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Photochromism of Phytochromes and Cph1 Requires Critical Amino Acids and Secondary Structure in the N-Terminal Domain

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Abstract The light perception and phototransformation of phytochromes are the first process of the phytochrome-mediated light signal transduction. The chromophore ligation and its photochromism of various site-specific and deletion mutants of pea phytochrome A and bacterial phytochrome-like protein (Cph1) were analyzed *in vitro*. Serial truncation mutants from the N-terminus and C-terminus indicated that the minimal N-terminal domain for the chromophore ligation spans from the residue 78 to 399 of pea phytochrome A. Site-specific mutants indicated that several residues are critical for the chromophore ligation and/or photochromism. Histidine-324 appears to serve as an anchimeric residue for photochromism through its H-bonding function. Isoleucine-80 and arginine-383 play a critical role for the chromophore ligation and photochromism. Arginine-383 is presumably involved in the stabilization of the Pfr form of pea phytochrome A. Apparently, the amphiphilic α-helix centered around the residue-391 is in the chromophore pocket and critical for the chromophore ligation.

Key words: Phytochromes, photochromism, chromophore, zinc-blot, Cph1

The photochromism of phytochromes is manifested by the photoreversible isomerization of the chromophore between the red light-absorbing Pr form (λ_{max} 666 nm) and the far-red light-absorbing Pfr form [13–15, 23]. The free chromophore is capable of photoisomerization in solution [5, 6], but does not exhibit a photoreversible spectral shift that is characteristic of photochromic proteins [21].

Until recently, phytochromes had been thought to exist only in higher plants. After complete genomic DNA analysis of the cyanobacterium *Synechocystis*, a novel discovery was made; the chromosome of cyanobacterium encodes a phytochrome-like protein (Cph1) [17]. The N-terminal domain of Cph1 is highly homologous to all known phytochrome N-terminal domains [23], whereas the C-terminal domain encodes a histidine kinase [28]. When Cph1 is expressed in *E. coli*, the apoprotein is highly soluble in the cytosol even though plant apophytochromes are usually not. The Cph1 apoprotein incorporates a chromophore *in vitro* just as wild-type apophytochrome does in plants. The Cph1 holoprotein shows Pr/Pfr photoconversion, which is a characteristic of plant phytochromes [16, 28].

Recombinant apophytochromes have been produced in *E. coli* and yeast. The recombinant apophytochromes have been shown to incorporate bilin chromophores *in vitro* to yield a photoreversible product. When phycocyanobilin (PCB) is used to reconstitute apophytochrome *in vitro*, the holophytochrome exhibits blue-shifted Pr and Pfr absorption maxima [12, 15, 22]. However, when holophytochrome is reconstituted with phytochromobilin (PΦB), it is spectrally indistinquishable from native phytochrome [9]. The PCB has been used for the apophytochrome photoassay, because the PCB-ligated holophytochrome has shown biological activity in the *hy*1 and *hy*2 long hypocotyl mutants of *Arabidopsis* [24].

An analysis of deletion mutants showed that the catalytic site for chromophore attachment resides in the N-terminal domain of phytochromes. The minimally required N-terminal size for photoreversibility has been determined by serial truncation of phytochrome [12]. N-terminal truncation of oat phytochrome A to residue 70 and deletion of the C-terminus from residue 399 allowed chromophore attachment to yield a photoreversible product [7]. However, truncation to residue 80 of rice phytochrome A abolished the photochromism as well as chromophore ligation [26].

In order to study the specific interaction between the chromophore and apophytochrome, several deletion mutants and various site-specific mutants have been generated, and

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chromophore ligation and photochromism were tested. Previous studies had shown that only H324 from among the five conserved residues (D309, R318, H321, H324, and Q326; C323 is the chromophore binding site) was critical for the photochromic properties of phytochrome A. Mutants H324R and H324L showed a detectable level of chromophore ligation by zinc-blot analysis at higher temperature, but without detectable photochromism [11]. In the present study, the importance of the residue H324 as well as many other residues for photochromism was further examined.

