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In vitro assembly of apophytochrome and apophytochrome deletion mutants expressed in yeast with phycocyanobilin

(plant photoreceptor/recombinant phytochrome/phytochrome biosynthesis)

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ABSTRACT **Recombinant pea type I phytochrome apoprotein expressed in yeast is shown to assemble *in vitro* with phycocyanobilin to produce a photoreversible phytochrome-like adduct. As an initial investigation of the amino acid sequence requirements for chromophore incorporation, three *phyA* gene product deletion mutants were produced in yeast. Truncation of the N-terminal tail to residue 46 demonstrates that this region is not critical to bilin attachment, but a deletion mutant lacking 222 amino acids from the N terminus failed to yield holophytochrome *in vitro*, under the same conditions. A mutant comprising a deletion of the C terminus to residue 548 showed bilin incorporation and red/far-red photoreversibility, indicating that bilin–apophytochrome assembly still occurred even when the entire C-terminal domain was truncated.**

Phytochrome mediates many morphogenetic and developmental light responses in plants (1, 2). The chromoprotein is a homodimer with a monomer molecular mass of ≈120 kDa (3–7) and with each monomer having one linear tetrapyrrolic chromophore attached to a cysteinyl residue by a thioether linkage (8). This chromophore is very similar in structure to phycocyanobilin (PCB) and to mammalian bile pigments. Phytochrome can exist in two photo-interconvertible forms, P_r and P_{fr}. The P_r form (λ_{max} = 666 nm) is synthesized in the dark. P_r is reversibly photoconverted to the P_{fr} form (λ_{max} = 730 nm), the initial event that triggers a wide variety of morphogenetic changes in plants (1, 2). Phototransformation involves a $Z \rightarrow E$ isomerization of the chromophore (9–11). This process alters mutual interactions between the chromophore and the protein, inducing conformational changes in both protein and chromophore (12, 13). The involvement of the 6-kDa N-terminal region of the polypeptide in these conformational changes and in the stabilization of the P_{fr} chromophore is well documented (2, 14, 15).

The apoprotein of phytochrome (PHYA) has been obtained from plants in which the bilin biosynthesis was inhibited (16–18). By using such a preparation, PCB could be incorporated *in vivo* and *in vitro* with formation of a photoreversible adduct (19, 20). Based on assembly studies using *in vitro*-translated PHYA, it was proposed that the covalent attachment of the chromophore is an autocatalytic process (21).

Recombinant pea PHYA was expressed in *Saccharomyces cerevisiae* (22). We report here that this apoprotein assembles *in vitro* with PCB in the absence of any other plant protein to produce a photoreversible phytochrome-like adduct. Furthermore, to investigate the amino acid sequence requirements for phytochrome assembly, deletion mutants have been constructed and expressed in yeast. We present an analysis of these phytochrome mutants in terms of chromophore incorporation and spectral characteristics.

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MATERIALS AND METHODS

Phytochrome Constructs. The construction of full-length pea type I phytochrome (PHYA) cDNA in a yeast episomal plasmid pAA7 (23) under control of the *GAL7* promoter was described elsewhere (22).

Construction of Deletion Mutants. Standard recombinant DNA techniques (24) and commercially available enzymes and chemicals were used for the generation of the phytochrome deletion mutants (see Fig. 3). The pea phyA cDNA constructs pPP30 and pPP800 used here were as described (24). Mutant PHYAΔ2–45 was produced by deletion of the *Sal* I–*Xho* I fragment of pPP30. The *Bam*HI–*Nco* I fragment of the newly obtained plasmid was used to replace the corresponding fragment in pPP800. For construction of mutant PHYAΔ4–225, two complementary oligonucleotides, TCGACCATGGCCATGCATGCATG and CATGCATGGCCATGG, were used to substitute the *Sal* I–*Sph* I fragment of pPP30 yielding a plasmid designated pPP60. The *Nco* I–*Sph* I fragment of pPP60 was replaced with the corresponding fragment in pPP800 and the *Bam*HI–*Sph* I fragment of the resulting plasmid was used for substitution of the analogue fragment of pPP800. The C-terminal deletion mutant PHYAΔ549–1124 was generated by insertion of a synthetic DNA segment coding for an in-frame stop codon in the unique *Ava* III site of pPP800 as follows: two complementary oligonucleotides, TTGATCTAGAG and AATTCTCTAGATCAATGCA, were used to replace the *Ava* III–*Eco*RI fragment of pPP800. The resulting mutant was identified by screening the recombinant clones for the new *Xba* I restriction site contained within the synthetic DNA fragment. For each mutant, the open reading frame was ligated into pAA7 (23) for expression in yeast.

