# Photochromic Biliproteins from the Cyanobacterium *Anabaena* sp. PCC 7120: Lyase Activities, Chromophore Exchange, and Photochromism in Phytochrome AphA†

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ABSTRACT: Photochromic biliproteins can be switched by light between two states, initiated by *Z*/*E* photoisomerization of the linear tetrapyrrole chromophore. The cyanobacterium *Anabaena* sp. PCC 7120 contains three genes coding for such biliproteins, two coding for phytochromes (a*phA/B*) and one for the α subunit of phycoerythrocyanin (*pecA*). (a) a*phA* was overexpressed in *Escherichia coli* with N-terminal His and S tags, and the protein was reconstituted by an optimized protocol with phycocyanobilin (PCB), to yield the photochromic chromoprotein, PCB-AphA, carrying the PCB chromophore. (b) AphA chromophorylation is autocatalytic such as in other phytochromes. (c) AphA chromophorylation is also possible by chromophore transfer from the PCB-carrying biliprotein, phycocyanin (CPC). The autocatalytic transfer is very slow, and it is enhanced more than 100-fold by catalysis of PCB:CpcA lyase and α-CPC as donor. (d) Through deletion mutations of *aphA*, a short sequence IQPHGV [amino acids (aa) 26−31] was found essential for the lyase activity of AphA, indicating an interaction of the N terminus with the chromophore-binding domain around cysteine 259. (e) A motif of at least 23 aa, starting with this sequence and located ∼250 aa N terminal of the chromophore-binding cysteine, is proposed to relate to the lyase function in plant and most prokaryotic phytochromes. (f) Long-range interactions in AphA are further supported by blue-shifted absorptions (≤12 nm) of both the Pr and Pfr forms of truncated chromoproteins.

The genomic sequence of the cyanobacterium, *Anabaena* sp. PCC 7120 (*2*), contains three genes annotated to code for proteins that are post-translationally chromophorylated by linear tetrapyrroles to yield photochromic chromoproteins. Two belong to the phytochrome photoreceptor family (AphA and AphB),[1] and the third one, PecA, is the α subunit of a light-harvesting pigment, phycoerythrocyanin (PEC). The *aphA* gene of 2.3 kbp encodes a protein of 765 amino acids (aa) (AphA) homologous to plant phytochromes and to "classical" cyanobacterial phytochromes such as Cph1 (748 aa) of *Synechocystis* PCC 6803 (see refs *1*, *3*, and *4*). The putative linear tetrapyrrole chromophore-binding site of AphA is cysteine 259, while the same position in AphB is replaced by leucine. The *pecA* gene encodes α-PEC. Like the entire *pec* operon of *Anabaena* sp. PCC 7120, *pecA* is homologous to that of *Mastigocladus laminosus* (*5*, *6*), the only system whose encoded proteins have been studied in some detail (*7*−*15*).

Phytochromes were originally thought to exist only as sensory photoreceptors for (primarily) red and near-infrared light in plants and algae. Presently, genes coding for phytochromes and phytochrome-related proteins have also been identified in photosynthetic prokaryotes (cyanobacteria

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[1] Abbreviations: *Pigments*: PCB, phycocyanobilin; PEB, phycoerythrobilin; PΦB, phytochromobilin; PVB, phycoviolobilin# (There are two terms for this chromophore in the literature, phycobiliviolin (*14*) and phycoviolobilin (*7*); 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, while the chromophores attached to the apoproteins are characterized as addition products. *Proteins and genes*: *aphA*, phytochrome gene of *Anabaena* sp. PCC 7120, which is homologous to "classical" cyanobacterial (1), and plant phytochrome genes AphA, phytochrome-like apoprotein encoded by *aphA*; AphA(X-Y), truncated AphA extending from amino acid "x" to amino acid "y"; CpcA/B, apoproteins of α- and *β*-CPC, respectively; CpcE and CpcF, subunits of PCB-CpcA-lyase; CPC, phycocyanin, α/*β*-CPC, chromophorylated CpcA/B, respectively; PecA, apoprotein of α-PEC; PecE and PecF, subunits of PVB-PecA-lyase; PEC, phycoerythrocyanin, α-PEC, chromophorylated PecA; Pr and Pfr, phytochrome in the red and far-red absorbing states, respectively. *General*: aa, amino acid(s); KPP, potassium phosphate buffer; TX-100, Triton X-100; $\Delta\Delta A_{x/y}$, amplitude of photochemical signal with difference maxima at *x* and *y* nm, normalized to maximum absorption of the chromophore in the *Z* configuration (see ref 7 for details).

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and purple bacteria), nonphotosynthetic eubacteria, and fungi. On the basis of mainly sequence homologies and the photochemical properties of some of the proteins reconstituted with bilin chromophores, it is accepted that prokaryotic phytochromes are more diverse regarding function, chromophore type, and chromophore attachment, with one line being closely related to the eukaryotic phytochromes of plants and algae (*1*, *3*, *4*, *16*−*24*).

Expression in *Escherichia coli* of genes coding for the latter group of classical phytochromes, like *cph1* of *Synechocystis* sp. PCC 6803, produces apoproteins of ∼85 kDa, which can be reconstituted in vitro with phycocyanobilin (PCB) and related bilins (*3*, *17*−*19*, *25*−*27*). The photoisomerization dynamics of the Cph1-PCB adduct exhibit very similar features as those described for plant type-A phytochrome (*19*, *28*). Full-length and 3′-truncated *cph1* were also coexpressed in *E. coli* with *Synechocystis* sp. PCC 6803 *ho1* (sll1184) and *pcyA* genes, encoding two biosynthesis genes for the chromophore, viz., heme oxygenase and phycocyanobilin:ferredoxin oxidoreductase, respectively (*29*, *30*). This system produces holo phytochrome in situ using heme produced by the host. The system is suggested not only to facilitate the genetic analysis of holoprotein assembly, photochromic activity, and biological function, but also to be potentially useful, e.g., to regulate gene expression artificially by light in nonplant cells (*31*).

They are also significant with respect to questions concerning biochemical and biophysical properties of plant phytochromes (*32*−*34*). Cph1, the first prokaryotic phytochrome studied in detail, is a light-regulated histidine kinase that mediates red and far-red reversible phosphorylation of a small response regulator (see refs *35* and *36* for leading references). Cyanobacterial phytochromes have been implicated as photoreceptors in the regulation of cAMP-mediated processes (*37*), in light−dark and dark−light transitions (*38*), circadian rhythm (*39*), and complementary chromatic adaptation (*4*).

Similar bilin chromophores are bound to phytochromes and the cyanobacterial light-harvesting biliproteins, and they are in most cases also bound covalently in a similar fashion via a thioether bond to cysteine. They differ, however, in the way the chromophores are attached to the protein. Ligation is usually an autocatalytic process in phytochromes (see refs *3* and *4*) but requires specific lyases in the phycobiliproteins, of which to date only a few have been characterized (reviewed in ref *40*). The PCB:CpcA lyase from *Synechococcus* sp. PCC 7002 has been studied in some detail (*41*, *42*). The heterodimeric enzyme (CpcE/F) attaches PCB selectively and reversibly to cysteine 84 of α-CPC. In the homologous PVB:PecA lyase from *M. laminosus* (*5*, *6*), the lyase activity is coupled to an isomerase function, thereby producing the PVB chromophore during the attachment process (*10*, *11*). The *pec* operon of *Anabaena* sp. PCC 7120 contains two homologous genes (*pecE/F*) (*2*), expected to catalyze the same ligation−isomerization reaction. Lyases have also been implicated in chromophore release from phycobiliproteins (*43*). It should be noted that chromophore ligation is also a practical problem in biliprotein research and in their potential applications (e.g., see refs *29* and *30*).

Little is known about the function of the lyases on the molecular level. Bilins can add spontaneously to thiols, including the cysteines, eventually bearing the chromophores in biliproteins, albeit in a less specific fashion. Chaperone-like functions have therefore been discussed for the lyases (*40*, *44*). Homology searches indicate, on the other hand, the presence of genes homologous to *cpcE* in organisms not known to contain phycobiliproteins, including archaea (*45*−*47*), plants (*48*−*50*), and even in a virus (*51*), as well as a distant relationship to karyopherins (*52*). The cyanobacteria also have to cope with the distribution of the bilin chromophore, PCB, on their phytochromes and on the biliproteins, which are present in vastly different amounts. Several protein regions seem to be involved in the autocatalytic chromophorylation of phytochromes (see the Discussion and refs *3* and *4* for leading references). No homologous motifs have hitherto been found in the phycobiliprotein lyases.