MATERIALS AND METHODS

Preparation of Mutants Open Reading Frame (ORF) of Pea Phytochrome A

A wild-type full-length cDNA of pea phytochrome A was cloned in pBluescript (Stratagene, CA, U.S.A.) and named as pPP800 [26]. Full-length mutated and wild-type *phyA* genes were cleaved out from pPP800 by using BamHI/SalI and ligated to the BglII/SalI-digested yeast expression vector pAA7 [1, 12], because BamHI and BglII generate complementary cohesive ends to ligate to each other. All mutations were confirmed by restriction enzyme mapping and DNA sequencing. N-terminal and C-terminal deletions and their site-specific mutations were generated by using the polymerase chain reaction (PCR)-directed gene replacement [19].

For the generation of pea *phyA* Δ(N-74)I80L, Δ(N-74)I80V, and Δ(N-74)I80F mutants, extra BamHI sites and starting codons (ATG) were inserted upstream of *phyA* specific oligos that were complementary to the target sites of *phyA*. The PCR reactions were carried out with mutant sense oligos, and antisense oligos homologous to the region just downstream of the NcoI site (674 bp). The PCR-based mutation strategy was also used to generate the following mutants;

Pea *phyA* D(400-C)[DR383, R383K, R383E, R383Q, R383L]

Cph1 (N-315)[DR303, R303K, R303E, R303Q, R303L]

Cph1 [(N-315), (N-299), (12-315), (24-315), (12-315)I26L, (12-315)I26V, (12-315)I26F, (12-315)I26D]

The Transformer Site-Directed Mutagenesis Kit (Clontech, CA, U.S.A.) was used for the generation of pea full-length mutants *phyA*[(A88P), (L144P), (I169P), (D248P), (S333P)]. Mutagenesis was carried out by following the protocol provided by the company. All pea *phyA* mutants were cloned into the pAA7 yeast expression vector, and all Cph1 mutants were cloned into the pQE30 *E. coli* expression vector (Qiagen, CA, U.S.A.). Cph1 mutants were designed to have a BamHI site upstream of the start codon and a SalI site immediately downstream of the stop codon. BamHI/SalI double-digested Cph1 mutants DNA were cloned into BamHI/SalI-digested pQE30. The starting codon of Cph1 mutant DNA was also designed to match in-frame to the reading frames of pQE30 vector to avoid a frame shift of the Cph1 gene.

Expression of Mutant Phytochromes

The protease-deficient *S. cerevisiae* strain BJ5465 (*ura*3-52, *trp*1, *leu*2Δ1, *his*3Δ200, *pep*4::HIS3, *prb*1Δ1.6R, *can*1, GAL) (Yeast Genetic Stock Center, CA, U.S.A.) was used for expression of phytochrome mutants that were cloned in the pAA7 yeast expression vector. About 1 μg of each mutant DNA construct was transformed into BJ5465 strain using the alkali-cation-based yeast transformation kit (Bio 101, CA, U.S.A.). Dozens of transformants were generated and selected on minimal medium lacking uracil for the host auxotrophy. Phytochrome mutants expression in yeast were carried out by following the method of Bhoo *et al.* [3].

The Cph1 mutant genes were constructed in the pQE30 *E. coli* expression vector and expressed in the strain of M15[pREP4] (Nal^S Str^S rif^S, lac^- ara^- gal^- mtl^- F^- $recA^+$ uvr^+). The protein expression was carried out according to Kang *et al.* [18]. Expressed proteins in yeast and *E. coli* were disrupted using the Ultra-Turrax T25 Homogenizer (Janke & Kunkel, Germany) or the beadbeater. For cells disruption with the beadbeater, 0.1 mm zirconia/silica beads for *E. coli* cells and 0.5-mm diameter beads for yeast cells were used in 2-ml microfuge tubes. Total protein extract clarified by centrifugation was then used for chromophore ligation. Poly-histidine-tagged Cph1 mutant proteins were further purified by a one-step purification method [20].