Expression in *S. cerevisiae*. The expression constructs were amplified in *Escherichia coli* and purified prior to transformation of *S. cerevisiae* KN380 (25, 26). Transformants were selected for on a minimal synthetic medium (27) containing 2% (wt/vol) glucose, leucine (30 mg/liter), tryptophan (30 mg/liter), adenine (20 mg/liter), 0.67% yeast nitrogen base without amino acids. The transgenic yeast were cultured in liquid minimal medium for 24 hr at 30°C. A 25-ml culture was used to inoculate 500 ml of complete medium [1% yeast extract/2% (wt/vol) Bacto peptone/2% (vol/vol) glycerol] and cells were incubated for 24 hr prior to induction with 2% (wt/vol) galactose. After a 4-hr incubation in the presence of the inducer, yeast cells were harvested by centrifugation (2500 × *g* for 5 min at 4°C), washed with distilled H_2O, and stored at −70°C.

Abbreviation: PCB, phycocyanobilin.

Apoprotein (PHYA) Preparations. Typically 10 g of pelleted yeast cells were thawed, resuspended in 20 ml of ice-cold extraction buffer [100 mM Tris·HCl/2 mM EDTA/28 mM 2-mercaptoethanol/4 mM phenylmethylsulfonyl fluoride/leupeptin (1 μg/ml), pH 8.0], and mixed with 50 g of ice-cold glass beads (450–600 μm). Disruption was performed using a rotary cell homogenizer (Braun, Melsungen, F.R.G.) at 4°C in four 15-sec cycles with 30-sec intervals. After disruption, the extract was decanted and the glass beads were washed twice with 15 ml of extraction buffer. The homogenate was clarified (40,000 × *g* for 20 min at 4°C) and ammonium sulfate was added to the supernatant to 40% saturation. The precipitation was carried out at 4°C for 1 hr and the proteins were pelleted by centrifugation (40,000 × *g* for 20 min at 4°C). The pellet was solubilized in 5 ml of resuspension buffer [50 mM Tris·HCl/2 mM EDTA/1 mM dithiothreitol/2 mM phenylmethylsulfonyl fluoride/leupeptin (1 μg/ml), pH 7.8], dialyzed against the same buffer for 2 hr at 4°C, and centrifuged (40,000 × *g* for 30 min at 4°C). The supernatant was used for reconstitution experiments.

PCB Isolation. C-Phycocyanin chromophores from *Spirulina platensis* (Sigma) and *Synechococcus lividus* (a generous gift from Robert MacColl, New York State Department of Health, Albany) were isolated under green safety light by methanolysis basically as described (28). After HPLC purification, the chromophores were lyophilized and desalted on an ISCO C_{18} guard column equilibrated with 10 mM trifluoroacetic acid. After washing with 10 mM trifluoroacetic acid, the chromophores were eluted with a 70% (vol/vol) acetonitrile step gradient in 10 mM trifluoroacetic acid. Two peaks were observed, suggesting two components in the major peak that was collected from the first HPLC purification. Upon closer inspection of our HPLC profile, which was similar to the profile in ref. 28, a very slight shoulder was observed on the left side of the main collected peak. Furthermore, the peak maximum was not in the center but was on the right side of the peak, suggesting two components were eluted. Possibly, two components were separable in our conditions because we used a C_{18} guard column instead of the C_4 guard column used in ref. 28. Thus, we had to use a larger amount of acetonitrile in contrast to ref. 28 to elute the chromophores from the C_{18} column. The first major component eluted was blue with absorption maxima of 369 and 684 nm in 5% (vol/vol) HCl in MeOH. The later eluting component was green and showed absorption maxima at 362 and 680 nm. Both species were lyophilized again and stored as small samples at −70°C.