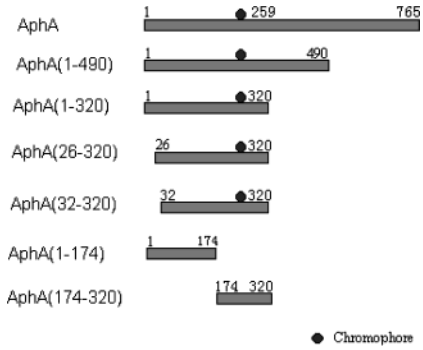


FIGURE 1: Schematic representation of AphA expression constructs. A dot indicates that the protein ligates the chromophore autocatalytically to cysteine 259 at the putative binding site. See EMBL entry PHYA_ANASP, (Q9LCC2) for the sequence. N- and C-terminal aa are as follows: AphA, M1:S765; AphA(1−490), M1:R490; AphA(1−320), M1:E320; AphA(1−175), M1:L175; AphA-(26−320), I26:E320; AphA(32−320), L32:E320; and AphA(174−320), M174:E320.

We are interested in the characterization of the phycobiliprotein lyases, as well as of the autocatalytic lyase activities embedded in phytochromes (*11*, *12*). Here, we report on chromophore attachment to phytochrome AphA of the cyanobacterium *Anabaena* sp. PCC 7120, as well as to N- and C-terminally truncated AphA. Several methods of phytochrome reconstitution will be addressed, including the autocatalytic assembly of apo phytochrome with free PCB and the chromophore transfer from CPC to apo phytochrome, autocatalytically and in the presence of PCB:CpcA lyase (CpcE/F).

## MATERIALS AND METHODS

*Cloning and Expression Procedures.* The *aphA* gene of *Anabaena* sp. PCC 7120 and *cpcA*, *cpcE*, and *cpcF* genes of *M. laminosus* PCC 7603 were isolated from genomic DNA by PCR using specific primers and *Taq* polymerase or *Pfu* DNA polymerase (MBI Fermentas, Shenzhen, People's Republic of China). Eight primers ($P_1$−$P_8$) were designed for generating full-length AphA (765 aa, 87 kDa) and six truncated proteins (Figure 1): the C-terminally truncated fragments AphA(1−490), AphA(1−320), and AphA(1−175), as well as the C- and N-terminally truncated fragments AphA(26−320), AphA(32−320), and AphA(174−320). Six primers ($P_9$−$P_{14}$) served to amplify *cpcA*, coding for the apo α subunits of the biliproteins, as well as *cpcE/F* coding for PCB:CpcA lyase.

Following combinations of the expression products, the 5′ and 3′ primers were used to generate the various genes:

*aphA*, $P_1$ and $P_5$; *aphA*(1−490), $P_1$ and $P_6$; *aphA*(1−320), $P_1$ and $P_7$; *aphA*(1−175), $P_1$ and $P_8$; *aphA*(26−320), $P_2$ and $P_7$; *aphA*(32−320), $P_3$ and $P_7$; *aphA*(174−320), $P_4$ and $P_7$; *cpcE*, $P_9$ and $P_{10}$; *cpcF*, $P_{11}$ and $P_{12}$; and *cpcA*, $P_{13}$ and $P_{14}$.

All 5′-terminal primers except $P_{13}$ have a *Sma*I site (*CCCGGG*) upstream of the natural start codon (ATG, shown in bold), which in the final constructs is no longer used and expressed as Met. $P_{13}$ was designed upstream of the natural start codon. All 3′-terminal primers have a *Xho*I site (*CTCGAG*) and a stop codon except for $P_{10}$ and $P_{12}$, which were designed downstream of the natural stop codon. $P_1$, 5′-TCA*CCCGGG*AGGCTTTTT**ATG**AGAATAGACGT-AG-3′; $P_2$, 5′-GTA*CCCGGG***ATG**ATTCAACCACATGGTG-TGCTTT-3′; $P_3$, 5′-TAA*CCCGGG***ATG**CTTTTAGTTTTGG-AAGAACC-3′; $P_4$, 5′-GGG*CCCGGG*TTTGATCGGGTA**AT-G**TTATATAAAT-3′; $P_5$, 5′-CAC*CTCGAG*TATCGGCTT-TATTATCC**TCA**ACTAA-3′; $P_6$, 5′-ATT*CTCGAG***TTA**AC-GCCAAGGTAGAGAGGTGAG-3′; $P_7$, 5′-ATC*CTCGAG*T-TCTGT**TTA**CTCTCTGGCGGA GAT-3′; $P_8$, 5′-CCC*CTC-GAG*CGAATTT**TTA**TAACATTACCCGATC-3′; $P_9$, 5′-TGT*CCCGGG*GCATTGGTC**ATG**ACAGAAGCA −3′; $P_{10}$, 5′-GGGCTCGAGCGGCAATTAAAGTGGGAAT −3′; $P_{11}$, 5′-ATACCCGGGATACTCCTGACC**ATG**ACTGC −3′; $P_{12}$, 5′-ACC*CTCGAG*TTATCTTGAGAGTGGAACAAA-3′; $P_{13}$, 5′-TAA*CCCGGG*ACACCAATTACTGACGCTATT-3′; and $P_{14}$, 5′-GGC*CTCGAG*TAT**CTA**GCTGAGAGCGTTGAT-3′.

The PCR of *aphA* with *Taq* polymerase was run for 30 cycles (94 °C for 90 s, 48 °C for 90 s, and 72 °C for 150 s) and with one additional incubation at 72 °C for 5 min. When *Pfu* DNA polymerase was used for full-length *aphA*, the PCR was run for 30 cycles (94 °C for 60 s, 45 °C for 80 s, and 72 °C for 360 s) and with one additional incubation at 72 °C for 9 min. For truncated *aphA*, the PCR was run with the same enzyme for 30 cycles (94 °C for 60 s, 45 °C for 80 s, and 72 °C for 200 s) and with one additional incubation at 72 °C for 5 min. The PCR of the other genes with *Taq* polymerase were run for 30 cycles (94 °C for 90 s, 48 °C for 90 s, and 72 °C for 90 s) and with one additional incubation at 72 °C for 5 min. All PCR products were digested with *Sma*I and *Xho*I and then ligated into the cloning vector *pBluescript* SK(+) (Stratagene, Shanghai, People's Republic of China), digested with the same restriction enzymes. Ligated products were transformed into *E. coli* strain TG1. After sequence verification, the gene fragments were subcloned into *pET*30a (Novagen, Schwalbach, Germany) via *Eco*RV and *Xho*I digestion. In some PCR products of full-length *aphA* with *Taq* DNA polymerase, a random mutation (coding for a K41N replacement) was found in the routine sequencing control, and a different one (V31L) in *AphA*(1−320). Both were absent when *Pfu* polymerase was used for amplification. The *pET*30a-derived expression vectors were transformed into *E. coli* strain BL21 for their overexpression. All genetic manipulations were carried out according to standard protocols (*53*). Via the *pET*30a vector, a 5-kDa peptide bearing His and S tags plus thrombin and enterokinase sites has been fused N-terminally to all expression products.

The transformed BL21 cells containing a gene from *Anabaena* sp. PCC 7120 were cultured in LB medium at 37 °C overnight, and an inoculum (2 mL) of the culture was transferred into 100 mL of liquid RB medium (0.5% NaCl, 0.5% yeast extract, 1% tryptone, and 0.2% glucose at pH 7.5), supplemented with kanamycin (30 $\mu$g/mL). The culture was incubated at 30 °C until $OD_{600}$ reached 0.6 and was transferred to 20 °C for 1 h before induction with 1 mM isopropyl $\beta$-D-thiogalactoside (IPTG). The induction time was 6 h, if not noted otherwise. The cells were centrifuged at 12000*g* for 3 min at 4 °C and were washed twice with water and once with the buffer (50 mM Tris-HCl and 300 mM NaCl at pH 7.8). After centrifugation, the pellet was resuspended in 1/20th of the original culture volume of the ice-cold lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 0.2 mM mercaptoethanol, and 0.1% Triton X-100 at pH 7.8) and sonicated for 1 min, with Branson model 450 W, 45 W for cell lysis.

Genes *cpcA*, *cpcE*, and *cpcF* from *M. laminosus* were inserted as described above for *aphA*. Plasmid-transformed *E. coli* strains were used to prepare CpcA, CpcE, and CpcF as reported previously (*52*).

*PCB Isolation.* Methanol-washed *Spirulina platensis* (spray-dried, Behr, Bonn, Germany) was refluxed in methanol and purified by chromatography on silica RP8 (ICN, Eschwege, Germany) (*11*). The PCB concentration was determined spectroscopically in methanol containing HCl (2%), using an extinction coefficient of $\epsilon = 37\,900\ M^{-1}\ cm^{-1}$ (*54*).

