Phytochrome Photochromism Probed by Site-Directed Mutations and Chromophore Esterification

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Phytochromes are remarkable photochromic proteins; they serve as light sensors and/or switches for red/far-red light-induced morphogenic and developmental responses such as germination, flowering, and gene expression in plants; for review, see refs 1−3. The photochromism of phytochromes is manifested by the photoreversible isomerization between the red light absorbing Pr form (λ_{max} 666 nm) and the far-red light absorbing Pfr form (λ_{max} 730 nm).[4−6] The free chromophore is capable of photoisomerization in solution,[7,8] but does not exhibit photoreversible spectral shifts characteristic for the photochromic proteins.[9] To identify the critical amino acid residues, among 1124 residues, that confer photochromism to phytochromes is a challenging task. Our strategy began with systematic N- and C-terminal truncations of phytochrome A (phyA for holophytochrome A, PHYA for apophytochrome A, and *PHYA* for PHYA gene) and site-directed mutagenesis (SDM) in the vicinity of the chromophore site.[10,11] We are now in a position to target SDM at specific amino acid residues involved in the photochromism of phyA. We also probed the role of the propionate side chain charges on the photochromism. For this study, we used phycocyanobilin (PCB) for covalent reconstitution of holoprotein [hereafter, phyA unless otherwise specified], as described previously.[9−11]

Of the ten mutants generated by substituting the five conserved amino acid residues (D309, R318, H321, H324, and Q326) in the vicinity of the chromophore−cysteine-323, only the H324 residue was identified as being critical for the photochromic properties of phyA. Mutants H324R and H324L showed a detectable level of chromophore ligation by Zn^{2+}-blot at a higher temperature, but without detectable photochromism.[11] The critical importance of H324 for photochromism is reconfirmed with mutant H324G (Table 1). Mutant H324F autocatalytically ligated the PCB chromophore, but failed to display photochromism. However, mutant H324Q assembled the chromophore efficiently and exhibited the characteristic photochromism. This "retention/gain of function mutation" is not surprising since glutamine residue with its steric requirement and H-bonding groups similar to that of histidine can often substitute the latter residue in wild type proteins with retention or enhancement of activity.[13]

Substitution mutants of other highly conserved amino acid residues including Y327F, S322V, and P320M in the immediate vicinity of the Cys-323 chromophore site were normal in their autocatalytic lyase activity photochromic behaviors. Our previous study also showed rather passive roles of other amino acid residues within the chromophore vicinity, except for His-324.[11] The phototautomeric mechanism for the phytochrome photochromism on the basis of serine-322 as an anchimeric catalytic residue has been proposed recently.[14] Our results using S322V mutant in Table 1 indicate that this residue does not play a critical role for photochromism in phyA.

Truncation from the N-terminus to residue 80 abolished the photochromism of rice phyA.[15] In the present study, deletion mutants from the N-terminus to residues 74 and 78 both exhibited normal lyase activity and photochromism. However, deletion to residue 81 abolished the ligation and photochromism (Table 1). The charge-altering K78E mutation and the hydrophobicity-imposing Q81L mutation within the N-terminus−74 deletion mutant retained the photochromic properties (Table 1). However, within the N-terminus−81 segment, isoleucine-80 turned out to be the critical residue, delineating the critical boundary for the peptide segment involved in photochromism. Isoleucine-80 is conserved for all phyA−E from different plant species, whereas its 100% conserved neighbor glutamine-81 is not critical for photochromism.

