefore also investigated the interactions of the phycoviolobilin adduct **5a** with the lyase CpcS and the apoprotein CpcB. In both cases, complex formation was supported by absorption and fluorescence spectroscopy (Figures S2−S4 in the Supporting Information). The resulting PVB−CpcB complex (**5c**) had an absorption ($\lambda_{max}$ = 574 nm) and intense fluorescence ($\lambda_{max}$ = 589 nm) resembling those of the P570 form of α-phycoerythrocyanin.[19] However, it lacked the characteristic photochemistry, possibly as a result of binding to the "wrong" apoprotein.

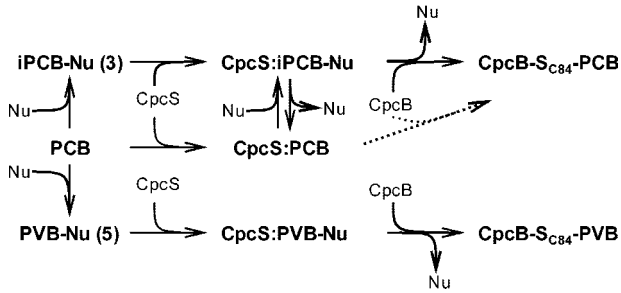
Stumpe et al.[12] have speculated on theoretical grounds that the initial nucleophilic attack of **1** occurs at C-10. Vinylogous substitution of the Rubin intermediate **2** (*2,22H,10Nu*, Scheme 1) would then generate the iso-PCB system **3** (*2,22H,3$^1$Nu*) as a precursor for both chromophores **4** (*4,5H,3$^1$Nu*) and **5** (*2,3H,3$^1$Nu*). While we were unable to isolate rubins from the reaction mixture, their formation is indicated by the frequent observation of minor but distinct absorptions near 430 nm (see also Figure 2).[13] With the

***Scheme 1.*** Products of Nucleophilic Addition to Phycocyanobilin (**1**)[a]




[a] Asterisks indicate newly generated asymmetric centers. Nu = nucleophile, Im = imidazolyl. CpcB is the apoprotein of the $\beta$-subunit from *Anabaena* PCC7120 with an accessible binding site at C-84 (the second binding site was blocked by C155I mutation).[6e] Colors and intensities of structures approximate those of the compounds. Structures **3a**−**d**, **5a**, and **5b** were identified in this study, structures **4** and **5c** by Lagarias et al.[11a] and Bishop et al.,[11b] respectively. The intermediacy of **2**[12] still needs verification.

***Scheme 2.*** General Scheme of Nucleophilic Addition Reactions Relevant to Lyase Function[a]




[a] Nu = nucleophile, iPCB = isophycocyanobilin; for other abbreviations, see Scheme 1.

assumption of their intermediacy, as suggested by Stumpe et al.,[12] the complete reaction sequence is summarized in Scheme 1. The course of the ensuing azafulvene rearrangement of **3** to **4** (plus isomerization to **5** in the case of the isomerizing lyases) might be determined by appropriate positioning of acidic amino acid residues near ring A. Such site selectivity by properly directed protonation has also been proposed for another bilin biosynthetic reaction, the reduction of the common bilin precursor biliverdin by a family of ferredoxin-dependent reductases.[20]

On the basis of the studies conducted here with the lyase CpcS, a general reaction mechanism for the different bilin lyases of cyanobacteria can be envisioned (Scheme 2). Spontaneous thiol addition leads to adduct mixtures carrying chromophore **3** or **5**. Formation of **5** is inhibited by the nonisomerizing lyase CpcS. In this case, chromophore **3** is probably bound to a histidine (or possibly a cysteine) of the protein. Histidine has been implicated as essential in chromophore addition not only to phycocyanin but also to phytochromes.[4] Transient binding to histidine is also favored by the lability of the adduct; the chromophore is released during proteolysis. Catalysis by E/F-type lyases requires thiols, in contrast to the thiol inhibition observed with S- and T-type lyases.[6f,8a] In this case, the nonisomerizing lyases would again be selective in favoring the formation of *2,22H,3*$^1$*Nu* adducts **3**, from which the chromophore would then be transferred to the acceptor protein, with concomitant back-isomerization to *2,3-H,3*$^1$*Nu* adducts **4**. The isomerizing lyases would instead inhibit formation of **4**, thereby favoring formation of *4,5H,3*$^1$*Nu* adducts **5**, from which the chromophore would then be transferred to the apoprotein without further (net) isomerization. In the proposed reaction sequence, therefore, the chromophore would be isomerized by all of the lyases, only transiently with the "nonisomerizing" lyases but irreversibly with the isomerizing lyases of the E/F type.

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**Supporting Information Available:** (a) Materials and methods; (b) spectral data for compounds **3a**−**d**, **5a**, and **5b**; (c) Figure S1 relating to the formation of imidazole−isophycocyanobilin adducts **3c** and **3d**; and (d) Figures S2−S4 relating to the interaction of **5a** with the lyase CpcS and the apoprotein CpcB. This material is available free of charge via the Internet at http://pubs.acs.org.

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(7) Nomenclature: In the biliprotein literature, 3-H,3$^1$-thiol addition products such as **4** are often termed the same as the parent $\Delta$3,3$^1$ bilins, such as **1**. The term phycocyanobilin is used here exclusively for the free chromophore **1**. The addition products are treated as derivatives of the former (i.e., as 3$^1$-nucleophile adducts) and distinguished by the positions of hydrogens in the tetrapyrrole system (see Scheme 1).
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(13) Rubinoid adducts to C-10 have frequently been found.[14] Their intermediacy is supported by characteristic absorptions near 430 nm during enzymatic[9b] and non-enzymatic chromophore[15] attachment, which are obvious, too, in Figure 1. MO calculations also favor attack at C-10 but indicate significant reactivities at other sites, including C-4 and C-6.[16] These addition products can also yield chromophore **3** by vinylogous substitution. No spectral evidence for such intermediates has been found, but the absorptions may be buried under those of **3** and **5**.
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