Chromophore Ligation and Photochromism Assay

All experiments were carried out under green safe light conditions. The PCB sample was prepared in dimethyl sulfoxide (DMSO), with the DMSO concentration not exceeding 0.5% of total volume. The light source for photoconversion was a 200-W quartz-halogen lamp equipped with 656 nm and 730 nm interference filters (Corion, MA, U.S.A.) for red and far-red light sources, respectively.

Pea mutant apophytochromes expressed in yeast were clarified by centrifugation from the disrupted yeast powders. Clarified Cph1 mutant protein extracts were used directly for the chromophore ligation. Six-hundreds μl each of mutant protein extracts was mixed with 1 μl of 1.5 mM HPLC-purified PCB. The mixture was incubated at 4°C for 1 h on the orbital shaker. After 1 h of incubation, 100 μl of the PCB mixture was mixed with SDS sample buffer for zinc blot and Western blot analyses, and 500 μl was used for the photochromism assay. The ligation mixture was photoconverted with a Cole-Parmer (Niles, IL, U.S.A.) illuminator equipped with an optical fiber. The sample was illuminated with red and far-red lights for 30 s and 60 s, respectively. The wavelength resolution of the spectrophotometer (HP 8452A Diode Array UV/VIS

Spectrophotometer, DE) was 2 nm. Data points were recorded from 550 nm to 800 nm. The differential spectrum was obtained by subtracting the Pr form of the spectrum from the Pfr form of the spectrum. A differential spectrum of wild type pea phytochrome A ligated with PCB was used as a control standard for all mutant phytochromes.

Zinc Blot and Western Blot Analyses

Phytochromes can be excited by near-UV or red light, as seen in the absorption spectra. The fluorescence of native phytochrome is very weak and is barely detected with the naked eye because of the structural flexibility of the chromophore. However, a zinc ion can induce and fix the cyclic structure of the tetrapyrrolic chromophore in the biliprotein [2]. Fixed cyclic chromophore changes the fluorescence polarization, and induces increased and intensified fluorescence. Therefore, zinc blot analysis is useful for detecting biliproteins.

For zinc-blot analysis, chromophore-ligated mutant phytochrome and Cph1 samples were resolved by SDS-PAGE and incubated in the zinc-containing buffer (20 mM zinc-acetate, 150 mM Tris-HCl, pH 7.0) for 20 min. The gel was illuminated with UV light in the dark, and the zinc fluorescence band was photographed using a 640-nm interference filter. The camera was exposed between 90–120 s with a maximum iris diaphragm.

For Western blot analysis, the protein samples were resolved again by SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad, CA, U.S.A.). The phytochromes were probed by using anti-pea phytochrome monoclonals. Cph1 mutants were probed by anti-penta-His monoclonal antibody (Qiagen, CA, U.S.A.), because the pQE30 vector produces six consecutive histidine residues in the N-terminus of recombinant protein. The alkaline phosphatase conjugated goat anti-mouse antibody (Promega, WI, U.S.A.) was used as a secondary antibody. Three percent BSA in TBS solution was used for blocking and the primary and secondary antibody reactions.

RESULTS AND DISCUSSION

In previous studies, five conserved amino acid residues (D309, R318, H321, H324, and Q326) in the vicinity of the chromophore-binding site (cysteine-323) were mutated to test their roles for phytochrome photochromism. Surprisingly, only residue histidine-324 was identified as being critical for the photochromic properties of phytochrome A. Mutants H324R and H324L showed a detectable level of chromophore ligation by zinc blot at a higher temperature, but without detectable photochromism [11]. The critical importance of the H324 residue for photochromism was reconfirmed with mutants H324G, H324F, and H324Q [3]. Mutant H324G showed no chromophore ligation at all, but mutant H324F autocatalyically ligated the chromophore without displaying photochromism. The result of H324F was exactly the same as in previous studies with H324R and H324L. However, mutant H324Q efficiently assembled the chromophore and exhibited characteristic photochromism. This "retention/gain of function mutation" is not surprising since the glutamine residue with its steric requirement and H-bonding groups similar to that of histidine can often substitute for the latter residue in wild-type proteins, with retention or enhancement of activity [4]. The kinetics of chromophore ligation of mutant H324Q showed a pattern similar to wild-type phytochrome A. As a result, histidine-324 appears to serve as an anchimeric residue for the phytochrome A photochromism through its H-bonding function [3].

