Mutational Analysis of the Pea Phytochrome A Chromophore Pocket: Chromophore Assembly with Apophytochrome A and Photoreversibility†

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ABSTRACT: Ten site-specific mutants of pea apophytochrome A were expressed in *Saccharomyces cerevisiae* and analyzed for chromophore assembly with apoprotein and photoreversible absorbance changes. The mutants constitute two specific changes for each of five conserved amino acid residues located in the microenvironment of the chromophore attachment residue, which is Cys-323 in pea phytochrome A. All mutant apophytochromes were autocatalytically able to covalently attach phycocyanobilin, indicating that there were no major structural perturbations in the apoproteins. However, the rate of chromophore ligation varied significantly among the mutants. Spectrally, the mutant holophytochromes are of three types: mutant phytochromes that are indistinguishable from the wild-type adduct, mutants with blue-shifted P_r and P_{fr} absorption maxima compared to the wild-type adduct, and mutants that are not photoreversible. From an analysis of the results, we concluded that the residues Asp-309, Arg-318, His-321, and Gln-326 are probably not catalytically involved in the chromophore ligation reaction, but some residues may play significant structural and stereochemical roles. Arg-318 might anchor the chromophore, as has been suggested [Partis, M. D., & Grimm, R. (1990) *Z. Naturforsch. 45c*, 987–998; Parker, W., et al. (1993) *Bioconjugate Chem.* (in press)]. The conserved Gln-326, three residues downstream from the chromophore attachment site, is not electrostatically critical for the spectral integrity and photoreversibility of phytochrome, but this residue is sterically important to the lyase activity. It appears that the role of the five amino acid residues in the N- and C-terminal vicinities of the chromophore binding Cys-323 is structural rather than catalytic for the ligation reaction.

Many aspects of plant photomorphogenesis are controlled by the phytochromes, a family of red/far-red photoreversible light receptors (Thomas & Johnson, 1991; Furuya, 1993). Pea phytochrome A (PhyA[1]) is a homodimer of two identical subunits with a molecular mass of ≈121 kDa (Nakasako et al., 1990). Each subunit bears a covalently bound tetrapyrrolic chromophore (Lagarias & Rapoport, 1980). The bilin chromophore is ligated to the apoprotein in a stereospecific fashion through a thioether bond with a single cysteine thiol, located in the N-terminal half of the apoprotein (Lagarias & Rapoport, 1980). PhyA accumulates in dark-grown pea seedlings in its red light absorbing form, P_r. Following absorption of light by the chromophore, a cis/trans isomerization around the C_{15}–C_{16} double bond of the chromophore takes place (Rüdiger et al., 1983; Rospendowski et al., 1989; Fodor et al., 1990). This induces conformational changes in both the apoprotein and the chromophore [Sommer and Song (1990) and references therein]. The final product of this photochemical reaction, P_{fr}, which absorbs maximally at around 730 nm, triggers photomorphogenic processes and is thereafter rapidly degraded [reviewed by Quail (1991)].

Until recently, little was known about the process of phytochromobilin (PΦB) synthesis and its attachment to apophytochrome. Terry and Lagarias (1992) have shown that the final step in the synthesis of PΦB, occurring within the chloroplasts, is the reduction of biliverdin IXα to yield PΦB. The final step in the synthesis of phytochrome is the attachment of the chromophore, which appears to be an autocatalytic process (Lagarias & Lagarias, 1989). Recombinant apophytochrome has been produced in both *Escherichia coli* (Tomizawa et al., 1991) and *Saccharomyces cerevisiae* (Ito et al., 1991; Wahleithner et al., 1991; Deforce et al., 1991; Li & Lagarias, 1992; Kunkel et al., 1993) to study chromophore ligation. In either case, the apophytochromes were shown to incorporate bilin chromophores *in vitro* to yield a photoreversible product. When phycocyanobilin (PCB) is used to reconstitute phytochrome *in vitro*, the resultant holoprotein exhibits blue-shifted P_r and P_{fr} absorption maxima (Wahleithner et al., 1991; Deforce et al., 1991; Li & Lagarias, 1992; Kunkel et al., 1993). When PΦB is used to reconstitute phytochrome *in vitro*, the adduct is spectrally indistinguishable from native phytochrome (Cornejo et al., 1992).

The lyase activity was shown to be confined to the N-terminal domain of phytochrome, and the first 45 amino acids are not required (Deforce et al., 1991). Cherry et al. (1992, 1993) have refined this result and demonstrated that the autocatalytic lyase activity resides between amino acid residues 70 and 398. The N-terminal domain alone is not biologically active (Boylan & Quail, 1991); actually, deletion

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[1] Abbreviations: DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraaceticacid; PCB, phycocyanobilin; PΦB, phytochromobilin; *PHYA*, gene of wild-type phytochrome A; *phyA*, mutant gene of phytochrome A; PHYA, wild-type apophytochrome A; phyA, mutant apophytochrome A; PhyA, optically active phytochrome A; PMSF, phenylmethanesulfonyl fluoride; P_r, red light absorbing form of phytochrome; P_{fr}, far-red light absorbing form of phytochrome; ΔA, difference in absorbance;

