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Chromophore Topography and Secondary Structure of 124-Kilodalton *Avena* Phytochrome Probed by Zn^{2+}-Induced Chromophore Modification†

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ABSTRACT: The relative extent of chromophore exposure of the red-absorbing (P_r) and far-red-absorbing (P_{fr}) forms of 124-kDa oat phytochrome and the secondary structure of the phytochrome apoprotein have been investigated by using zinc-induced modification of the phytochrome chromophore. The absence of bleaching of P_r in the presence of a 1:1 stoichiometric ratio of zinc ions, in contrast to extensive spectral bleaching of the P_{fr} form, confirms previous reports of differential exposure of the P_{fr} chromophore relative to the P_r chromophore [Hahn et al. (1984) *Plant Physiol. 74*, 755–758]. The emission of orange fluorescence by zinc-chelated P_{fr} indicates that the P_{fr} chromophore has been modified from its native extended/semi-extended conformation to a cyclohelical conformation. Circular dichroism (CD) analyses of native phytochrome in 20 mM Tris buffer suggests that the P_r-to-P_{fr} phototransformation is accompanied by a photoreversible change in the far-UV region consistent with an increase in the α-helical folding of the apoprotein. The secondary structure of phytochrome in Tris buffer, as determined by CD, differs slightly from that of phytochrome in phosphate buffer, suggesting that phytochrome is a conformationally flexible molecule. Upon the addition of a 1:1 molar ratio of zinc ions to phytochrome, a dramatic change in the CD of the P_{fr} form is observed, while the CD spectrum of the P_f form is unaffected. Analysis of the bleached P_{fr} CD spectrum by the method of Chang et al. (1978) reveals that chelation with zinc ions significantly alters the secondary structure of the phytochrome molecule, specifically by increasing the β-sheet content primarily at the expense of α-helical folding. Further evidence of a zinc-induced conformational change in phytochrome has been obtained with proteolytic digestions of chelated and nonchelated P_{fr}. We propose that chelation with zinc ions at the phytochrome chromophore cyclizes the semiextended P_{fr} chromophore, which reduces/eliminates the interactive forces between the chromophore and the polypeptide. Once these forces are attenuated, the phytochrome molecule undergoes a conformational rearrangement, resulting in a secondary structure similar to that of the phytochrome apoprotein, which appears to differ substantially from that of the holoprotein.

Phytochrome is a tetrapyrrolic chromoprotein that serves as the primary photoreceptor for several light-mediated developmental responses in plants. There are two photoreversible forms of phytochrome, the red light absorbing P_r[1] form and the physiologically active far-red-absorbing P_{fr} form. Phytochrome undergoes the following photoreversible transformations upon irradiation with red or far-red light [for reviews, see Schäfer and Briggs (1986), Quail et al. (1986), and Furuya (1987)]:

$$P_{r(\text{red-absorbing form})} \underset{730\text{ nm}}{\overset{660\text{ nm}}{\rightleftharpoons}} P_{fr(\text{far-red-absorbing form})} \rightarrow \text{morphogenic and developmental responses}$$

Although the exact structure of native phytochrome and the mechanism of its action have not yet been elucidated, the chromophore topography and conformational differences between the two forms have been studied extensively [for detailed reviews, see Lagarias (1985) and Song (1988)]. Although considerable progress has been made in these areas, major discrepancies have arisen in attempts to quantitatively assess the accessibility of the chromophore and to predict the secondary structure of phytochrome. For example, while methods based on the amino acid sequence of phytochrome predict a substantial amount of β-pleated sheet structures, particularly at the chromophore attachment site, methods based on CD analyses have frequently failed to predict any β-sheet structures in the phytochrome conformation.

The present study used zinc ions, which are known to chelate the tetrapyrroles in phycocyanins and phytochrome (O'hEocha, 1963; Furuya et al., 1965; Lisansky & Galston, 1974), to probe

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[1] Abbreviations: HA, hydroxyapatite; CD, circular dichroism; P_r, red-absorbing form of phytochrome; P_{fr}, far-red-absorbing form of phytochrome; kDa, kilodaton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; ME, 2-mercaptoethanol; PMSF, phenylmethanesulfonyl fluoride; PEI, poly(ethylenimine); EG, ethylene glycol; KPB, potassium phosphate buffer; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; UV–visible, ultraviolet–visible; ANS, 8-anilinonaphthalene-1-sulfonate.