***In Vitro* Reconstitution and Adduct Analysis.** All manipulations were carried out under green safety light. PCB and biliverdin IXα (Porphyrin Products, Logan, UT) stock solutions were prepared in dimethyl sulfoxide, the final amount not exceeding 0.5%. The bilin concentrations were determined as described (29, 30).

Difference spectra were obtained by subtracting the spectrum recorded after saturating red light from the spectrum recorded after saturating far red light. The light source was a 200-W quartz–halogen lamp and actinic light was generated using 656-nm and 730-nm interference filters (Corion, Holliston, MA). The fluence rates and illumination times were 72 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ and 30 sec for red light and 59 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ and 60 sec for far red light.

SDS/PAGE was carried out as described (31). Zn^{2+}-induced fluorescence of biliproteins (32) was visualized under UV irradiation after incubation of the gel in 20 mM Zn^{2+}/150 mM Tris·HCl, pH 7.0, and photographed using a 640-nm interference filter (Corning). For immunoblot analysis, anti-pea phytochrome monoclonal antibodies mAP5 and mAP13 (33) were used as primary antibodies and alkaline phosphatase-conjugated goat anti-mouse (Promega) was used for detection. The amount of apoprotein was quantified by reflectance densitometry after immunoblot analysis. Pea phytochrome isolated from dark-grown seedlings was used as a standard.

RESULTS

Formation of Wild-Type Pea PHYA-PCB Adduct. After incubation of a preparation of heterologously expressed recombinant pea PHYA at 4°C in the presence of 5 μM PCB (green component) for 5 min, a red/far red photoreversible product was detected (Fig. 1, trace A), whereas no photoreversible product was present in the purified PCB (Fig. 1, trace B) or the chromophoreless apoprotein preparation (data not shown). No increase in the difference absorption ($\Delta\Delta A$) was obtained by increasing the PCB concentration or prolonging the incubation period, indicating that the reaction was complete. The absorption maxima of the adduct were at 709 nm for the P_{fr} form and at 650 nm for the P_r form. Both peaks are blue-shifted compared with phytochrome obtained from pea (2). A hypsochromic shift is expected due to the slightly shorter conjugated structure of PCB compared to phytochromobilin (34).

To examine the covalent assembly of protein with chromophore, we investigated the Zn^{2+}-induced fluorescence of biliproteins after SDS/PAGE (32). PCB was added to the apoprotein preparation to a final concentration of 5 μM. After a 5-min incubation at 4°C, the reaction was stopped by adding SDS/PAGE sample buffer containing 2-mercaptoethanol and boiling the samples for 2 min. When Zn^{2+}-dependent fluorescence was analyzed (Fig. 2*B*), a single fluorescent band was visible at a molecular mass corresponding to that of the immunostained apophytochrome (Fig. 2*A*). The gel in Fig. 2*C* is the same as in Fig. 2*B* except that it was Coomassie blue stained. The fact that only a single fluorescent band was visible at ≈120 kDa indicates that the assembly reaction was specific for the apoprotein. The $\Delta\Delta A$ did not decrease after several P_{fr}–P_r photocycles or storage for 16 hr at 4°C. By contrast, the chromophoreless apophytochrome was unstable as it gradually aggregated over time when kept at 4°C (assayed by gel filtration). No photoreversibility (data not shown) or Zn^{2+}-induced bilin fluorescence (Fig. 2*B*, lane 2) was detected when the apoprotein preparation was incubated