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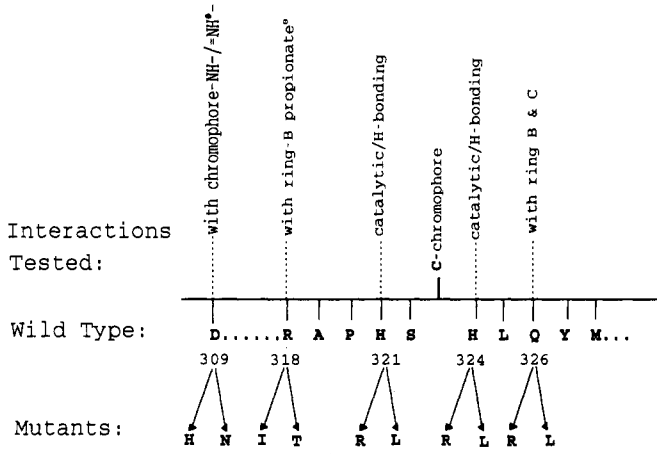

FIGURE 1: Pea phytochrome A chromophore pocket and the amino acid substitutions analyzed in this study.

of as few as 35 amino acids from the C-terminus and 69 amino acids from the N-terminus causes the loss of function of PhyA (Cherry et al., 1992, 1993), whereas internal deletion of residues 37–46 does not abolish the biological activity of PhyA (Quail, 1991).

In order to probe the specific interactions between the chromophore and the apoprotein, we have studied the effects of single amino acid replacements of conserved residues in the vicinity of Cys-323 (Figure 1). The chromophore attachment cysteine has been substituted previously by serine, and this abolishes the photoreversibility and biological activity of oat phytochrome A (Boylan & Quail, 1991) in transgenic *Arabidopsis*. We have expressed 10 site-specific mutants of pea PhyA in *S. cerevisiae* and have analyzed them for their kinetics of chromophore attachment and spectral characteristics of the PCB–holophytochromes. The results are discussed in the light of phytochrome structural models.

MATERIALS AND METHODS

Preparation of the Mutant Pea phyA Open Reading Frames. Standard recombinant DNA techniques were carried out as described elsewhere (Sambrook et al., 1989). The mutations are abbreviated as follows: R318T, R318I, H321L, H321R, H324L, H324R, Q326L, Q326R, D309N, and D309H. Mutagenesis was carried out utilizing the Amersham *in vitro* mutagenesis system (Arlington Heights, IL) using the 965-bp *Nco*I/*Ava*III fragment of *PHYA* in M13 as a template. For all of the mutants, the presence of the mutation and absence of second site mutations was verified by sequencing (Sanger et al., 1977) of the entire 965-bp *phyA* fragment. This fragment was used to replace the nonmutated fragment in pea *PHYA*. The identities of the newly generated open reading frames were verified by restriction mapping and sequencing. The open reading frames were ligated between the GAL7 promotor and the GAL7 terminator in the *S. cerevisiae* expression vector pAA7 (Abe et al., 1991). Each mutation was verified by sequencing (Sanger et al., 1977).

Purification of Phycocyanobilin. PCB was purified from *Spirulina mexicana* (Sosa Texcoco, Mexico City, Mexico) essentially as described previously (Kunkel et al., 1993). The PCB preparation was subjected further to reversed-phase HPLC as described elsewhere (Cornejo et al., 1992) using an ISCO HPLC system (ISCO, Lincoln, NE). The columns used were a Rainin Dynamax 300A C-18 column (Rainin, Woburn, MA) (10 × 250 mm), preceded by a C-18 guard column (ISCO, Lincoln, NE). The main peak eluent was collected, dried *in vacuo*, dissolved in DMSO, and stored at −70 °C in small aliquots. PCB was quantified as described previously (Cole et al., 1967).

Expression in S. cerevisiae and Apophytochrome Preparations. The expression constructs were transformed into the protease-deficient *S. cerevisiae* strain BJ2168 (*MAT*a, *prc*1-407, *prb*-1122, *pep*4-3, *leu*2, *trp*1, *ura*3-52) (Yeast Genetic Stock Center, University of California, Berkeley, CA) using the lithium acetate method (Ausubel et al., 1989). For each mutation, two independent transformation reactions were carried out. Transformants were selected on minimal medium (Ausubel et al., 1989) lacking uracil but supplemented with leucine and tryptophan for the host auxotrophies. The transgenic yeasts were grown in liquid minimal medium for 30–34 h at 30 °C (Ausubel et al., 1989). This culture was used for the inoculation of complete medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, and 2% (v/v) glycerol) (25 mL of minimal culture/500 mL of complete medium) and was grown for another 20 h at 30 °C, prior to induction with galactose at 2% (w/v). Six hours following induction, the yeasts were harvested (2500*g*, 5 min, 4 °C), washed with buffer A (100 mM Tris-HCl/2 mM EDTA, pH 7.8), mixed with an equal volume of buffer A, frozen in liquid N_2, and stored at −70 °C. The disruption of the transgenic yeast cells was performed in liquid N_2 (Ausubel et al., 1989) to minimize the denaturation of apophytochrome, using a Ultra-Turrax T25 homogenizer (Janke & Kunkel, Germany) at ~20 000 rpm for 15 min while constantly adding liquid N_2 to replenish the evaporated N_2. Following disruption, the liquid N_2 was evaporated and the powder was stored at −70 °C.