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the chromophore topography/accessibility and the secondary structure of phytochrome. The results suggest that the P_{fr} chromophore is preferentially accessible to Zn^{2+} ions and that in addition to the conformational differences between P_r and P_{fr}, a third distinct conformation, that of the phytochrome–Zn^{2+} complex, exists.

Materials and Methods

Materials. Hydroxyapatite (HA)[1] was prepared in the laboratory as previously described (Hahn et al., 1984a). Centricon 30 microconcentrators were purchased from Amicon (Danvers, MA). Oat seeds (*Avena sativa L.*, CV Garry oat) were obtained from Agriculver (Trumansburg, NY). Ammonium sulfate (ultrapure, special enzyme grade) was purchased from Schwarz/Mann (Orangeburg, NY). Acrylamide, bis(acrylamide), and Coomassie brilliant blue were from Bio-Rad Laboratories (Richmond, CA). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). Ethylene glycol and zinc chloride (reagent grade) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). All other chemicals, including trypsin, α-chymotrypsin, and papaya crude protease, were purchased from Sigma Chemical Co. (St. Louis, MO). All solutions and buffers were prepared with deionized doubly distilled water.

Photoconversion of Phytochrome. Phytochrome photoconversions were carried out using a Cole Parmer low-noise illuminator (Model 7141-50) combined with either a far-red cutoff filter (Ealing 26-4457; 1.8 kW/m^2) or a 660-nm interference filter (Oriels C572-6600; 8.3 W/m^2). The phytochrome isolation was performed under a gold lamp (F40G0, Gold; General Electric) at 4 °C.

Purification of Phytochrome from Oat Tissues. Undegraded 124-kDa phytochrome was purified from etiolated oat seedlings as modified from previously described procedures (Vierstra & Quail, 1983; Chai et al., 1987b); 1.6 kg of 4-day-old etiolated tissue was illuminated with gold light for 10 min and homogenized in a Waring blender with 1.2 L of 100 mM Tris-HCl, pH 8.3, containing 140 mM ammonium sulfate, 10 mM EDTA, 50% (v/v) EG, 20 mM ME, 20 mM $Na_2S_2O_4$, and 4 mM PMSF. The homogenate was squeezed through four layers of cheesecloth, and the filtrate was stirred for 15 min with 10 mL of 10% PEI per liter of filtrate. After centrifugation at 11000*g* for 30 min, the pelleted phytochrome was suspended in 200 mL of 50 mM Tris buffer, pH 7.8, containing 5 mM EDTA, 25% (v/v) EG, and 14 mM ME. The solution was clarified by centrifugation at 24000*g* for 10 min and applied to a 2.5 × 10 cm HA column equilibrated with distilled water. The column was washed with 2 column volumes of the above resuspending buffer and 2 column volumes of 5 mM KPB, pH 7.8, containing 5 mM EDTA, 14 mM ME, and 25% (v/v) EG. Following elution with 60 mM KPB, pH 7.8, containing 5 mM EDTA, 14 mM ME, 25% (v/v) EG, and 2 mM PMSF, the fractions containing phytochrome were pooled and precipitated with 3.3 M ammonium sulfate (45 mL/100 mL of pooled phytochrome). After centrifugation for 20 min at 24000*g*, the phytochrome pellet was resuspended in 1 mL of 20 mM KPB with 1 mM EDTA, pH 7.8, per 2.5 units of phytochrome (1 unit of phytochrome, as P_r, per milliliter has A_{666} = 1.0 in a 1.0-cm path length cell). The resuspended phytochrome was centrifuged at 40000*g* for 5 min, and the supernatant was discarded. The phytochrome pellet was dissolved in 1 mL of 20 mM KPB with 1 mM EDTA per 3 units of phytochrome and centrifuged for 5 min at 40000*g*. The purity of the phytochrome preparations was verified by absorbance measurements (A_{666}/A_{280} = 0.8–1.14 after far-red irradiation) and SDS–polyacrylamide gel electrophoresis with Coomassie blue staining (a single band at 124 kDa).