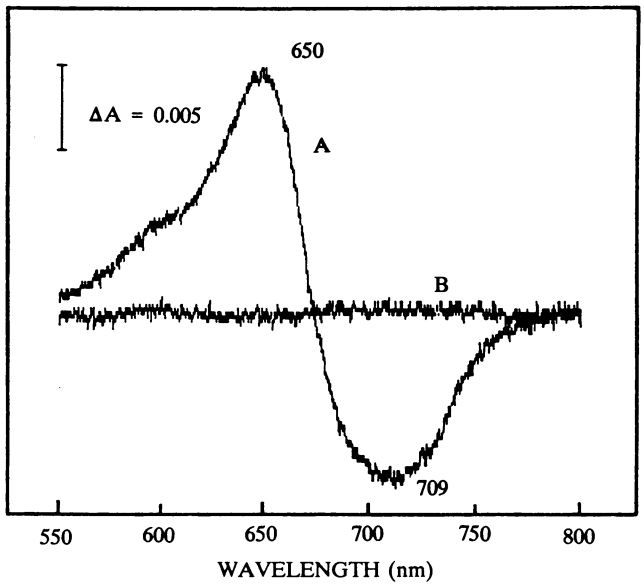

FIG. 1. Difference absorption spectrum (far red–red) of wild-type PHYA-PCB adduct and PCB only. Traces: A, wild-type PHYA was incubated in 50 mM Tris·HCl (pH 7.8) for 5 min at 4°C in the presence of 5 μM PCB and analyzed spectrophotometrically; B, 5 μM PCB only under the same conditions.

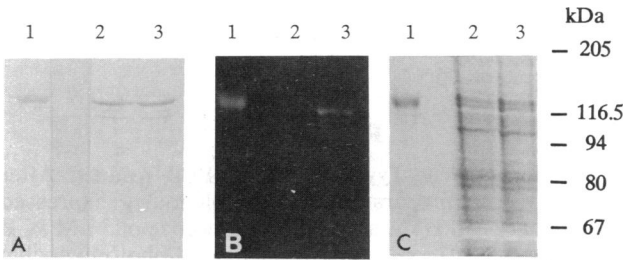

FIG. 2. Expression and Zn^{2+}-induced bilin fluorescence of PCB-treated recombinant pea PHYA. Proteins were separated on a 7.5% SDS/PAGE. (*A*) Pea PHYA was visualized on an immunoblot using monoclonal antibody mAP5 as primary antibody. (*B*) Zn^{2+}-induced fluorescence of biliproteins was visualized under UV irradiation. (*C*) Coomassie blue staining of the gel in *B*. Lanes: 1, 1 μg of *Avena* phytochrome; 2 and 3, PHYA preparation incubated in 50 mM Tris·HCl (pH 7.8) in the presence of 5 μM biliverdin IXα and 5 μM PCB, respectively. Molecular mass markers are on the right.

in the presence of 5 μM biliverdin IXα, indicating that, under the described conditions, this phytochromobilin analogue cannot be covalently joined with the pea PHYA expressed in yeast.

Assembly of Pea PHYA Deletion Mutants with PCB. Three deletion mutants were generated and expressed in *S. cerevisiae*. Partially purified PHYA preparations were analyzed for the presence of recombinant proteins by SDS/PAGE and on immunoblots (see Fig. 5*A*). The molecular weight of the expressed phytochrome deletion mutants was in each case close to the value expected on the basis of the length of the open reading frame that was cloned in the expression vector pAA7. This indicates that the mutant apoproteins produced in yeast correspond with the truncated phytochrome apoproteins depicted in Fig. 3.

N-terminal deletions. Mutant PHYAΔ2–45, comprising a deletion from the N terminus to amino acid 46, yielded a red/far red photoreversible product after incubation with 5 μM PCB at 4°C for 5 min with a maximum and minimum in the difference spectrum at 649 and 704 nm (Fig. 4*A*). The newly formed species involves a covalent adduct between the mutant apophytochrome and the chromophore, indicated by the Zn^{2+}-induced fluorescent band that was detected at the same position by immunoblot analysis (Fig. 5 *A* and *B*).

Mutant apophytochrome PHYAΔ4–225 is missing 222 amino acids from the N-terminal end. This mutant failed to incorporate PCB as shown by its lack of photoreversibility (Fig. 4*B*) or Zn^{2+}-induced bilin fluorescence (Fig. 5*B*). We have increased the PCB concentration and incubation time up to 30 μM and 30 min, respectively, and never obtained evidence of incorporation of PCB into this mutant apoprotein.