Kinetic Analysis of Covalent Chromophore Ligation. Typically, 4 g of disrupted yeast powder was mixed with 16 mL of ice-cold buffer B (100 mM Tris-HCl, 2 mM EDTA, and 25% ethylene glycol, pH 7.8) containing 1 mM DTT, 1 mM PMSF, and 5 μg/mL leupeptin. The mixture was stirred for 20 min at 4 °C and clarified (~2000*g*, 2 min, 4 °C). Aliquots were quickly frozen in liquid N_2 and stored at −70 °C. The protein concentration was determined (Protein Quantitative Assay, Stratagene, La Jolla, CA; Bradford, 1976), and the apophytochrome concentration was measured by Western blotting (Harlow & Lane, 1988) using the monoclonal antibodies mAP5 and mAP13 (Nagatani et al., 1987). Native pea PhyA was used as a standard. The yeast extract was diluted at 4 °C with buffer C (100 mM Tris-HCl, 2 mM EDTA, and 20% ethylene glycol, pH 7.8). The final phyA concentration was 50 nM for the mutants H324L and H324R and 25 nM for the wild-type PHYA and all other mutant phyAs. Fresh DTT was added to the reaction mixture at a final concentration of 1 mM. The PCB ligation reactions were carried out at 4 °C (unless specified otherwise) under green safelight. The reaction mixture (typically 1 mL) was stirred on ice to ensure immediate mixing of the HPLC-purified PCB, which was added from a DMSO stock. The amount of DMSO never exceeded 0.5%. At timed intervals, 100-μL samples were withdrawn from the ligation reaction, mixed with SDS–PAGE sample buffer, and immediately frozen in liquid N_2. The samples were boiled for 2 min and separated in duplicate on a 7.5% SDS–polyacrylamide gel. The Zn^{2+}-induced bilin fluorescence (Berkelman & Lagarias, 1980) was visualized as described before (Deforce et al., 1991). On each gel, a standard dilution series of PCB–PHYA was run to verify the linearity of the fluorescence with the concentration of PCB adduct. The adducts were quantified by a densitometric analysis of the negative image using IMAGE 1.44 scanning routines (National Institutes of Health, Bethesda, MD). Prestained (Bio-Rad, Richmond, CA) and fluorescent

(Integrated Separating Systems, Natick, CA) protein markers were used for molecular weight determinations.

Spectrophotometric Assays. Typically, 2 g of disrupted yeast powder was mixed with 2 mL of buffer C. DTT and leupeptin were added to the yeast extracts to final concentrations of 1 mM and 50 μg/mL, respectively. PCB (non-HPLC-purified) was added to these apoprotein preparations from a DMSO stock to make a final concentration of 10 μM, and the mixture was incubated for 60 min under green safelight at 22 °C for mutants H324R and H324L and at 4 °C for all of the other mutants. The samples were clarified (~7000*g*, 5 min, 4 °C), and the supernatant was analyzed at room temperature. The holophytochromes were photoconverted with a Cole-Parmer (Niles, IL) illuminator equipped with an optical fiber and red and far-red interference filters. The fluence rates were 11.67×10^{-3} W/cm^2 for 30 s and 1.21×10^{-3} W/cm^2 for 60 s for red and far-red irradiations, respectively. The wavelength resolution of the spectrophotometer is 2 nm. The data points at 654–658 nm, which were artifacts because of the high intensity of the deuterium lamp, were mathematically interpolated. The spectra were smoothed using the UNPLOTIT 4.0 automated digitizing system (Silk Scientific Corporation, Orem, UT). The concentrations of the photoreversible adducts were estimated according to Li and Lagarias (1992).

RESULTS AND DISCUSSION

Rationale for the Choice of PCB Chromophore and the Mutations. The replacement of PΦB by PCB in the phytochrome chromophore pocket results in a photoreversible adduct with blue-shifted P_r and P_{fr} absorption maxima of PhyA, which predominantly originate from the altered chromophore structure (Wahleithner et al., 1991; Deforce et al., 1991; Li et al., 1992) and that of PhyB (Kunkel et al., 1993). When authentic phytochromobilin is used to reconstitute oat PhyA, the adduct is spectrally indistinguishable from native PhyA (Cornejo et al., 1992). Also, Parks and Quail (1991) have shown that PCB–apophytochrome adducts are functional in the *hy1* and *hy2* long hypocotyl mutants of *Arabidopsis*, which are defective in chromophore biosynthesis. This indicates that the replacement of the authentic phytochrome chromophore PΦB by PCB, which can be more readily purified, does not significantly change the structure of the recombinant adduct and merely results in blue-shifted absorption maxima.

Ten mutants of pea *PHYA* were prepared (Figure 1). The mutated residues are amino acids located in the chromophore pocket which are conserved among all or most phytochromes (Quail et al., 1991). Phytochromes do not show a direct sequence homology with phycocyanin. However, the structures of the chromophores, as well as the absorption spectra of P_r and phycocyanin, are very similar. Further, both chromophores are covalently linked to the apoprotein through a thioether bond. The nature of the chromophore attachment reaction bears similarities as well. In PHYA (Lagarias & Lagarias, 1989) and at two out of three attachment sites in phycocyanin (Arciero et al., 1988), the chromophore attachment can occur autocatalytically *in vitro*. Also, apophytochromes readily incorporate the chromophore of phycocyanin, PCB, *in vitro* (Elich & Lagarias, 1989; Deforce et al., 1991; Wahleithner et al., 1991; Li & Lagarias, 1992; Kunkel et al., 1993). Phycocyanin is a globular, predominantly α-helical protein with no β-sheet structure (Schirmer et al., 1991). CD structural determinations for oat PhyA have indicated that PhyA is also mainly α-helical with little or no β-sheet structure [Sommer and Song (1990) and references therein].