Truncation from the N-terminus to residue 80 abolished the photochromism of rice phytochrome A [26]. In addition, deletion mutants from the N-terminus to residues 74 and 78 exhibited both normal lyase activity and photochromism. However, deletion to residue 81 abolished the ligation and photochromism, confirming the previous results [3]. The charge-altering K78E mutation and the hydrophobicity-imposing Q81L mutation within the Δ(N-74) deletion mutant retained the photochromic properties. However, from the mutant Δ(N-74)I80D, isoleucine-80 turned out to be the critical residue for phytochrome photochromism. Isoleucine-80 is conserved for all phytochromes A to E from different plant species as well as for *Synechocystis* phytochrome-like protein (Cph1), whereas its fully conserved neighbor glutamine-81 is not critical for photochromism [3].

To further study the role of the isoleucine-80, this residue was mutated to leucine, valine, and phenylalanine. Hydrophobicity-preserving I80L and I80V completely retained the chromophore ligation and photochromism, whereas I80F showed highly reduced lyase activity (Fig. 1). Results indicate that a hydrophobic side-chain at residue

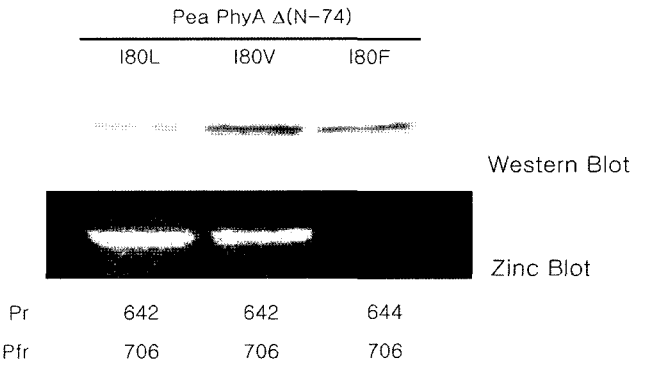

Fig. 1. Zinc blot and Western blot analyses of three PCB-ligated isoleucine-80 mutants.
Western blot was probed with monoclonal pea phytochrome specific antibody, mAP5. Numbers under the figure indicate the photochromic absorption wavelength at Pr and Pfr conformations.

80 in plant phytochrome A is required for photochromism. It is surprising that isoleucine without a hydrogen bonding or acid/base functional side-chain plays such a critical role for the photochromism of phytochrome A.

To study whether the function of isoleucine-80 is evolutionarily conserved, isoleucine-26 of Cph1 that corresponds to Ile-80 of plant phytochromes was mutated to the same residues (I26D, I26L, I26V, I26F). These Cph1 mutants showed exactly the same results of pea phytochrome A (data not shown). In conclusion, isoleucine-80, presumably within the chromophore pocket, is evolutionarily conserved, ranging from prokaryotic cells to plant cells, and is critical for phytochrome photochromism [27]. Previous deletion analysis showed that the N-terminal peptide from the N-terminus to residue 399 is minimally required for the photochromism of phytochrome A [8]. In this study, deletion from the C-terminus to upstream residue 400 showed normal photochromism. Interestingly, this deletion significantly enhanced the extent of PCB ligation, possibly due to cysteine-323 that is more exposed and/or reactive than in the full-length protein. Further deletion to residue 395 abolished the ligation and photochromism [3], confirming the previous results. At this point, secondary structures of the N-terminal domain of phytochromes A and B were predicted by using the protein sequence-based modeling software pSAAM (obtained from Dr. A. R. Crofts) [10]. The 12-residue segment, residues 385-396, was predicted to be a conserved buried amphiphilic α-helix in phytochromes A and B. This predicted secondary structure is conserved in plant phytochromes including bacterial Cph1 (Fig. 2).