*Phycobiliproteins.* CPC was prepared from *M. laminosus* by chromatography on DEAE cellulose (*13*). It was dissolved in potassium phosphate buffer (KPP) (100 mM at pH 7.1), dialyzed 3 times against KPP (1 M at pH 7.1), and centrifuged at 12000*g* for 10 min (*55*) to yield a precipitate of pure, microcrystalline CPC, which can be readily redissolved in KPP-NaCl buffer [50 mM, NaCl (500 mM) at pH 7.2]. α- and $\beta$-CPC were isolated by isoelectric focusing (*56*). All chromophores and phycobiliproteins were quantified by absorption spectroscopy as described (*57*, *58*).

*Reconstitution of Holoproteins: Autoassembly of Apo Phytochrome and PCB.* Three methods were used to prepare the holoproteins: (a) The first method used the 12000*g* supernatant of the sonicated *E. coli* cells overexpressing *aphA*. A total of 6 $\mu$L of the PCB solution (1 mM in DMSO) was added to 600 $\mu$L of the supernatant (end concentration of PCB 10 $\mu$M) and incubated at ambient temperature for 3 h. (b) In the second method, 6 $\mu$L of the PCB solution was added to 600 $\mu$L of the sonicated cell slurry and incubated at ambient temperature for 3 h. (c) In the third method, 6 $\mu$L of the PCB solution was added to 600 $\mu$L of the cell suspension and then cosonicated in an ice bath for 1 min and then incubated at ambient temperature for 3 h. All samples were centrifuged after incubation at 12000*g* for 15 min, to remove cell debris, and then applied to a $Ni^{2+}$-affinity column for purification before spectral analysis. The elution buffer for the affinity column (0.6 mL) included 1 M imidazole, 20 mM potassium phosphate (pH 7.2), and 500 mM NaCl. If not stated otherwise, the eluates containing the tagged chromoproteins were dialyzed against imidazole-free buffer before use.

*Reconstitution of AphA with CPC.* For reconstitution with CPC, 12 mL of cells overexpressing *aphA* were collected and washed twice with distilled water. They were resuspended in 0.6 mL of the ice-cold buffer [potassium phosphate (16 mM) and Tris-HCl (100 mM) at pH 8.0] containing NaCl (166 mM), $Mg^{2+}$ (5 mM), and mercaptoethanol (5 mM). The final concentration of AphA was 30 $\mu$M. A total of 10 $\mu$L CPC, α-CPC, or $\beta$-CPC (120 $\mu$M) was added, and the total

reaction volume was adjusted to 600 $\mu$L by adding distilled water. If desired, CpcE/F was added to a concentration of 10 $\mu$M. The mixture was sonicated for 1 min and incubated at 37 °C for 3 h. Before spectral analysis, the sample was centrifuged at 12000*g* for 15 min. Purification via Ni-affinity chromatography was done as described above for the proteins reconstituted with PCB.

*Determination of Extinction Coefficients of Reconstituted Holoproteins.* On the basis of the extinction coefficient of PCB in CPC in 8 M acidic urea of $\epsilon = 35\,500$ $M^{-1}$ $cm^{-1}$ (*58*, *59*), the extinction coefficients were determined as 74 000 $M^{-1}$ $cm^{-1}$ for AphA, 73 000 $M^{-1}$ $cm^{-1}$ for AphA(1−490), 70 000 $M^{-1}$ $cm^{-1}$ for AphA(1−320), and 70 000 $M^{-1}$ $cm^{-1}$ for AphA(26−320). All quantitative data of holoproteins including kinetic data were calculated with these extinction coefficients.

*Protein Assay.* Protein concentrations were determined by the Bradford assay (*60*), calibrated with bovine serum albumin. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) was performed by Laemmli's method (*61*). Proteins were stained with Coomassie brilliant blue, and chromophore-containing ones were identified by $Zn^{2+}$-induced fluorescence (*62*). SDS−PAGE gels were soaked in zinc acetate (1 M) for 1 h before detection and photographed under UV light (356 nm).

*Spectral Analyses.* The chromoproteins were investigated by UV−vis absorption spectroscopy (model Lambda 25, Perkin−Elmer, Shelton, CT). Photoreactions were carried out with a fiber-optic cold light (150 W, Volpi, Switzerland), equipped with suitable interference filters (15 nm of full width at half maximum). Spectra were recorded in the 300−900 nm range, after saturating irradiation with 650 or 700 nm of light for AphA reconstituted with PCB. Fluorescence spectra were recorded with a model F-4500 spectrofluorimeter (Hitachi, Tokyo, Japan).

## RESULTS

*Expression and Reconstitution of AphA with PCB.* Full-length and truncated AphA polypeptides were expressed with His and S tags to facilitate purification of the reconstituted products. All apoproteins, as well as their reconstituted holoproteins, were reasonably soluble, which has been reported for other cyanobacterial phytochromes (*17*, *63*), as long as expressions were maintained at low levels. However, induction with IPTG for 12 h as used in previous studies (*18*, *19*) resulted in the formation of inclusion bodies and poorer yields of reconstituted protein. This effect was most pronounced with the truncated proteins, irrespective of the reconstitution method used (Table 1). There was no such effect for reconstitution of full-length AphA using methods b and c. The three methods differ in the form in which the apoprotein was presented; crude extracts (methods a and b) gave considerably higher yields than purified AphA. Best results were consistently obtained by method c, using cosonication of PCB with AphA-containing cells induced for only 6 h, indicating that some factors from the cell debris may somehow assist the reaction.

All reconstitutions were monitored by absorption spectroscopy and light-induced absorption changes. As an example, the results of AphA(1−320) are shown in Figure 2; other reconstituted proteins (see below) gave similar spectra. Interactions between the chromophore and apoprotein are minimal after acidic urea denaturation, the absorption spectrum difference must then reflect the molecular structures of the PCB chromophore induced by the irradiation with light of different wavelengths. The characteristic absorption differences of reconstituted AphA were retained after denaturation of the two spectral forms with acidic urea (8 M) in the dark; the absorption maxima of Pr and Pfr forms were at 660 and 610 nm, respectively (Figure 3), corresponding to the absorption of PCB-cysteine adducts with 15*Z* and 15*E* configuration (*64*, *65*). Except for a small blue shift because of the ethyl group instead of a vinyl group at C-18, the spectra in the visible range are also well-comparable to those of plant phytochrome denatured in the respective state (*66*). The absorption increase of denatured Pr, as compared to Pfr, in the near UV was less pronounced. We ascribe this to interference from scattering, which was often observed in these samples. Covalent binding of PCB to apoprotein was verified via SDS−PAGE combined with $Zn^{2+}$-induced fluorescence (*62*) (Figure 4).

Table 1: Reconstitution Efficiencies of Full-Length and Truncated AphA with PCB[a]

| protein | plus | $c_{\text{aporotein}}$ ($\mu$M) in crude extract | $c_{\text{aporotein}}$ ($\mu$M) purified | relative yield (%) |
|---|---|---|---|---|
| AphA | PCB | 53 | 23 | 45 |
| AphA(1−490) | PCB | 51 | 23 | 51 |
| AphA(1−320) | PCB | 55 | 25 | 78 |
| AphA(26−320) | PCB | 61 | 27 | 76 |
| AphA(26−320) | PC + CpcE/F | 61 | 27 | 103 |
| AphA(26−320) | CPC | 61 | 27 | 98 |

[a] Comparison of the method using the crude extract of overexpressing *E. coli* cells and using the purified AphA proteins (see the Materials and Methods for details).

The absorption (Figure 5) and difference spectra (Figure 6) of full-size AphA-PCB and truncated AphA(1−490)-PCB, AphA(1−320)-PCB, and AphA(26−320)-PCB had similar characteristics in the visible and near-UV regions as reconstituted classical cyanobacterial phytochromes such as Cph1 (*17*−*19*). Quantitatively, the amplitude of the light-induced difference signal was only about 60% of that of Cph1-PCB (e.g., see ref *3*). A test using our irradiation setup with PCB-Cph1 gave nearly the same amplitude of the difference signal (90%) as that shown in ref *3*. We therefore consider the lower amplitude of AphA real. The maxima in the visible region were determined by 2nd derivative spectroscopy. They were shifted to the blue in the truncated chromoproteins (Figures 5 and 6 and Table 2). This blue shift increases with increasing truncation in AphA(26−320). While the direction is similar, the shift is somewhat more pronounced in Pfr ($\leq$10 nm) than in Pr ($\leq$6 nm), indicating a greater sensitivity of the former. Note that the blue shift is enhanced by imidazole (1 M), which is frequently used for elution of His-tagged proteins from Ni columns (Table 2).