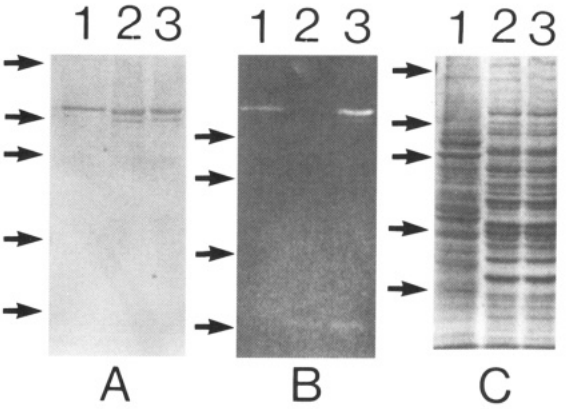

FIGURE 2: Expression and Zn^{2+}-induced bilin fluorescence of PCB-treated recombinant *Pisum sativum* PHYA. The proteins were separated by SDS–PAGE: lane 1, dark-grown pea plant extract (20 μg of total protein); lanes 2 and 3, yeast extract incubated in the absence or presence of 2.5 μM PCB (20 μg of total protein/lane). (A) Immunoblot. (B) Zn^{2+}-induced bilin fluorescence. (C) Coomassie blue staining. The molecular masses of the protein standards indicated on the left are 205, 116.5, 80, 49.5, and 32.5 kDa for the markers indicated in panels A and C and 95, 68, 39, and 29 kDa for the fluorescent markers indicated on panel B.

The chromophore pocket Arg-318 shows positional conservation with the chromophore pocket arginine in the C-phycocyanin α-subunit. It has been speculated that, as in phycocyanin, the positively charged arginine residue might interact directly with a propionate side chain of the phytochromobilin (Partis & Grimm, 1990; Quail et al., 1991; Parker et al., 1993). Therefore, we chose to change this residue to tyrosine and isoleucine. Partis and Grimm (1990) and Parker et al. (1993) have also proposed a direct interaction between the conserved glutamine three residues downstream of the chromophore attachment cysteine and both nitrogen atoms of the B and C rings of the chromophore, in analogy with phycocyanin. In the case of phycocyanin, the residue that occupies the same position is aspartate. In the crystal structure of phycocyanin, this residue is involved in an electrostatic interaction with the nitrogens of the B and C rings of the chromophore [e.g., Duerring et al. (1991)]. This was the premise for changing the conserved glutamine in the PHYA chromophore pocket to a hydrophobic amino acid, leucine, and a positively charged residue, arginine.

Because histidine has a pK_a near neutrality, it is often involved in the catalysis of enzymatic reactions where it can act as both an acid and/or a base at physiological pH values. The attachment of the cysteine 323 thiol to the 3-(*E*)-ethylidene double bond of the chromophore requires protonation of C_3 of the chromophore at some stage during the reaction. Therefore, we have chosen to change the two chromophore pocket histidyl residues, which are invariant in all phytochrome sequences, to arginine and leucine, respectively. The conserved Asp-309, which is the aspartate nearest to the chromophore attachment site, was changed to either asparagine or histidine to probe interactions between this residue and the ring nitrogens of the chromophore.

Kinetic Analysis of Chromophore Ligation. Yeast extracts were prepared as described in Materials and Methods. The apoprotein concentration in these extracts was, on average, 0.19% of the total protein concentration. Figure 2 , panel B, shows the Zn^{2+}-induced bilin fluorescence, which is only detected in apoprotein preparations that are incubated in the presence of PCB (compare lanes 2 and 3). From the immunoblot presented in Figure 2, panel A, it can be seen that the fluorescent band corresponds to the recombinant pea

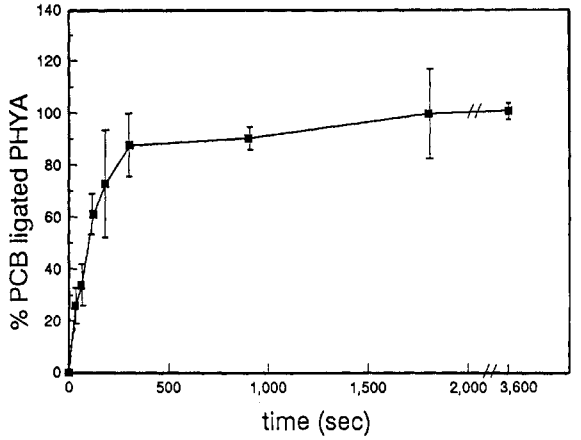

FIGURE 3: Kinetics of PCB ligation to *Pisum sativum* wild-type PHYA. The mean values for the % PHYA ligated with PCB were determined from three separate reactions are plotted ± the standard deviation.

PHYA that has incorporated PCB in a covalent fashion *in vitro*. A pea extract is shown in lane 1 for comparison. PHYA is a minor component of our yeast preparation. Clearly, the bilin incorporation is highly specific for the recombinant PHYA under our conditions.

The kinetics of the ligation reaction for wild-type PHYA was carried out in the presence of a 100-fold excess of PCB at 4 °C. The PHYA concentration was 25 nM and the PCB concentration was 2.5 μM. The percentage of PCB-ligated PHYA, at particular time intervals, was calculated relative to the amount of bilin-ligated PHYA detected at 60 min. The reaction kinetics was followed by measuring the Zn^{2+}-induced bilin fluorescence intensities that were obtained from samples on two protein gels for each reaction. Figure 3 shows the kinetics of the formation of the wild-type PHYA–PCB adduct, based on three separate ligation reaction runs. The logarithm of the fraction of remaining unligated PHYA, when plotted against time, decreases in a linear fashion during the initial period of the reaction (data not shown). The slope of this line is the pseudo-first-order rate constant (k_{obs}) for the formation of PHYA–PCB adducts. The reaction rate constants, k_{obs}, determined as described above, for each of the three individual wild-type PHYA ligation reactions at 4 °C were 9.4×10^{-3}, 6.0×10^{-3}, and 7.2×10^{-3} s^{-1} (average $k_{obs} = 7.5 \times 10^{-3}$ s^{-1}).