Within the twelve amino acid-spanning region, seven residues were completely conserved in all phytochromes A through E, and four residues were fully conserved in all phytochrome A sequences (Fig. 3). The helix-breaking mutation, Q391P, completely abolished the chromophore ligation. The helix-conserving mutation, Q391L, retained photochromism. When a part of the 12-residue helix was deleted (deletion to residue 395), photochromism was completely abolished. However, helix-preserving mutants F393A and H396L retained the chromophore ligation and photochromism as expected [3]. From these results, we propose that the amphiphilic 12-residue helix segment

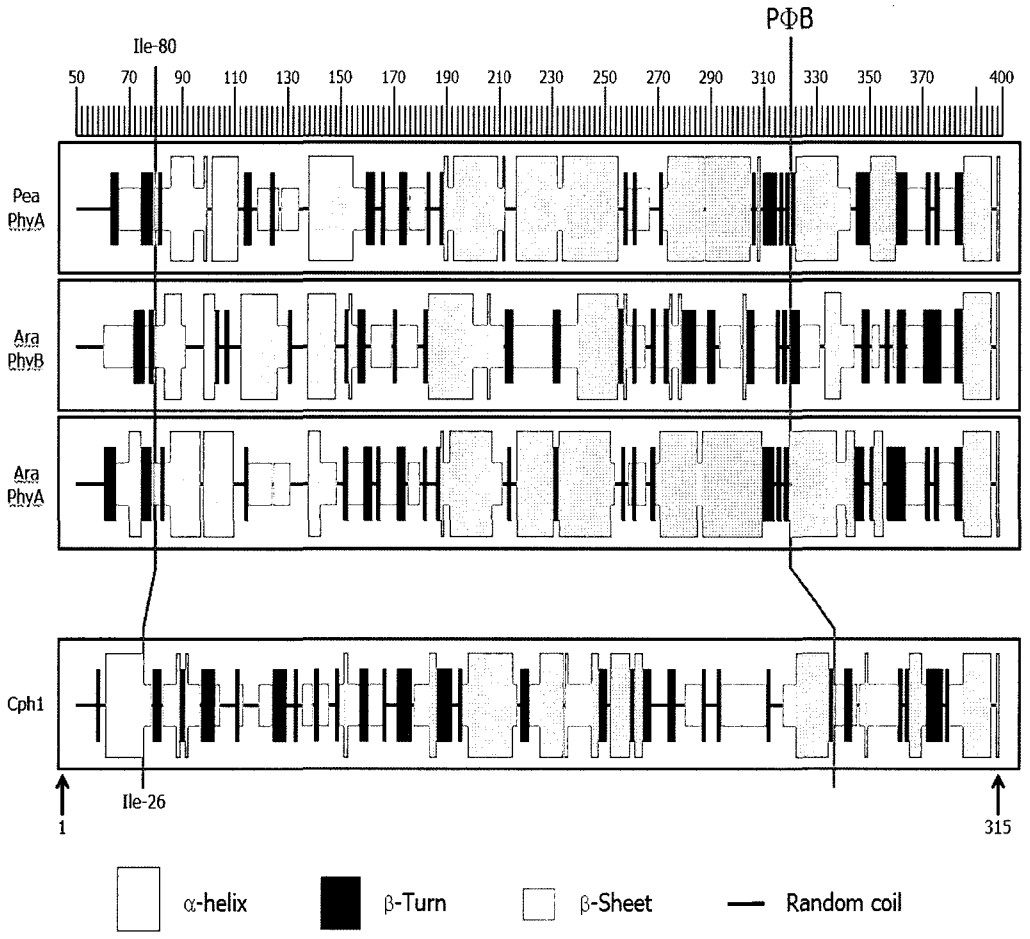

Fig. 2. Comparison of secondary structures of photochromic N-terminal domains of plant phytochromes and Cph1 that are predicted by the prediction program of pSAAM.