For full-length AphA-PCB, the absorption spectra of Pr and Pfr were separated, assuming that as in Cph1-PCB from *Synechocystis* sp. PCC 6803 (*18*) the percentage of $[\text{Pfr}]_{\text{max}} = 68\%$ after irradiation with red light. These spectra were very similar to those of Cph1 reconstituted with PCB (not shown). In the truncated PCB-containing chromoproteins, the Pr and Pfr bands overlapped more strongly, because of broadening (Figure 5), which is also a factor in type-II

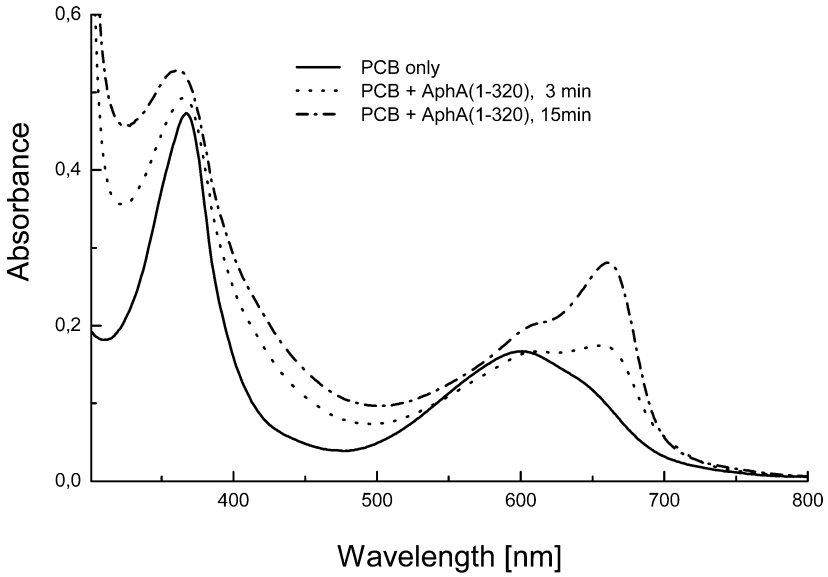


FIGURE 2: Changes in absorption spectra during auto-assembly of AphA(1−320) (25 $\mu$M) with PCB (5 $\mu$M). Spectra at $t = 3$ (- - -) and 15 min (-·-) after addition of PCB and at PCB (—) in the reconstitution buffer at the concentration used for reconstitution.

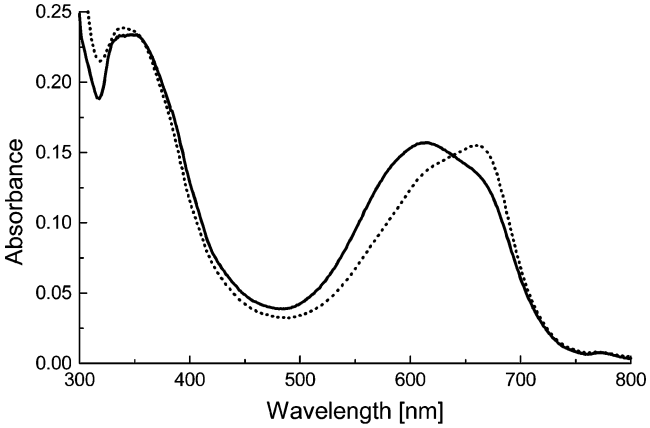


FIGURE 3: Absorption spectra of AphA(26−320) reconstituted with PCB, after denaturation with urea (8 M)/HCl (pH 2). The broken line corresponds to denaturation after saturating irradiation of the native sample with 700 nm of light, which contains mainly *Z*-PCB. The solid line corresponds to the sample denatured after saturating irradiation with 650 nm of light, which contains mainly *E*-PCB.

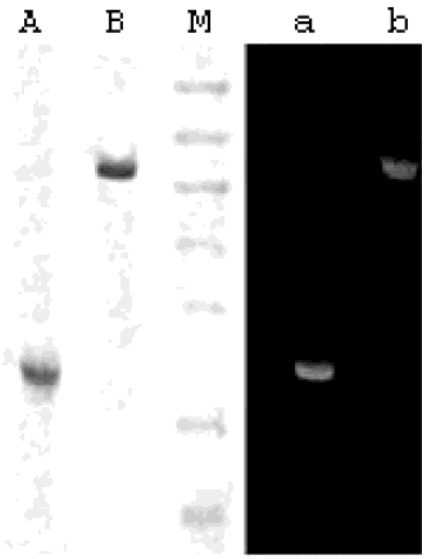


FIGURE 4: SDS−PAGE of reconstituted AphA(1−320) (lanes A and a) and AphA (lanes B and b), detected by Coomassie staining (right) and $Zn^{2+}$-induced fluorescence (62) under UV (left). Lane M, protein markers (from top to bottom: 180, 116, 84, 58, 48.5, 36.5, and 26.6 kDa).

photochemistry of α-PEC (*7*). Truncation also decreased the amplitudes of the photochemistry (Δ$\Delta A$ of Table 2), again, as expected for line-broadening. Attempts failed to separate the individual spectra of the truncated chromoproteins as done above for full-length AphA-PCB. The Pfr maxima differed considerably from those obtained from 2nd derivative or from the light-induced difference spectra (Table 2). Obviously, the assumption of a photoequilibrium with 68% Pfr is no longer correct, which we ascribe again to the line broadening. In the Pr form, all reconstituted AphA-PCB and fragments showed the typical fluorescence of phytochrome, which is lost in the Pfr form (not shown). This behavior is paralleled by α-PEC in the same species *Anabaena* sp. PCC 7120; it also fluoresces only in the *Z* form, absorbing around 570 nm, but not in the *E* form, absorbing around 500 nm.

None of the other three truncated proteins, AphA(174−320), AphA(1−175), and AphA(32−320), could be reconstituted with PCB, alone or in the presence of phycobilin lyases. The reconstitution of AphA(32−320) is shown in Figure 5f as an example for such a negative result. AphA-(174−320) corresponds to the highly conservative chromophore domain binding PCB. The failure in reconstitution indicates that (a) the binding site is no substrate for the PCB:CpcA or PVB:PecA lyases (see below on this activity) and (b) the truncated protein lacks a functional lyase domain. AphA(1−175) (which lacks the binding cysteine) and AphA-(32−320) could also not be reconstituted with PCB. Because AphA(1−320) and AphA(26−320) did reconstitute autocatalytically (see above), this indicates that the region of aa 26−31 is required for chromophorylation in our N-tagged AphA construct.

An intermolecular cooperation among two fragments was tested by generating, from AphA(1−320), the two proteins AphA(1−175) (i.e., aa 1−175), carrying the putative lyase activity, and AphA(174−320) (i.e., aa 174−320), carrying