The kinetic data obtained indicate that the ligation reaction of PCB to pea PHYA expressed in yeast obeys pseudo-first-order kinetics under the conditions of 100-fold excess PCB. When the ligation was carried out in the presence of 50-fold excess PCB with respect to the apoprotein, under otherwise identical conditions, the rate constants, which were determined for two separate reactions, were 9.5×10^{-3} and 7.0×10^{-3} s^{-1} (average $k_{obs} = 8.3 \times 10^{-3}$ s^{-1}). Because the rate constants, determined at two different chromophore concentrations are comparable, the chromophore concentration is not rate-limiting under our reaction conditions. The reaction rate constant compares well to the reaction rate constant of 9.8×10^{-3} s^{-1} for the incorporation of PCB by *A. sativa* PHYA under similar conditions (Li & Lagarias, 1992). However, the reaction is considerably slower compared to the incorporation of PΦB by *Avena* PHYA, for which a rate constant of 2.1×10^{-2} s^{-1} was measured (Li & Lagarias, 1992). The only structural difference between PCB and PΦB lies in the substituent of the D-ring, which is an ethyl group in the case of PCB and a vinyl group in the case of PΦB. This shortens the conjugated double bond system of the chromophore by a single double bond. Possibly, the reduced ligation rate for the incorporation of PCB originates from this smaller π-system, which might yield a less stable reaction intermediate. This would reduce the rate of thioether bond formation, which is the rate-limiting step in the ligation reaction. Alternatively, the substituent on the D-ring is directly involved in specific interactions with apophytochrome that are slightly modified when PCB is substituted for PΦB. The latter seems less likely. In the case of phycocyanin, the D-ring is relatively exposed to the solvent. Molecular modeling of the phytochrome chromophore pocket, using phycocyanin as a template (Parker et al., 1993), has suggested that, as with phycocyanin, the D-ring is exposed to the solvent and the D-ring vinyl substituent may not be involved in direct interactions with the apoprotein in phytochrome.

Table 1: Range of the Rate Constants Determined for the Bilin Ligation of PHYA Mutants[a]

mutant	k_{obs} (s^{-1})	k_{obs}^{M}/k_{obs}^{WT}	R^2
H321R	$(2.2–2.4) \times 10^{-3}$	0.29	0.97–0.99
H321L	$(3.5–4.6) \times 10^{-3}$	0.52	0.98–0.99
D309N	$(8.0–9.5) \times 10^{-3}$	1.1	0.96–0.99
D309H	$(2.6–3.5) \times 10^{-3}$	0.39	0.97–0.99
Q326R	$(4.9–5.3) \times 10^{-3}$	0.64	0.81–0.99
Q326L	$(0.58–1.2) \times 10^{-3}$	0.10	0.97–0.99
R318I	$(2.4–6.5) \times 10^{-3}$	0.47	0.98–0.99
R318T	$(1.3–2.3) \times 10^{-3}$	0.22	0.93–0.99

[a] The PCB concentration was 2.5 μM, and the temperature was 4 °C. k_{obs}^{WT} is the average rate constant for wild-type [average $k_{obs}^{WT} = 7.8 \times 10^{-3}$ s^{-1}; range, $(6.0–9.5) \times 10^{-3}$ s^{-1}]; k_{obs}^{M} is the average rate constant for mutant. R^2 is the correlation coefficient.

In the case of PCB ligation to PHYB, a pseudo-first-order rate constant of 2.8×10^{-2} s^{-1} was reported (Kunkel et al., 1993). The higher rate constant might originate from differences in the kinetic assay (spectroscopic quantitation of the adduct *versus* quantitation by Zn^{2+} fluorescence) and/or may reflect differences between pea PHYA and tobacco PHYB.

The *in vitro* ligation reaction of PCB to PHYA has provided us with a powerful tool to obtain information pertaining to specific interactions between the apophytochrome and its prosthetic group that are involved in the ligation reaction. The kinetics of the mutant apophytochrome–PCB ligations were determined essentially as for the wild-type PHYA. For the D309, R318, H321, and Q326 mutants, the percentage ligated mutant phytochrome was calculated relative to the ligated mutant product detected at 60 min. At this time point, the reaction was essentially complete for those eight mutants. The reaction rate constants were determined from the plots of the fraction of unligated PHYA *versus* time, as for the wild-type PHYA (see Table 1). For these mutants, the ligation reactions were performed in duplicate or triplicate.

The ligation reaction was very slow for both mutations at His-324. This is the amino acid residue immediately C-terminal to the chromophore attachment cysteine (see Figure 1). The percent PHYA that was ligated with PCB for these mutants was not determined relative to the PCB ligation product determined at 60 min reaction time, but it was determined from a standard curve based on the Zn^{2+} fluorescent wild-type PHYA–PCB adduct, which was generated on the same gel as the particular His-324 mutant. The reactions were carried out with an apophytochrome concentration of 50 nM instead of 25 nM. The PCB concentration was 5 μM. Under those conditions, we obtained rate constants for H324L of 0.45×10^{-4} and 0.10×10^{-3} s^{-1} at 4 and 22 °C, respectively. The reaction for H324R was even slower. The