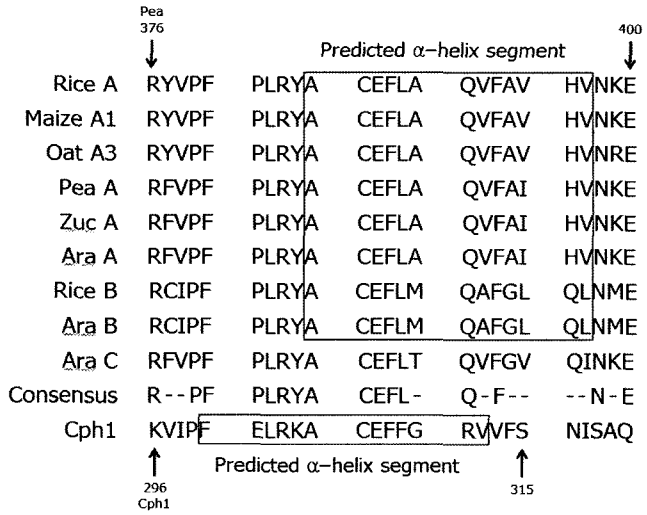

Fig. 3. Amino acid sequences from residues 376 to 400 of plant phytochromes compared with the Cph1 sequence.
Boxed segments are well conserved and predicted as α-helices from the pSAAM program.

plays a critical role for conferring the correct protein environment to the chromophore pocket.

Arginine-383 (303 in Cph1) was chosen for site-specific mutation from comparisons of amino acid sequences and predicted secondary structures of plant phytochromes and Cph1. This residue is conserved in all known phytochromes including Cph1 (Fig. 3). Even though this residue was just in front of the predicted α-helix in plant phytochromes, the corresponding arginine-303 of Cph1 was inside of the predicted α-helix. Because of its high homology and positively charged side-chain, arginine-383 was mutated to examine its role in photochromism. Of the five mutants generated by deletion and sustitution, mutants ΔR-383, R383E, and R383Q abolished the ligation. In contrast, mutants R383K and R383L showed markedly decreased lyase activity (Fig. 4).

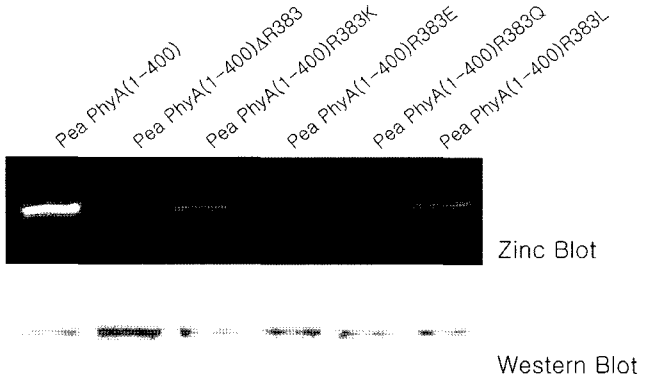

Fig. 4. Zinc blot and Western blot analyses of three PCB-ligated site-specific mutants of Arg-383.
Western blot was probed with monoclonal pea phytochrome specific antibody.