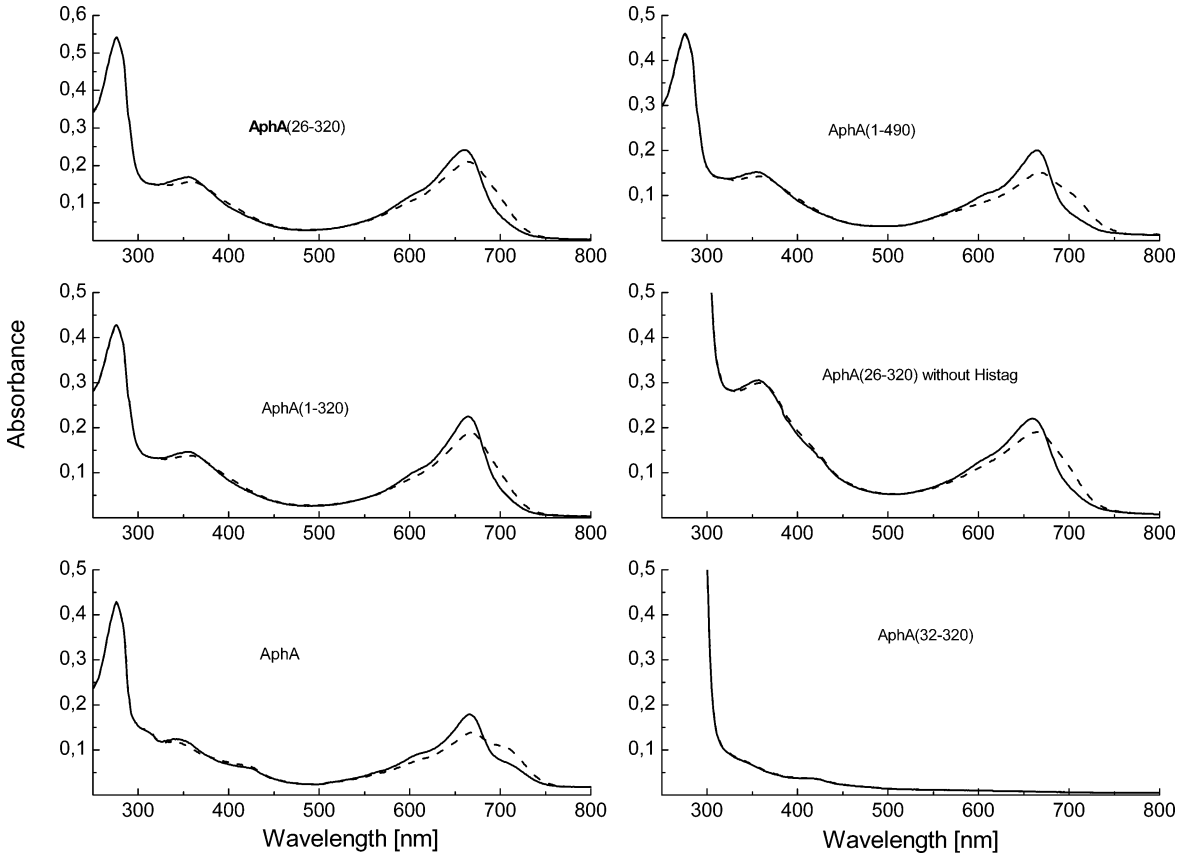


FIGURE 5: Absorption spectra of the reconstitution products of full-length and truncated AphA with PCB, after irradiation with 650 nm of light (- - -) and 700 nm of light (—). All products were purified over a $Ni^{2+}$-chelating column and dialyzed subsequently to remove imidazole.

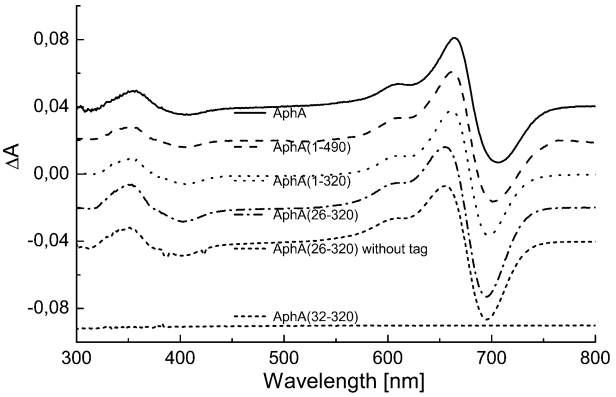


FIGURE 6: Ligh-induced difference spectra of full-size and truncated AphA reconstituted with PCB. Details are the same as in Figure 5.

the highly conserved chromophore-binding domain. When the two AphA fragments were incubated with PCB, no reconstitution product was formed, as judged from absorption and light-induced difference spectroscopy (not shown).

*Reconstitution of AphA with Phycocyanin and Its Subunits.* The PCB:CpcA lyase is known to catalyze the addition as well as the removal of PCB from CpcA (*42*). Considering the lyase activity of phytochromes, one may ask if phytochromes can also cleave their chromophores autocatalytically and possibly also catalyze the transfer to and from other biliproteins. While we have no evidence for the former, the latter idea was indeed verified by the chromophorylation of AphA and truncated AphA in the presence of CPC (summarized in Table 3). Product yields were determined from the photoactivities of the affinity-purified chromoprotein. They were generally low, but the difference spectra are qualitatively identical to those of chromophorylated phytochromes. As an example, the difference spectrum is shown of AphA(26−320) in Figure 7a (top, - - -). Obviously, apo-AphA is able to catalyze PCB release from CPC, albeit only slowly, because CPC is probably not the true substrate of phytochrome lyase activity. The activity of the truncated AphA(26−320) is almost twice as high as with full-length AphA (Table 3). Enzyme kinetics for the chromophore transfer have therefore been studied using this truncated acceptor. The results for this direct transfer from CPC to AphA(26−320), in the absence of any other protein, are shown in Figure 8a. The affinity for CPC is relatively high ($K_m = 2.6\ \mu M$), but the transfer rate is low ($v_{max} = 71$ pmole $min^{-1}$), resulting in $k_{cat} = 7.1 \times 10^{-6}\ min^{-1}$. Optimum chromophore transfer has been obtained at pH 7 and ∼37 °C (Figure 1 of the Supporting Information).

PCB transfer from CPC was considerably enhanced by addition of CpcE/F to the above reconstitution system. The reconstitution efficiency was improved about 4.5 times (see Figure 7a and also Figure 9), owing probably to CpcE/F catalyzing the release of PCB from CPC, with the released PCB being recruited by apo phytochrome, AphA. The reconstitution efficiencies of the different AphA fragments again depend on the length of apoprotein (Figure 7b and Table 3). In this case, the efficiency of the full size of AphA was only about 20%, compared to that of the most active fragment, AphA(26−320). The affinity of this truncated protein for CPC in the CpcE-catalyzed reaction is similarly high as for the uncatalyzed reaction ($K_m = 3.1\ \mu M$), but the transfer rate is increased by more than 2 orders of magnitude

Table 2: Spectral and Photochemical Properties of AphA and Its Truncated Derivatives, Reconstituted with PCB in the Absence and Presence of 1 M Imidazole

| | absorption spectra | | | difference spectra | | | |
|---|---|---|---|---|---|---|---|
| chromoprotein | $\lambda_{\lambda_{max},Pr}$ | $\lambda_{\lambda_{max},Pfr}$[a] | $\lambda_{\lambda_{max},Pfr}$[b] | $\lambda(\Delta A_{max})$ | $\lambda(\Delta A_{min})$ | $\Delta A_{max}/\Delta A_{min}$ | $\Delta\Delta A$ (%)[c] |
| AphA | 666/666 | 706/703 | 702/698 | 664/664 | 706/705 | 1.2/1.36 | 46/54 |
| AphA(1−490) | 665/665 | 704/698 | 687/687 | 662/663 | 702/702 | 1.1/1.13 | 41/50 |
| AphA(1−320) | 665/656 | 697/692 | 670/670 | 660/649 | 697/695 | 1.0/0.79 | 34/33 |
| AphA(1−320) (V31L) | nd/660[d] | nd/691 | nd/670 | nd/655 | nd/696 | nd/1.13 | nd/35 |
| AphA(26−320) | 660/654 | 696/695 | 670/675 | 655/646 | 695/693 | 0.68/0.6 | 37/52 |

[a] Maxima determined from second derivative spectra. [b] Maxima of component spectra, resolved assuming 68% Pfr in the equilibrium mixture (according to ref 18). [c] Amplitude of difference signal, normalized to absorption of Pr (see ref 7 for details). [d] nd = not determined.

Table 3: Yields of Photoactive Products from Full-Length and Truncated AphA (30 $\mu$M) Reconstituted with CPC or Its and Subunits, α- or $\beta$-CPC (2 $\mu$M), with or without the Addition of the PCB:CpcA Lyase, CpcE/F (10 $\mu$M)[a]

| | | | product yield | |
|---|---|---|---|---|
| acceptor protein | donor biliprotein | enzyme | absolute ($\mu$M) | relative (%)[b] |
| AphA | CPC | | 0.06 | 13 |
| AphA | CPC | CpcE/F | 0.19 | 42 |
| AphA(1−490) | CPC | CpcE/F | 0.21 | 48 |
| AphA(1−320) | CPC | CpcE/F | 0.39 | 88 |
| AphA(26−320) | CPC | | 0.11 | 24 |
| AphA(26−320) | CPC | CpcE/F | 0.45 | 100 |
| AphA(26−320) | α-CPC | | 0.08 | 18 |
| AphA(26−320) | α-CPC | CpcE/F | 0.44 | 98 |
| AphA(26−320) | $\beta$-CPC | | 0.06 | 13 |
| AphA(26−320) | $\beta$-CPC | CpcE/F | 0.06 | 13 |

[a] Reactions were carried out with the crude lysates of *E. coli* cells overexpressing full-length or truncated *aphA*, for 3 h in the presence and 6 h in the absence of CpcE/F. The samples were then centrifuged at 12000*g* for 15 min, and the amounts of photoactive products were calculated from the difference spectra and the extinction coefficients (determined in the Materials and Methods). [b] Maximum yield obtained with AphA(26−320) *plus* CPC and CpcE/F, was arbitrarily set to 100%. The concentration of photoactive chromoprotein in this sample was 0.45 $\mu$M, which corresponds to a yield of 22.5% with respect to the acceptor protein (see the Materials and Methods for details).