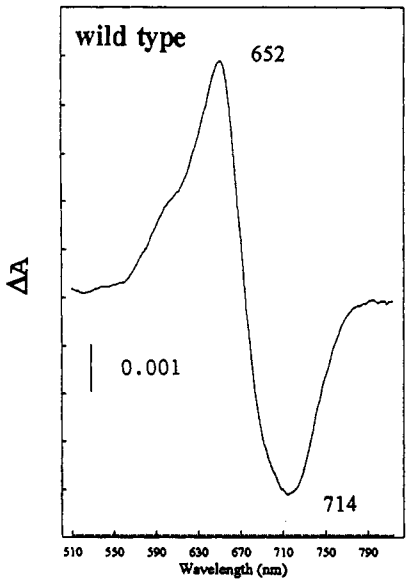

FIGURE 4: Difference absorption spectrum of the wild-type PHYA–PCB adduct. The positions for the absorption maximum and minimum are indicated on the spectrum.

rate constant for the bilin ligation of this mutant, k_{obs}, for a reaction at 22 °C was 0.48 × 10^{-4} s^{-1}. For the other eight mutants studied, the ligation reaction was close to completion at 60 min reaction time. Thus, there was no increase in the $\Delta\Delta A$ when it was spectrophotometrically assayed at times beyond 60 min. The Zn^{2+}-induced fluorescence was also close to saturation at 60 min.

The following reaction mechanism has been proposed for the bilin ligation to apophytochrome (Li & Lagarias, 1992), in analogy with the mechanism of PCB ligation to apophycocyanin:

$$\text{PHYA} + \text{PCB} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\text{PHYA---PCB}] \xrightarrow{k_2} \text{PHYA–PCB}$$

If the reaction proceeds in two steps, as presented above, the differences in ligation rates among the mutants may reflect a decrease in the rate constant for the formation of the thioether bond (k_2), which is the rate-limiting step. A reduced k_2 may result either from a change in the positioning of the chromophore and the thiol group with respect to each other or from a direct role for the changed residue in catalysis. Since all mutants are able to attach the chromophore covalently, albeit with altered kinetics, the latter seems less likely. Alternatively, the mutations may result in changes in the association/dissociation constants (k_1/k_{-1}), or all steps of the ligation reaction may be affected.

Spectrophotometric Assays. Except for the His-324 mutants, the wild-type PHYA and all mutant PHYAs yielded a red/far-red photoreversible product when incubated in the presence of PCB (see Figures 4 and 5). The holophytochromes could be photocycled several times between P_r and P_{fr}, indicating that the adducts were fully photoreversible. The spectra in Figures 4 and 5 represent data from a single line of yeast transformants. When a second set of independent transformants was analyzed, the results were consistent with the spectra presented here. The wild-type absorption maxima were at 648–652 nm for P_r and at 712–716 nm for P_{fr}. This suggests that the PHYA structure is more nativelike, compared to the PHYA in previous preparations which displayed P_r and P_{fr} peaks at around 650 and 709 nm (Deforce et al., 1991).

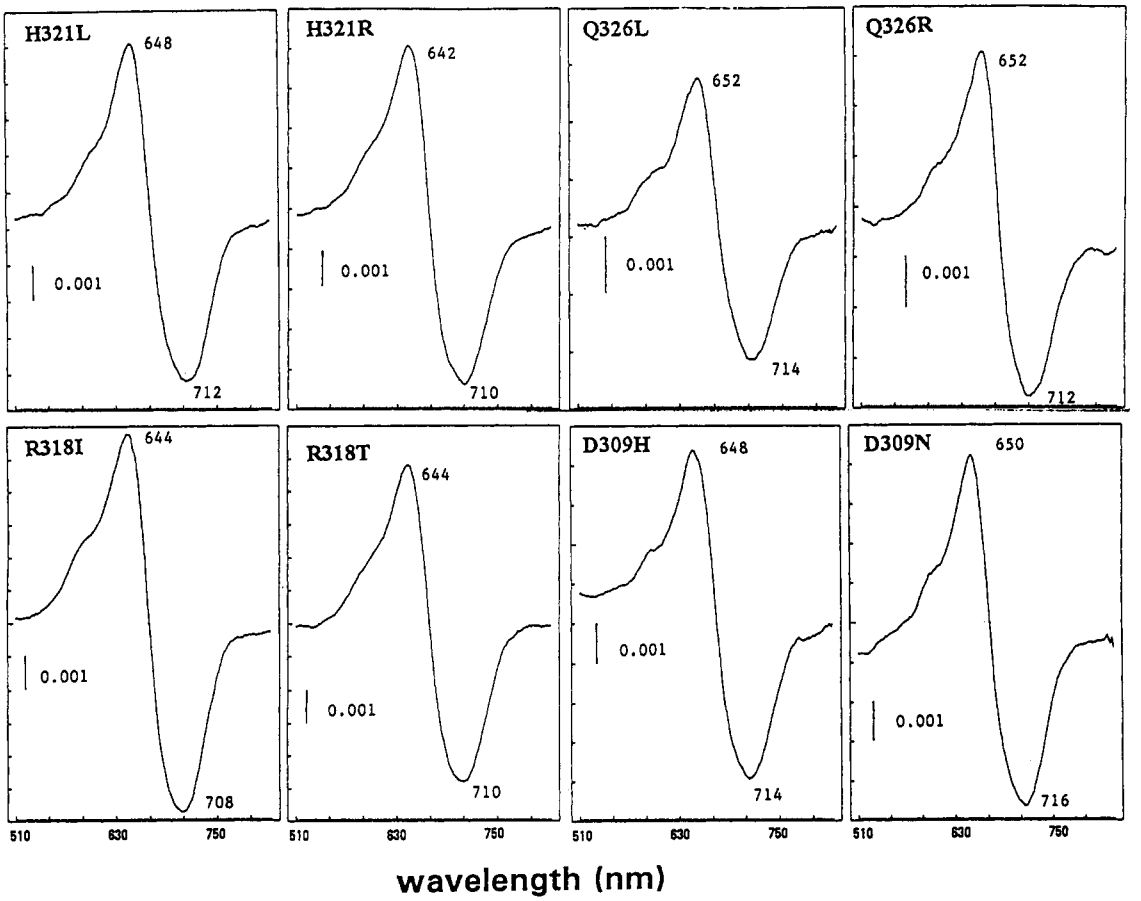

FIGURE 5: Difference absorption spectra of the D309H, D309N, H321L, H321R, R318I, R318T, Q326, Q326R mutant PHYA–PCB adducts. The positions for the absorption maximum and minimum are indicated on the spectra.