Previous deletion analysis showed that the N-terminal peptide from the terminus to residue 399 is minimally required for the photochromism of phyA.[16] Deletion from the C-terminus to the upstream residue 400 showed normal photochromism, compared to native phyA (Table 1). Interestingly, this deletion significantly enhanced the extents of PCB ligation, possibly due to Cys-323 which is more exposed and/or reactive than in the full length protein. However, its rate of ligation was actually slower (0.53 compared to the wild type rate 1.00 measured after 2 min of incubation). Further deletion to residue 395 abolished the ligation and photochromism. Within the twelve amino acid-spanning region, residues 385−396, seven residues are 100% conserved in all phyA−E, and four residues are fully conserved in all phyA's. The 12-residue segment is also predicted to be 100% conserved for a buried amphiphilic α-helix in all phyA−E, according to protein sequence analysis (modeling software, pSAAM, was obtained from Dr. A. R. Crofts). The helix-breaking mutation, Q391P, completely inhibited chromophore ligation; thus, no photochromism is observable, whereas the helix-conserving mutation, Q391L, retained the photochromism. When part of the 12-residue helix is deleted (deletion from the C-terminus to residue 395), the photochromism is completely abolished. However, helix-preserving mutants F393A and H396L retained the chromophore ligation and photochromism, as expected. Data in Table 1 suggest that the amphiphilic 12-residue helix segment plays a critical role for conferring the photochromic properties to the PCB−phyA molecule. [It is not

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Table 1. Summary of the Phycocyanobilin Ligation and Photochromism of Pea Wild Type and Mutant Phytochromes A

mutant phytochrome A	ligation[a]	λ_{max}(Pr)	λ_{max}(Pfr)
wild type phyA	1.00	650	712
phyA Y327F	1.17	648	704
phyA H324F	0.52		
phyA H324G			
phyA H324Q	1.20	654	716
phyA S322V	0.98	648	704
phyA P320M	1.18	650	712
phyA Δ(N-74)	1.04	646	710
phyA Δ(N-78)	1.02	644	704
phyA Δ(N-81)			
phyA Δ(N-74)K78E	1.12	644	698
phyA Δ(N-74)I80D			
phyA Δ(N-74)Q81L	1.00	646	708
phyA Δ(395-C)			
phyA Δ(400-C)	3.64	644	698
phyA Δ(400-C)Q391P			
phyA Δ(400-C)Q391L	3.36	646	698
phyA Δ(400-C)F393A	3.30	644	698
phyA Δ(400-C)H396L	3.52	646	698

[a] Relative extents of the ligation of mutant PHYA's (1 mL each) with PCB (5 μL of 2 mM PCB) after 1 h at 4 °C are based on the quantified values (within 10% errors) of Zn^{2+}-induced fluorescence,[12] relative to wild type PHYA. Relative initial rates determined after 2 min of incubation were as follows: WT 1.00, Y327F 1.72, H324F 1.45, H324Q 0.90, S322V 1.42, Δ(N-78) 0.35, Δ(N-74)K78E 2.80, Δ(N-74)Q81L 1.27, Δ(400-C) 0.53, Δ(400-C)F393A 2.05, Δ(400-C)H396L 0.52.

possible to resolve the effects of mutations into the chromophore ligation and the photochromism in PCB−phyA; however, H324R, H324L,[11] and H324F autocatalytically conjugated the chromophore but without photochromism.]

It is remarkable that Ile-80 without a hydrogen-bonding or acid/base functional side chain plays such a critical role for the ligation and photochromic properties of phyA. However, in the case of phytochromes, additional residues not identified here are likely to be involved in the chromophore−apoprotein interactions, affecting the absorbance maxima for Pr and Pfr species. For example, both N- and C-terminal deletions cause blue shifts, especially for Pfr (Table 1), due to either a conformational effect of the deletions or loss of the residues/peptide segments in contact with the chromophore and/or its binding domain. However, it appears to us that relatively few residues and at least one short stretch of amphiphilic α-helix are specifically involved in the autocatalytic ligation and photochromism in phyA. [A qualitative model for the phyA molecule incorporating the present results depicts His-324 interacting with ring A and ring D in the Pr form, whereas Ile-80 preferentially interacts with ring D/vinyl group (ethyl in PCB−phyA) in the Pfr form. See the Supporting Information for the phyA chromophore pocket based on a molecular model of the presently identified amino acid residues.]