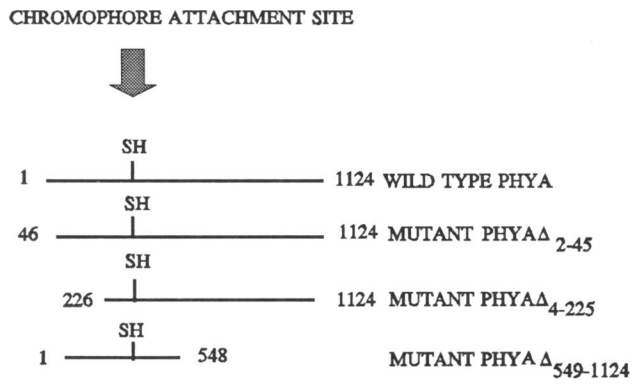

FIG. 3. Structure of PHYA and its deletion mutants.

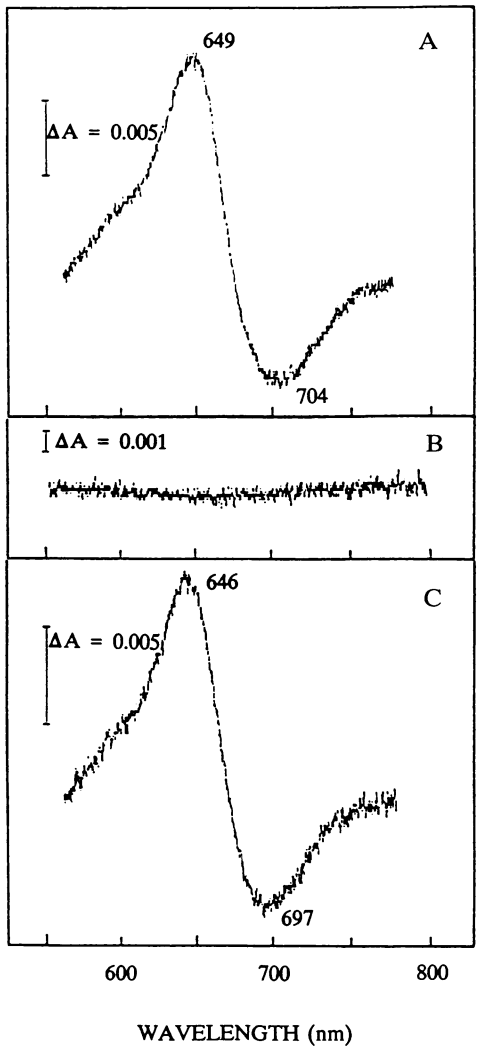

FIG. 4. Difference absorption spectra (far red–red) of PHYA deletion mutants incubated in 50 mM Tris·HCl (pH 7.8) in the presence of 5 μM PCB for 5 min at 4°C. (*A*) Mutant PHYAΔ2–45. (*B*) Mutant PHYAΔ4–225. (*C*) Mutant PHYAΔ549–1124.

C-terminal deletion. When the entire C-terminal domain was deleted (mutant PHYAΔ549–1124), chromophore incorporation was observed. This phytochrome-like species was photoreversible with P_r and P_{fr} absorbance peaks at 646 nm and at 697 nm, respectively. A fluorescent band was detected after SDS/PAGE and incubation in the presence of Zn^{2+} at the same position as the recombinant PHYAΔ549–1124 product detected by immunoblot analysis (Fig. 5 *A* and *B*). A Coomassie blue-stained duplicate of the gel in Fig. 5*B* is shown in Fig. 5*C* on which the recombinant phytochrome polypeptides can be observed. When we examined various mutant apophytochrome preparations and various chromophore preparations, the absorption maxima of the generated photoreversible products were within 2 nm of the above reported values. The $\Delta A_{fr}/\Delta A_r$ for mutants PHYAΔ2–45 and PHYAΔ549–1124 was ≈0.5 in both cases, which was significantly lower than for the wild-type pea PHYA-PCB adduct.