($v_{max}$ = 10.3 nmole min$^{-1}$), resulting in a $k_{cat} = 1.05 \times 10^{-3}$ min$^{-1}$ (Figure 8b). Optimum chromophore transfer under catalysis of CpcE/F was again obtained at temperatures around 37 °C, but the pH optimum is shifted to pH 8 (Figure 1 of the Supporting Information).

CPC is comprised of two subunits, α and $\beta$. It has been shown that CpcE/F acts on α-CPC about 10 times faster than on $\beta$-CPC (*42*). Reconstitution was therefore tested using the subunits as donors for AphA(26−320). The results, using equal amounts of apoproteins, are summarized in Table 3. The reconstitution efficiency of AphA with $\beta$-CPC was low, in the absence as well as in the presence CpcE/F. Only a residual light-induced difference signal is obtained in both cases (Figure 7). This slow, CpcE/F-independent reconstitution is again ascribed to the lyase activity of AphA. The uncatalyzed reconstitution efficiency with α-CPC as the donor is similarly low, but it becomes as high as with integral CPC in the presence of CpcE/F. These variations in the chromophore transfer efficiencies to AphA(26−320) catalyzed by CpcE/F agree with the well-established preference of the latter for α-CPC (*42*). This preference is further supported by the absorption changes observed during the reaction. The absorption maxima of α-CPC, $\beta$-CPC, and CPC were at 618, 605, and 614 nm, respectively. After incubation of CPC ($\lambda_{max}$ = 614 nm) with AphA(26−320) and CpcE/F,

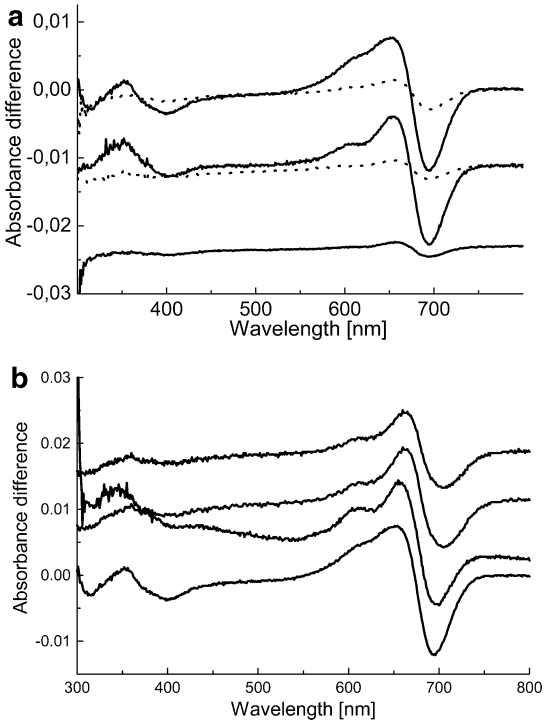


FIGURE 7: (a) Difference spectra of AphA(26−320) reconstituted with CPC and its subunits, in the absence (···) and presence (—) of CpcE/F. Top, donor = CPC; center, donor = α-CPC; and bottom, donor = $\beta$-CPC. Donor and acceptor concentrations were the same in all cases (see the Materials and Methods). (b) Difference spectra of full-size and truncated AphA reconstituted with CPC in the presence of CpcE/F. From top to bottom: AphA, AphA(1−490), AphA(1−320), and AphA(26−320).

there was an increase of absorption at 650 nm because of chromophorylation of the phytochrome (Figure 9), and at the same time, the absorption maximum of the donor chromoprotein shifted to 606 nm. This is close to that of $\beta$-CPC ($\lambda_{max}$ = 605 nm), indicating a selective loss of chromophore from the α subunit. A quantitative analysis of these absorption changes using the known extinction coefficients (*67*) indicates that PCB supplied by α-CPC was quantitatively transferred to AphA(26−320*)*. We also finally tested α-PEC as the donor, which contains the PVB chromophore that is isomeric to PCB and more stably attached (*44*). Reconstitution of AphA(26−320) is not possible at all with this chromoprotein, as judged from the absence of any photoactivity (not shown).

## DISCUSSION

The bacterial phytochromes have considerably widened our view on these photosensory pigments (*1*, *24*, *68*).

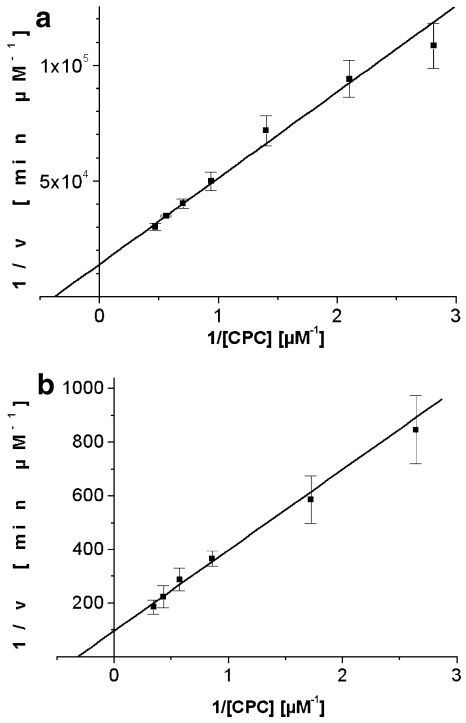


FIGURE 8: Kinetics of the chromophore transfer reaction with CPC as the donor substrate for AphA(26−320) (27 $\mu$M), (a) in the absence and (b) in the presence of PCB:α-CPC lyase (9.8 $\mu$M). Lineweaver−Burke plot resulting in the following parameters: (a) $K_m = 2.63 \pm 0.4$ $\mu$M, $v_{max} = 71 \pm 6.5$ pmol min$^{-1}$, $k_{cat} = (7.1 \pm 0.58) \times 10^{-6}$ min$^{-1}$ and (b) $K_m = 3.1 \pm 0.7$ $\mu$M, $v_{max} = 10.3 \pm 1.5$ nmol min$^{-1}$, $k_{cat} = (1.05 \pm 0.17) \times 10^{-3}$ min$^{-1}$.

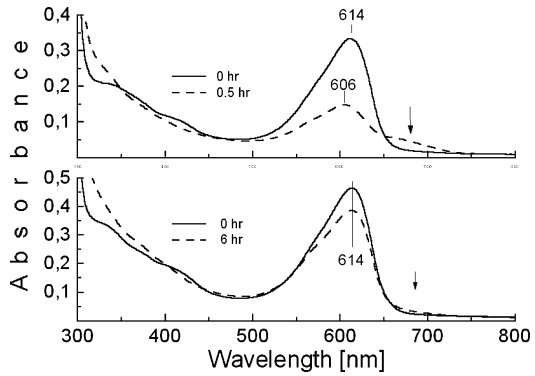


FIGURE 9: Absorption spectra of phycocyanin before (—) and after (- - -) chromophore transfer from CpcA to AphA(26−320), in the presence of CpcE/F (top) and in the absence of CpcE/F (bottom).

Structurally, the phytochrome family seems to be comprised of two distinct groups, which are distinguished by their chromophore binding; plant phytochromes and the classical cyanobacterial phytochromes (Cph1 type according to ref *24*) bind a chromophore carrying a 3-ethylidene group at C-3 (PCB and PΦB) to a cysteine located approximately 260 aa from the N terminus. Some cyanobacterial phytochromes and those of many other photosynthetic and nonphotosynthetic bacteria (Cph2 type according to ref *24*) bind a chromophore carrying a 3-vinyl group, at C-3$^1$, viz., biliverdin (*69*−*71*), and here, a conserved cysteine close to the N terminus (Cys-20) has recently been proven as the binding site (*1*). The result obtained here with AphA clearly group it to the classical phytochromes: (i) it lacks the N-terminal binding motif but carries the one around cysteine 259; (ii) chromophore binding is retained if the N terminus is truncated by 26 aa; and (iii) the 3-ethylidene bilin, PCB, is bound autocatalytically.

*Chromophore Ligation.* Three aspects related to the chromophore are characteristic for phytochromes, as compared to most phycobiliproteins: (a) the autocatalytic chromophore ligation, (b) the *Z*/*E* photoisomerization of the chromophore, and (c) the strong red shift of the *E*-chromophore in Pfr. Members of the other major class of biliproteins, the phycobiliproteins, generally lack these properties, with the notable exception of α-PEC; it is photochromic too but lacks the red shift of the *E* isomer (*7*) and needs a lyase for chromophore attachment (*11*). The situation is further complicated by the failure of some phytochrome-like proteins (RcaE and ClkA) to bind the chromophore properly in vitro (*4*, *39*, *74*) and, on the other hand, by the observation of the typical photochemistry with noncovalently bound chromophores (*75*−*77*).