To demonstrate the role of the two negative carboxyl side chains of the chromophore in the ligation and photochromic properties of phytochromes, we investigated the ligation of PCB methyl esters to PHYA and the far red − red difference spectra of their adducts (Figure 1). The two propionate side chains of PCB appear to contribute to the photochromism of phyA. When both carboxyl groups are methylated, the chromophore ligation and photochromism were inhibited, according to Zn^{2+}-fluorescence assay and parts C and F, respectively, of Figure 1. [A reviewer pointed out that Figure 1F could be explained by autocatalytic exchange of the dimethyl PCB with PCB. Although this is possible, lack of any effect of the second step addition of PCB to monomethyl PCB−phyA (Figure 1E) suggests otherwise.] PCB monomethyl ester formed the covalent adduct with apoprotein, but without photochromism (Figure 1B,E). Therefore, the role of the propionate side chains may be to position and maintain the conformation of the

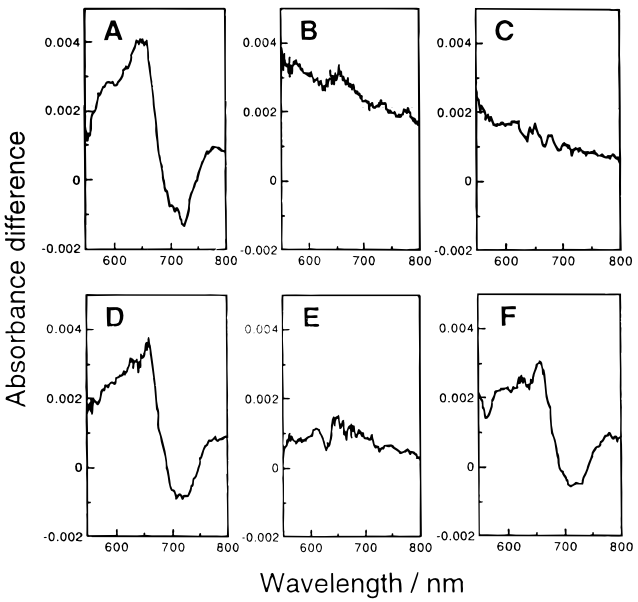

Figure 1. Photochromism assays for PCB−phytochrome monomethyl and dimethyl esters. Apophytochrome A (PhyA) was obtained as $(His)_6$-tagged PHYA. The far red − red difference spectra (A−C) of the supernatant solutions, which were obtained after incubation of $(his)_6$-tagged PHYA with PCB, monomethyl PCB, and dimethyl PCB at 0 °C for 30 min, were recorded as shown. (A) PCB−phyA, (B) monomethyl PCB−phyA, (C) dimethyl PCB−phyA. To determine whether mono- and/or dimethyl esters inhibit the autocatalytic ligation of PCB to the apoprotein, the two-step incubation experiment was carried out in which PCB reacted with the apoprotein after incubation of mono- and dimethyl esters with the apoprotein. The far red − red difference spectra (D−F) were recorded after two-step incubation. In the first step, PCB (D), monomethyl PCB (E), and dimethyl PCB (F) were incubated with $(His)_6$-tagged PHYA for 15 min, and after addition of PCB to each incubated solution, the mixture was incubated for another 15 min.

chromophore electrostatically. Monomethylation disrupts the chromophore orientation necessary for photochromism without affecting its ligation. However, the dimethyl ester disrupts both ligation and photochromism, probably due to either loss of electrostatic bonding or steric hindrance.

In conclusion, the remarkable photochromism of phyA 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 the phyA photochromism through its H-bonding function. Surprisingly, the isoleucine-80 presumably within the chromophore pocket plays a catalytic role for chromophore ligation and photochromism in phyA. Apparently, the chromophore pocket entails not only the Ile-80 peptide chain but also the amphiphilic α-helix backbone centered around Gln-391, reminiscent of the amphiphilic helices in other tetrapyrrole/heme-containing proteins. According to the cysteine-topographic map (unpublished results), these two peptide segments are buried, consistent with the present finding that they form a critical part of the chromophore pocket.

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Supporting Information Available: Experimental details and figures showing the Zn^{2+}-fluorescence and Western blot images, probability profiles of the N-terminal peptide segment containing Ile-80 for a β-sheet and residues 350−410 for an α-helix, and molecular models of Pr and Pfr (18 pages). See any current masthead page for ordering and Internet access instructions.

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