DISCUSSION

Wild-Type Pea PHYA-PCB Adduct. The heterologous expression of type I pea phytochrome apoprotein (PHYA) in the eukaryote *S. cerevisiae* is advantageous with respect to the post-translational processing. Whereas the apophytochrome expressed in *E. coli* is denatured into inclusion

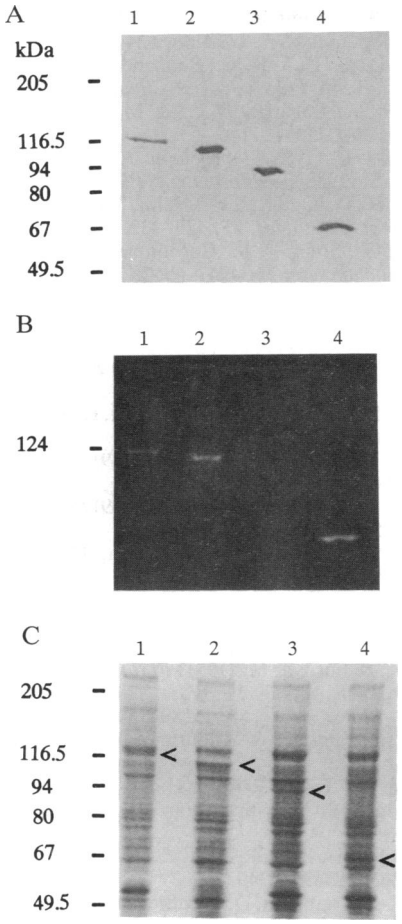

FIG. 5. Expression and Zn^{2+}-induced bilin fluorescence of wild-type PHYA and PHYA deletion mutants incubated in 50 mM Tris·HCl (pH 7.8) in the presence of 5 μM PCB for 5 min at 4°C. Proteins were separated on 7.5% SDS/PAGE. (*A*) Immunoblot using monoclonal antibody mAP13 as primary antibody. (*B*) Zn^{2+}-induced fluorescence of biliproteins was visualized under UV irradiation. (*C*) Coomassie blue staining. Lanes: 1, wild-type PHYA; 2, PHYAΔ2–45; 3, PHYAΔ4–225; 4, PHYAΔ549–1124. Molecular mass markers are on the left.

bodies (24), the recombinant protein produced in yeast has the structure that allows dimerization (22) and, as we show here, covalent assembly with a bilin prosthetic group. PCB is rapidly attached to this apoprotein *in vitro* and the holoprotein thus produced shows the red/far-red photoreversibility, which is characteristic of phytochrome. The absorption maxima of the *in vitro*-produced phytochrome are blue-shifted in both P_r and P_{fr} forms. This blue shift must predominantly originate in the shortened π-system of the chromophore but other factors such as altered post-translational modifications of the apoprotein may also contribute to spectral shifts, either bathochromic or hypsochromic. The blue shift shown in Fig. 1 is consistent with previously reported spectral characteristics of PCB assembled *in vivo* or *in vitro* with apophytochrome obtained from bilin-deficient plants (19, 20). Similar difference spectra were obtained when the blue PCB component was used instead of the green PCB component.

The P_{fr} form of the newly synthesized phytochrome shows a decreased absorption of far-red light since the ratio of the maximal reversible absorbance change ($\Delta A_{fr}/\Delta A_r$) was only 0.77, which is lower compared to the reported value for native phytochrome (35, 36). This is characteristic of phytochrome in which the protein structure is altered by limited denaturation (so called P*) (37) or by partial proteolysis (38), but not of phytochrome generated in seedlings with a PCB prosthetic group. The $\Delta A_{fr}/\Delta A_r$ of such nonnatural phytochrome was similar to that of native phytochrome (19). Also, the recombinant apoprotein migrates as a somewhat shorter molecule (≈1 kDa) than the native pea phytochrome on SDS/PAGE. We suggest that the structural properties of the recombinant PHYA obtained from *S. cerevisiae* are slightly different from the structural properties of native PHYA produced in plants. For example, the post-translational processing of the apoprotein could be different in yeast compared to plants or the apoprotein could be gradually denatured during the extraction procedure. The chromophore incorporation occurred very rapidly in the absence of any other plant protein, confirming earlier data that no plant enzymes are required to carry out the reaction *in vitro* (21). Our preparation may contain yeast enzymes or cofactors that participate in the attachment of the bilin to the apoprotein,[§] although we were able to obtain holophytochrome when recombinant PHYA, purified by immunoprecipitation, was incubated in the presence of PCB (data not shown). It has been proposed that the assembly of phytochrome is an autocatalytic process *in vivo* (21). Knowledge about the site at which the reaction takes place within plant cells and the free chromophore concentration at this site should help to address this hypothesis further.