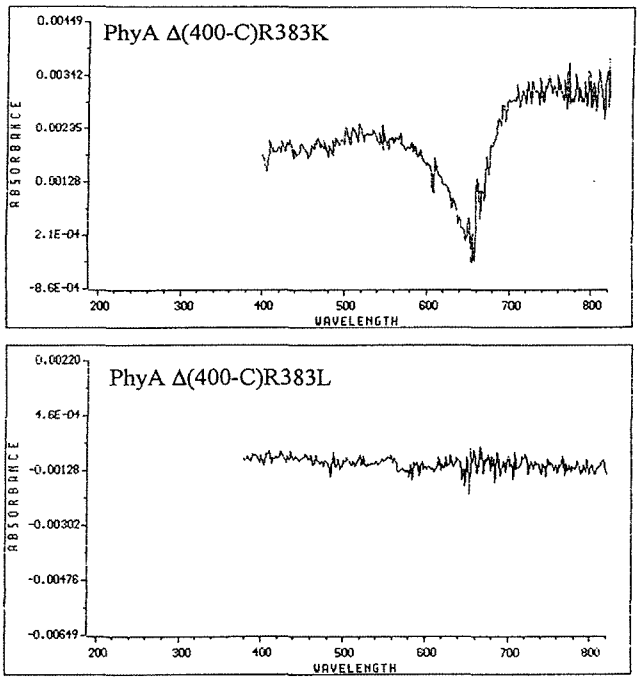

Fig. 5. Differential spectra of mutants pea phytochrome A Δ(400-C) R383K and Δ(400-C) R383L.
Spectra were taken after 30-sec irradiation of PCB-ligated mutant phytochromes with 660 nm of red light.

The mutant R383L showed no detectable photochromism, but R383K showed altered photochromism (Fig. 5). The mutant R383K showed a photoconversion from Pr to Pfr with red light illumination. However, the photoconverted Pfr form of R383K mutant was unstable, since the Pfr peak was immediately bleached after photoconversion (Fig. 5). The same results were obtained from the similar mutants of Cph1 [Cph1(N-315)ΔR303, R303K, R303E, R303Q, R303L]. Together with these results, we speculate that a

Table 1. The PCB ligation and photochromism of Cph1 deletion mutants and site-specific mutants of pea phytochrome A.

Mutant proteins	Rel. extents of PCB ligation*	Photochromism
Cph1 (1-315)	1.00	Yes
Cph1 (1-299)	---	No
Cph1 (12-315)	1.03	Yes
Cph1 (24-315)	1.02	Yes
Pea PhyA A88P	---	No
Pea PhyA I144P	0.15	Yes
Pea PhyA I169P	---	No
Pea PhyA D248P	0.34	No
Pea PhyA S333P	0.67	Yes

*Relative extents of the PCB ligation were based on the quantified values (within 10% errors) of zinc-induced fluorescence, relative to Cph1 (1-315) for Cph1 deletion mutants, and relative to wild-type pea PhyA for pea PhyA mutants.

positively charged side-chain in addition to a short stretch of hydrophobicity is needed at this residue for photochromic properties. However, the photochromic role of this arginine residue must be addressed with further refined experimental data. Truncation mutations indicated that the minimally required Cph1 segment for photochromism is residue 24-315 (Table 1). The boundaries of this segment are evolutionarily well conserved in plant phytochromes. The minimal photochromic domain starts at several residues upstream of the highly conserved isoleucine (residue-80 in plant phytochrome and residue-26 in Cph1) and ends at a predicted α-helix (residue-399 in plant phytochrome and residue-312 in Cph1).

The secondary structure prediction analysis of plant phytochromes and Cph1 showed that secondary structures of the photochromic domain are well conserved (Fig. 2). To study these predicted conserved secondary structures, five predicted secondary structures were disrupted by substituting a single residue in the middle of each predicted secondary segment with proline (A88P, L144P, I169P, D248P, S333P). The mutants L144P and S333P showed a decreased chromophore ligation with detectable photochromism. However, mutants A88P and I169P abolished the chromophore ligation. The mutant D248P showed a decreased chromophore ligation without detectable photochromism (Table 1). These preliminary results should be refined by further studies.

In conclusion, the remarkable photochromism of phytochromes can be partly described in terms of only a small number of specific amino acid residues. Histidine-324 appears to serve as an anchimeric residue for photochromism through its H-bonding function. Isoleucine-80 and arginine-383 are presumably within the chromophore pocket and play a critical role for the chromophore ligation and photochromism. The functions of these two residues might be conserved during the evolution of phytochrome. Apparently, the chromophore pocket entails the amphiphilic α-helix backbone centered around residue 391, reminiscent of the amphiphilic helices in other tetrapyrrole/heme-containing proteins.

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