Some progress has been made recently in separating the aforementioned three effects and relating them to specific sections of the phytochrome protein. Using truncated or mutated cyanobacterial phytochromes (Cph1 and Cph2 from *Synechocystis* PCC 6803), Wu and Lagarias (*3*) have identified 2000 by site-directed mutagenesis and truncations a PAS domain (P3) with about 130 aa, which carries the lyase activity but leads to a photochemically incompetent chromophore. Insertion into this of a stretch of about 23 aa N-terminally adjacent to the binding cysteine 259 conveys some basic photoactivity, and full photoactivity requires more C-terminal parts of the protein. This domain is N-terminal in Cph2 but preceded by approximately 150 aa in many bacterial phytochromes. In AphA, it extends from aa 153−310, and it is therefore retained in most of the truncated proteins studied here. This includes in particular AphA(32−320), which does not reconstitute. The lyase domain includes an essential glutamate (*3*) (E189 in AphA), as well as 20 aa neighboring N-terminally the chromophore-binding cysteine (*4*), which is also essential for photoactivity (*3*). We therefore conclude that in AphA the lyase domain P3 is not sufficient for chromophore binding. Because the slightly longer AphA-(26−320) shows autocatalytic chromophore binding, an N-terminal motif IQPHGV starting at isoleucine 26 is required for lyase activity, which is absent in Cph2 (there is no interference from the N-terminal tags, see below).

In phytochromes from higher plants, a charged residue (generally histidine) C-terminal to and next to the binding cysteine is necessary (*28*, *72*) for chromophore binding and, in a more stringent fashion, for photochemistry. This histidine is present in many bacterial phytochromes as well, including AphA. It also seems to be required in phytochromes binding biliverdin at C-20, which often lack the conserved cysteine of classical phytochromes (*1*, *9*), pointing to an interaction of these two regions during chromophore attachment. Such an interaction is further supported by mutation studies, in particular with PhyA from pea (*73*). Besides a putative short amphiphilic helix located about 80 aa C-terminal of the chromophore-binding cysteine 323, it also requires a region approximately 240 aa N-terminal of the chromophore-binding cysteine. In particular, isoleucine 80 was essential for both lyase activity and photochemistry.

Our results support the importance of the latter region; the IPQHGV-segment (aa 26−31), whose presence was necessary for lyase activity in AphA, is located at a similar

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AphA:        26-IQPHGvLLVLEEpgLkIlQVSnNtwgi
AphB:        27-IQPHGvLLVLQEvdLtIlQVSnNtfni
PhyA(pea):   80-IQPFGcLLaLdEktckvvaysenapem
Cph1:        26-IQPHGlvvVLQEpdLtIsQISaNctgi
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FIGURE 10: Common lyase motif for plant and bacterial phytochromes. The leading aa have been proposed to be essential in both plants (*73*) and cyanobacteria (this paper). Motif search has been done with the "MUSCA" multiple sequence alignment tool (http://cbcsrv.watson.ibm.com/Tmsa.html), using the "structural character equivalency set". With the chemical nature equivalency set, the motif is even extended by 17 aa acids longer. Similar results were obtained with other programs. The sequences shown are, from top to bottom, AphA, AphB, PhyA from pea, and Cph1.

distance N-terminal from the chromophore and has a high similarity (IPQFGC) to pea PhyA. Isoleucine 26 of the former is homologous to isoleucine 80 of the latter. We are aware that most of our proteins contain an N-fused tag region, which may influence folding and function of the protein, in particular when these functions involve aa close to these tags. We feel relatively confident, however, that there is no such interference. On the basis of the rapid reconstitution and the properties of the chromoprotein, AphA and all its lyase-active fragments behave like other phytochromes, despite the relatively large tag. Also, the critical construct AphA(26−320) has been produced without tags and shows identical lyase activity and photochemistry of the chromoproteins as the tagged ones (Figures 5 and 6).

A distinct role of this segment in the autoassembly of phytochromes, is further supported by the ligation difference between AphA(1−320) and AphA(1−320) (V31L) (Table 2). According to GOR IV prediction (*78*), these six aa are in a random-coil region. Similar sequences are found in a number of other phytochromes, including both members of the PΦB- or PCB-binding plant-like group and of the new (*1*) biliverdin-binding bacterial group. Examples of the former are Cph1 from *Synechocystis* sp. PCC 6803 (Q55168) with a homologous sequence near the N terminus (note that it is also contained in all lyase-active mutants of this protein investigated by Wu and Lagarias (*3*) to identify the lyase domains), as well as PhyE from *Pharbitis nil* (P55004) and PhyB from *Arabidopsis thaliana* (P14713), where the homologous sequences are more C-terminal beginning at aa 78 and 114, respectively. Examples of the second group are AphB from *Anabaena* sp. PCC 7120 (Q9R6X3), BphP from *Deinococcus radiodurans* (Q9RZA4) and BphP from *Pseudomonas aeruginosa* (Q9HWR3). Similarities in this region are recognized by all sequence analysis tools that we tested. An example is shown in Figure 10; using the IBM "MUSCA" alignment program with the structural equivalency set for the aa, a 23 aa motif is produced, which begins with the aforementioned isoleucine, and is common to plant and classical cyanobacterial phytochromes. It even extends 17 aa further using the "chemical nature equivalency set"; therefore, the 23 aa motif is probably a conservative estimate. In many phytochromes including AphA, this motif is not in a classified domain, while in others it is in a domain classified as PAS [domain P2 in the nomenclature of Montgomery and Lagarias (*24*)], albeit not a chromophore-binding one (on the basis of Expasy search 21.4.04).

A model has been proposed from studies with PhyA from pea (*73*) in which the N-terminal protein region containing the critical isoleucine 80 and the C-terminal amphiphilic region interact with different pyrrole rings of the chromophore, the latter with ring A and the former with ring D, including in particular the C-18 substituent. This substituent is different in the native chromophores of plant and classical cyanobacterial phytochromes; PΦB present in the former contains a 18-vinyl group, and PCB present in the latter contains an 18-ethyl-group. Interestingly, this chromophore difference is paralleled by a sequence difference in the N-terminal pattern identified above; in particular, the sequence IPQHGV found essential in this study is present in many bacterial phytochromes, including nonclassical ones, while the corresponding sequence with two replacements, IPQFGC, is characteristic for plant phytochromes. We note that in vitro both classes of proteins bind PCB as well as PΦB and show the characteristic photochemistry of phytochromes, as well as the fact that most studies on cyanobacterial phytochromes used in vitro reconstitution of proteins expressed in *E. coli*. The identification of a PCB chromophore in holo-*Cph1* isolated from *Synechocystis* sp. PCC 6803 (*79*) lends support to this differential preference for the two chromophores in vivo and may point to some more subtle differences in the chromophore−protein interactions, either with respect to the details of the photoreaction or the ensuing signal transduction.

Interestingly, this N-terminal region is absent in Cph2, which was studied in detail by Wu and Lagarias (*3*) to disentangle the protein regions required for ligation and photochemistry. Here, the P3 domain is immediately N-terminal. Because this phytochrome binds PCB and forms photoactive products, one might therefore expect it to be replaced by another domain now located more C-terminal. We have been unable, however, to spot with reasonable significance a homologous pattern anywhere else in the sequence. We note, however, another difference among Cph2 and the other phytochromes, which is the second binding site for a chromophore near the C terminus. Originally proposed on the basis of sequence homologies, autocatalytic chromophore binding to a truncated Cph2 lacking the N-terminal binding site has been verified by in vitro reconstitution with PCB, but the resulting chromopeptide was photochemically inactive (*3*). One might then expect full-length Cph2 to bind two chromophores, one active and one inactive, resulting in a strongly reduced photochemical signal. Unfortunately, the published data are inconclusive concerning this possibility. Wu and Lagarias (*3*) show only absorption spectra of truncated proteins containing either one of the binding sites, and Park et al. (*19*) show only a difference spectrum of reconstituted full-length protein. The latter authors did show, however, that a full-length mutant lacking cysteine 129 of the conventional binding site does not bind a chromophore to the C-terminal motif, seemingly in contradiction with the successful reconstitution of this site in a C-terminal fragment (*3*). When the chromophore transfer activities among biliproteins discussed in the following section are taken into account, this contradiction may be resolved if one of the binding sites is only transiently occupied and transfers the chromophore to the other. Clearly, more work is required to characterize these two sites and there interaction.