This most likely results from the more gentle liquid N_2 yeast disruption technique employed here. The blue-shifted peaks for the wild-type PHYA preparation (compared to native PhyA) are consistent with what was observed earlier for the PCB-ligated oat PHYA [e.g., Wahleithner et al. (1991) and Li and Lagarias (1992)] and originate from differences in the chromophore structure. The $\Delta A_r/\Delta A_{fr}$ for different wild-type spectra varied between 1.03 and 1.25.

The mutants D309H, D309N, Q326R, Q326L, and H321L were spectrally wild-type-like. The mutants H321R and both mutants for Arg-318, R318I and R318T, exhibited P_r and P_{fr} absorption maxima that were blue-shifted slightly compared to those of the wild-type adduct. By all other spectral criteria, such as the $\Delta A_r/\Delta A_{fr}$, the peak shape and width, and the P_r and P_{fr} extinction coefficients (see below), they exhibited wild-type spectral characteristics. This indicates that the perturbations, brought about by these mutations, are minor. An interesting observation is that the rate of chromophore ligation is reduced for the mutants H321L, D309H, and Q326R and especially for Q326L, while their spectroscopic characteristics are wild-type-like. This suggests that the bilin–apophytochrome interactions during the ligation reaction are not necessarily the same as the interactions that contribute to the P_r spectral integrity.

Fodor et al. (1989) have demonstrated that the N of the chromophore C ring is protonated and positively charged in phytochrome. If Q326 is involved in a direct interaction with the chromophore at that position, changing Q to either L (nonpolar) or R (positively charged) is anticipated to perturb the spectral integrity of P_r, P_{fr}, or both. Because the spectra for both mutants are essentially wild-type-like, an electrostatic interaction between Q and the ring nitrogens of the chromophore seems unlikely. However, this residue is sterically important for the autocatalytic lyase activity of the protein, as a substitution of this residue with Arg, and especially with Leu, affects the kinetics of the ligation reaction (Table 1). It is interesting that there is a single exception to the absolute conservation of Gln-326. The corresponding position in maize PhyA is occupied by a positively charged lysine (Quail et al., 1991). The oscillator strength ratios for the visible and the Soret bands for P_r (Parker et al., 1993) indicate that a partly extended conformation for the chromophore is more compatible with the spectral data compared to a fully extended linear conformation. In the case of phycocyanin, the chromophore is fully extended [e.g., Duerring et al. (1991)]. Perhaps the interaction between the aspartate and the B and C ring nitrogens is critical to holding the chromophore in the extended conformation in the case of phycocyanin. Thus, since the phytochrome chromophore is more likely semicyclic, a similar interaction might not be present.

When the positively charged functionality at position 318 is changed, as for R318I and R318T, both the P_r and P_{fr} absorption maxima are blue-shifted slightly. If indeed the R318 anchors the chromophore through an interaction with a chromophore propionate, as was proposed (Partis & Grimm, 1990; Parker et al., 1993), our data show that this hydrogen bonding is not an absolute requirement for either chromophore attachment or photoreversibility.

Under the assumption that the extinction coefficients for the reconstituted holophytochromes are identical to the extinction coefficients of native P_r and P_{fr}, we determined the percentage of PHYA that could be ligated to PCB. This was, on average, 70% and 61% for the mutants Q326R and Q326L, respectively, and 84% or higher for all other photoreversible holophytochromes. This indicates that the extinction coefficients of both P_r and P_{fr} for the photoreversible mutants cannot be very different from those of the wild-type adducts or native phytochrome A.

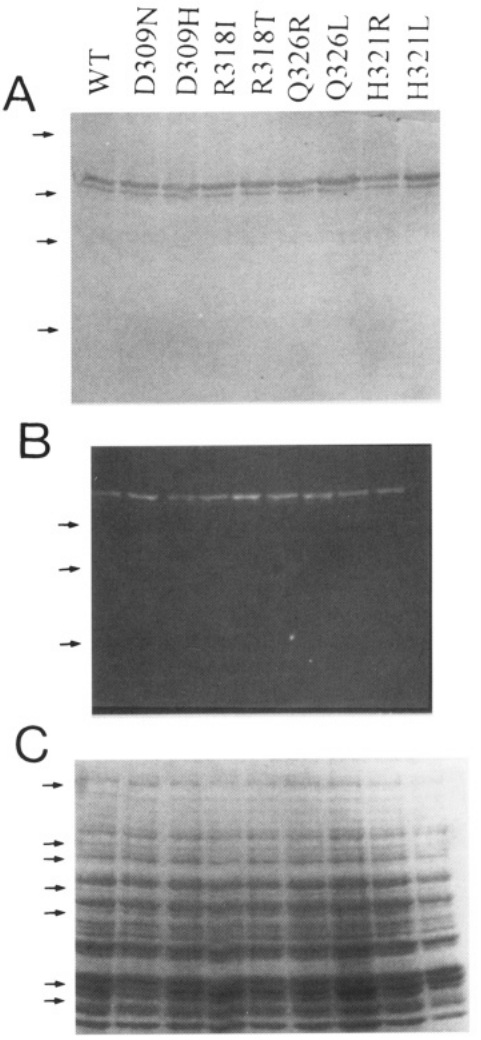

FIGURE 6: Immunoblotting (A), Zn^{2+}-induced bilin fluorescence (B), and Coomassie blue staining (C) of wild-type and mutant holophytochrome A preparations. Following spectrophotometric assays, the proteins were separated by SDS–PAGE; 40 ng of photoreversible holophytochrome was loaded per lane. Prestained molecular mass markers (panel A) were at 205, 116.5, 49.5, and 32.5 kDa. Fluorescent protein markers (panel B) were at 95, 68, 39, and 29 kDa. All markers were visible in panel C.