Assembly of PHYA Deletion Mutants with PCB. A deletion of the N-terminal tail to residue 46 does not interfere with the rapid covalent linkage of PCB to the apoprotein. The resultant mutant holophytochrome is red/far-red-photoreversible and displays a P_{fr} maximum blue-shifted by ≈5 nm compared to the wild-type hybrid phytochrome (compare Fig. 1 and Fig. 4*A*). This is in good agreement with the spectral characteristics of a "large" phytochrome lacking the 6-kDa N-terminal tail; i.e., blue-shifted P_{fr} peaks have been reported in *Avena* (39) and *Pisum* (40) phytochromes. Although this 6-kDa fragment is important for the stabilization of the P_{fr} chromophore, we show here that it is not crucial for the self-assembly of PHYA with PCB *in vitro*. When 222 amino acids are deleted from the N terminus, PCB cannot be joined to the apoprotein *in vitro* under the described conditions. This suggests that the protein segment ranging from amino acids 46 to 225 is either directly involved in the assembly process or stabilizes a protein domain that participates in the attachment of the chromophore. By insertion of a stop codon at position 549, we have generated an N-terminal domain of PHYA roughly corresponding to a "small" phytochrome including the entire N terminus. *In vitro*, PCB can be joined rapidly to this monomeric N-terminal domain to yield a photochromic product (Figs. 4*C* and 5). The P_{fr} peak in this mutant was especially blue-shifted compared to the wild-type adduct or the mutant PHYAΔ2–45 adduct. Previously, a 39-kDa pea phytochrome fragment from amino acids 65 to 414 was shown to be photoreversible between P_r and a bleached form (40). This and the results obtained with the mutant PHYAΔ549–1124 suggest that a protein segment confined between the amino acids 414 and 548 stabilizes the P_{fr} chromophore directly or indirectly. The data presented above demonstrate that a photoreversible chromophore–apoprotein conjugate can be formed when the entire C-terminal domain is truncated.

The analysis of the percent apoprotein that will attach the chromophore is based on two assumptions. (*i*) The extinction coefficient of the newly synthesized full-length or mutant P_r is equal to that of native P_r. (*ii*) Under saturating red light, 87% of the P_r is photoconverted to P_{fr}, as with native phytochrome (41). Thus we determined that 77% of the full-length apoprotein, but only 52% and 14% of the mutant apoprotein PHYAΔ2–45 and PHYAΔ549–1124, respectively,

[§]As pointed out by one of the referees, we cannot rule this out.

yielded a photoreversible product. These numbers are probably underestimates of the actual values. (*i*) PCB has a shorter conjugated structure than phytochomobilin and is thus expected to have a lower extinction coefficient. (*ii*) More importantly, the reconstituted P_r and P_{fr} peaks are closer together than in the native pea phytochrome. Thus, the spectral overlap between the reconstituted P_r and P_{fr} is increased, which results in a decrease in the percent of P_{fr} under saturating red light. The spectral overlap is different for the three reconstituted proteins (smallest for PHYA-PCB and largest for PHYAΔ549–1124-PCB). The underestimation of the percent chromophore incorporation is consequently greatest for PHYAΔ549–1124. Additionally, a visual inspection of the Zn^{2+}-induced fluorescence intensity (Fig. 3*B*) shows that the percent incorporation for the deletion mutants is likely to be higher than the calculated values.

The possible difference in the yield of photoreversible assembled product between the full-length and deletion mutants has several plausible explanations. We observed that the mutant apoproteins aggregated quite rapidly over time. Also, the $\Delta A_{fr}/\Delta A_r$ ratio for both mutant-PCB adducts (Fig. 5) is lower than for the wild-type adduct. This indicates that the deletion mutants might be less stable than the wild-type PHYA. Our preparation may also contain anomalously folded stable conformers that do not attach the chromophore or yield a nonphotoreversible adduct.

In conclusion, the yeast expression system has proven to be a valuable approach to the study of the assembly of apophytochrome and apophytochrome deletion mutants with PCB *in vitro*. This system should be a useful tool to study the protein–chromophore interactions, underlying the red/far-red photoreversibility unique to phytochrome, by site-specific mutations.

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