In summary, our results support the model proposed by Bhoo et al. (*73*) for PhyA from pea that a motif approximately 250 aa N-terminal of the chromophore-binding

cysteine is required for lyase activity and extends it to a large number of prokaryotic phytochromes and phytochrome-like proteins. when taken together with the mutation studies with the relatively homologous Cph1 (3) and viewing Cph2 as an exception, it then appears that the lyase activity in the plant and the Cph1-type phytochromes involves interactions between the N-terminal sequences found here in the P2 domain and the more C-terminal 130 aa motif around the chromophore-binding site in the P3 domain (nomenclature of ref *22*). Interactions between the N-terminal domain and the chromophore have been well-documented for plant phytochromes (e.g., see ref *80*), where the N-terminal 6-kDa region exerts a red shift on the chromophore. The latter is, however, not the domain that is required for chromophore attachment but rather precedes the motif discussed here; in pea, for example, it ends already 20 aa N-terminal from the isoleucine 80, starting the motif identified in this study. Interestingly, the motif always contains histidine or cysteine, aa known to interact with or capable of covalent binding to PCB, respectively. It is therefore conceivable that in the native protein this motif interacts with the chromophore-binding site around cysteine 259, in a fashion which is complementary to the interaction of the conserved histidine in this region with the N-terminal cysteine 20 recently identified as the binding aa in the "nonclassical" phytochromes (*1*). A direct test of such an interaction failed; no chromoprotein was obtained by incubation of PCB joined with the two fragments AphA(1−175) (i.e., aa 1−175), carrying the putative lyase activity, and AphA(174−320) (i.e., aa 174−320) carrying the highly conserved chromophore-binding domain. The integrity of the protein therefore seems crucial for the autoassembly. Tertiary structure elements may of course be changed critically in the fragments, as compared to the folding of the respective sections in the integral protein. Because there is presently no phytochrome structure known, this negative experiment is therefore not conclusive.

*Chromophore Exchange.* Besides the autocatalytic chromophore ligation of phytochromes, the reconstitution experiments with AphA and its truncated apoproteins indicate a new activity, i.e., chromophore exchange from CPC to phytochrome. Absorption and difference spectra of the purified chromoproteins obtained with CPC as the chromophore donor were the same as those of the products reconstituted with free PCB. Therefore, apo phytochromes not only catalyze ligation of the chromophore (*72*), but also can cleave the bound chromophore from other biliproteins. In view of the high concentrations of PCB-bearing phycobiliproteins in cyanobacteria, it is conceivable that this reaction might occur in vivo too. The reversibility of chromophore ligation has also been implicated in biliprotein degradation, although specialized lyases such as NblB may be responsible for this process in vivo (*43*).

Chromophore exchange from CPC to AphA is strongly enhanced by the addition of the PCB:CpcA lyase and CpcE/F. Previous studies have demonstrated that CpcE/F constitute the lyase for α-CPC, catalyzing the ligation of the PCB chromophore with apo-α-CPC (=CpcA) at Cys-α-84, and also its cleavage (*42*). When CpcE/F was present in the apo phytochrome/CPC chromophore exchange system, the reconstitution efficiency was increased by almost an order of magnitude. Obviously, the release of PCB from CPC catalyzed by CpcE/F, cooperates with the autocatalytic ligation of AphA. As judged from the absorption spectra (Figure 9) and the experiments with isolated subunits, this CpcE/F-dependent reaction preferentially involves α-CPC as the chromophore donor, as expected (*81*). It is currently not clear if any free chromophore is released in the process, because the catalytic mechanisms are unknown for both CpcE/F and the intrinsic phytochrome lyase. However, the latter process seems to be unlikely in view of the failure of CpcE/F to increase binding of PCB to AphA.

The results open the possibility that phytochromes may not only be sensory receptors for free bilin chromophores (see ref *24* for leading references), but also that chromophores may be exchanged among different biliproteins, in particular among phytochromes and phycobiliproteins. While heterologous in vivo reconstitution systems have been established for phytochromes (*24*) as well as for phycobiliproteins (see ref *82*), little is presently known, to our knowledge, on the metabolic flow of tetrapyrroles and the distribution among the two reservoirs of vastly different sizes in cyanobacteria.

*Spectra and Photochemical Competence of Products.* The absorption spectra of the AphA reconstitution products had the characteristics of plant phytochromes, albeit with all absorption bands shifted to shorter wavelengths because of the different chromophores. This is similar to results obtained with other cyanobacterial phytochromes such as Cph1 (*17*−*19*). The *Z*/*E* photoisomerization is fully reversible; irradiation of the *Z* isomer ($\lambda_{max}$ = 650 nm) with 650 nm of light resulted in a new absorption band of the *E* isomer at about 700 nm. Also, all truncated AphA fragments, which reconstitute autocatalytically, show full Pr and Pfr reversibility, as observed for Cph2 (*3*). Absorption and difference spectra were also independent of the reconstitution procedures and purification, as long as imidazole was removed from the solution after Ni-affinity chromatography.

While the type of absorption and difference spectra was retained, the exact positions of the absorption and difference maxima are affected by truncation of the peptide chain. The Pr absorption maximum of full-length AphA-PCB is at 666 nm, but that of the shortest autocatalytic fragments AphA-(26−320)-PCB was at 660 nm. The respective Pfr absorptions blue-shift somewhat more from 706 to 696 nm, and the other-truncated proteins had maxima between these extremes. The difference must be related to an effect of the apoprotein on the chromophore, the details of which are still largely unknown on the molecular level. Experiments on plant phytochromes have long since recognized this point. An N-terminal segment of 6 kDa confers a red shift to phytochrome; its removal results in a small blue shift of 3−4 nm (*80*, *83*). Similar shifts have also been observed for truncated Cph2 (*3*). To our knowledge, no such influences as were seen in these studies with AphA have been reported previously for C-terminal sections of the protein.

We finally note that AphA(26−320) is photochemically competent, with a $\Delta\Delta A$ amounting to 80% of the full-length chromoprotein. This can be compared with the Cph2 (N197) construct showing only 5% of the photoactivity of full-length Cph2. This construct terminates 68 aa C-terminal of the binding cysteine 129, while the photochemically competent AphA(26−320) construct terminates 61 aa C-terminal of the binding cysteine 259. While the sequences are reasonably homologous up to about 40 aa C-terminal of the binding

cysteine (*3*), this homology stops more "downstream". This indicates that the region 40−60 aa C-terminal of the chromophore-binding cysteine is quite important for the photoactivity of the chromoprotein. This region is already part of another PAS domain, P4 in the nomenclature of Montgomery and Lagarias (*22*), who recently summarized the evidence that this region interacts with ring D of the chromophore in phytochrome. When taken together with the results discussed above on the lyase activity, this would suggest that domain P3 interacts both during ligation and in the holoprotein with ring A, while ring D is transferred from domain P2 involved in the ligation to domain P4 in the photochemically competent holoprotein.

According to the "BLAST 2 SEQUENCES" method (*84*), no homology motif was found between the chromophore-binding domains of cyanobacterial phytochrome and α-PEC, let alone the whole proteins. This is surprising because both bind their chromophores covalently to cysteines, both have at least the *Z*-chromophores in a similar conformation and protonation state, and both show basically similar photochromism. From the studies of PVB-PecA and its truncated photochromic chromoproteins from *M. laminosus* (*12*) and *Anabaena* sp. PCC 7120 (Figure 2 of the Supporting Information) and of PCB-AphA and its truncated photochromic chromoproteins from *Anabaena* sp. PCC 7120, it is clear that two rather different photochromic systems can be generated in good yield from the same PCB chromophore. In PVB-PecA, the photochromic system comprises three conjugated pyrroles generated by the isomerase activity of PecE/F, which covers the spectral region around 500−600 nm. In this case, a very small photochromic chromoprotein (13 kDa) could be engineered (*12*). In the PCB-AphA system, the more extended conjugation systems cover the spectral range around 650−750 nm. It is expected that modifications of the apoproteins and the chromophores can further expand this spectral range and adjust the photophysical properties.

## SUPPORTING INFORMATION AVAILABLE

pH and temperature dependence of the chromophore transfer reaction from CPC to AlphA(26−320) in the absence and presence of CpcE/F. Absorption and difference spectra of PecA reconstituted with PCB in the presence of PecE/PecF. Absorption after irradiation with 500 and 570 nm of light and difference absorption (2 figures). This material is available free of charge via the Internet at http://pubs.acs.org.

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