Figure 6 shows the immunoblot, Zn^{2+}-induced bilin fluorescence, and Coomassie blue staining of the wild-type and mutant apoprotein preparations following the spectrometric assays. A fluorescent band comigrated with the immunostained PHYAs. This indicates that the ligation *in vitro* was covalent and specific for the phytochrome apoproteins. A second, weakly fluorescent band at 114–116 kDa, corresponds with a degradation product of the apoprotein, as can be seen from the Western blot.

The mutations of His-324 to either L or R yielded phyAs which incorporated the chromophore at a much slower rate compared to wild-type PHYA and did not yield a photoreversible product when irradiated with 660-, 620-, or 600-nm actinic light. Figure 7, lanes 2 and 3, shows the Zn^{2+}-induced fluorescence and immunostaining of these two histidine mutant preparations, following incubation at 22 °C in the presence of 10 μM PCB for 60 min. A wild-type PCB–PHYA preparation is shown in lane 1 for comparison. The mutant with the slowest rate of incorporation is the H324R mutant.

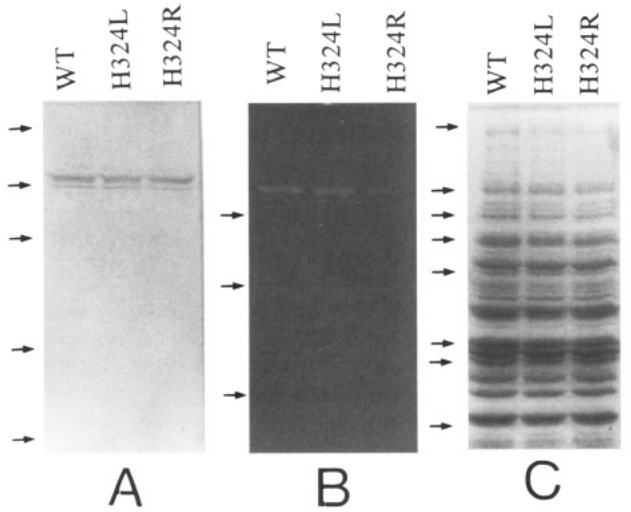

FIGURE 7: Immunoblotting (A), Zn^{2+}-induced fluorescence (B), and Coomassie blue staining (C) of mutant H324L and H324R preparations incubated at 22 °C in the presence of 10 μM PCB. A wild-type preparation was loaded in lane 1 for comparison. Prestained molecular mass markers were at 205, 116.5, 49.5, and 32.5 kDa (panel A). Fluorescent molecular mass markers were at 95, 68, 39, and 29 kDa. All markers were visible in panel C.

Under the conditions described above, a minimum of ~15% of the mutant PHYA incorporated PCB, as determined by Zn^{2+} fluorescence assays. Therefore, it was possible that the concentration of bilin-ligated phyA was below the detection limit in our spectrophotometric assays. We therefore diluted a wild-type preparation, incubated at room temperature for 60 min, to 15% of its original concentration as a control. Under these conditions, we were still able to detect the red/far-red photoreversible product reliably.

Because the His-324 is immediately adjacent to the chromophore attachment site, the effect of the substitutions at this site might be rather indirect, arising from a structural perturbation at the chromophore attachment site. However, a direct catalytic role for this residue in the bilin ligation and/or the photoreversibility of phytochrome is a distinct possibility. For the ligation reaction of the H324L mutant, the Arrhenius activation energy is approximately 7.2 kcal/mol; the enthalpy and entropy of activation for the bimolecular ligation reaction are 6.6 kcal/mol and −31 eu, respectively. The negative entropy of activation suggests a structural reorganization in the autocatalytic reaction.

In summary, we have analyzed the kinetic and spectral consequences of introducing single amino acid substitutions in pea phytochrome A within the conserved chromophore pocket. An important concern with mutagenic work pertains to the effect of changing residues on the overall structure of the protein. The fact that all mutants specifically attach the chromophore, whereas 99.8% of the other yeast proteins present in our preparation do not react with PCB under our conditions, indicates that the mutational changes did not cause major perturbations in the gross structure of apophytochrome. This observation indicates that the thiol group at position 323 in all mutants can attack the ethylidene double bond, yielding a covalent linkage between the apoprotein and the chromophore. Therefore, it seems reasonable to conclude that the residues that we changed (D309, R318, H321, H324, and Q326) are not directly but rather indirectly involved in the catalysis of chromophore ligation. The H324 mutants are severely affected, both in their kinetics of bilin ligation and in their spectral characteristics. Since this residue is located immediately adjacent to the chromophore attachment site, the inhibition is most likely due to an altered positioning of Cys-323 and PCB. R318 might anchor the chromophore *via* a bilin propionate, but a direct electrostatic interaction between Q326 and the B and C ring nitrogens of the chromophore does not seem likely.

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