Addition of Zinc Chloride and Spectroscopic Measurements. From this step, all manipulations involving phytochrome were performed under green safety lights as described by Hahn et al. (1984a). Immediately before the addition of $ZnCl_2$, 2 mL of the phytochrome solution was placed into a Centricon 30 microconcentrator and centrifuged at 2500*g* for 15 min. The filtrate was discarded, and 1.5 mL of 20 mM Tris, pH 7.8, was added to the tube, and the solution was centrifuged at 2500*g* for 20 min. Additional 20 mM Tris was added, and the centrifugation step was repeated 2 more times. Following centrifugation, the phytochrome was loaded onto a Sephadex G-25 buffer exchange column (1.0 × 18.0 cm) equilibrated with 20 mM Tris, pH 7.8. Following elution, the phytochrome was diluted to A_{666} = 0.45 with 20 mM Tris, divided into two 2-mL portions, and photoconverted to either P_r or P_{fr} by irradiation with a far-red cutoff filter or a 660-nm interference filter, respectively.

3.4 μL of zinc chloride from a 1 mM stock solution was added to 1 mL of the P_r and P_{fr} solutions in 0.5-μL increments, resulting in a 1:1 stoichiometric ratio of zinc ions to phytochrome molecules. The concentration of phytochrome was calculated from an extinction coefficient of 1.32×10^5 M^{-1} cm^{-1} (Lagarias et al., 1986). Although previous studies used as much as 6.5×10^3 zinc atoms for each phytochrome molecule (Lisansky & Galston, 1974), we found that bleaching could be achieved at a 1:1 ratio, provided that the phosphate buffer was completely replaced with Tris. After each incremental addition of zinc ions, the solution was thoroughly mixed. When the addition of the 3.4 μL of $ZnCl_2$ was complete, absorbance measurements were taken at 5 -min intervals at room temperature with a Hewlett-Packard 8451A spectrophotometer. Control solutions, with the addition of 20 mM Tris, pH 7.8, instead of $ZnCl_2$, were treated in the same manner.

Fluorescence Measurements. Fluorescence measurements were made on a Shimadzu RF-540 spectrofluorophotometer. The fluorescence of the above P_{fr} solution with the addition of a 1:1 stoichiometric ratio of $ZnCl_2$ and the P_{fr} solution with the added 20 mM Tris buffer was measured. In addition, the fluorescence of a 1-mL sample of a 3.4 μM BSA solution was measured before and after the addition of 3.4 μL of 1 mM $ZnCl_2$.

Circular Dichroism. CD spectra of the P_r and P_{fr} forms of 124-kDa oat phytochrome (A_{666}/A_{280} = 1.14) in 20 mM KPB, pH 7.8, with 1 mM EDTA and oat phytochrome in 20 mM Tris, pH 7.8, with and without added $ZnCl_2$ were recorded on a JASCO-J600 spectropolarimeter calibrated with ammonium *d*-10-camphorsulfonate (Takakuwa et al., 1985). Reproducibility of results was regularly checked with standard substances, such as bovine serum albumin (Barnes et al., 1972) and myoglobin (Yang et al., 1986). Phytochrome concentrations of 0.6–1.2 μM and 2-mm path-length cells were used. Data obtained from four repetitive scans were averaged by the instrument's computer. Once the spectrum was obtained for one form of phytochrome, the phytochrome was photoconverted in the cell compartment in situ to the other form for a second set of CD scans. The samples were regularly checked for signs of precipitation by monitoring the absorbance at 280 nm. Measurements were recorded at 1-nm intervals from 190 to 310 nm. CD spectra of BSA and myoglobin controls were taken before and after the addition of varying amounts of $ZnCl_2$.

Mean residue ellipticity was calculated from

$$[\theta]_\lambda = -[33(M/100)(A_L - A_R)]/lc$$

where $[\theta]_\lambda$ = the decimolar ellipticity at a given wavelength,

Table I: Analysis of the Secondary Structure of Reference Proteins and Phytochrome with and without the Addition of Zinc Chloride According to the Method of Chang et al. (1978)[a]

protein	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)
myoglobin	79.0	0.0	3.5	17.5
α-chymotrypsin	7.0	48.5	5.0	39.5
BSA	55.0	18.0	2.5	24.5
P_r[b]	52.5	0.0	21.1	26.7
P_{fr}[b]	55.3	0.0	18.8	26.0
P_r[c]	51.2	0.0	24.3	24.5
P_{fr}[c]	54.5	0.0	21.5	24.0
P_r[d]	45.5	0.0	21.0	33.5
P_{fr}[d]	50.5	0.0	17.5	32.0
$P_r + Zn^{2+}$	45.5	0.0	21.0	33.5
$P_{fr} + Zn^{2+}$	28.0	39.5	7.5	25.0

[a] Ellipticity data were taken at 1-nm intervals in the range of 190–240 nm, unless specified otherwise. Reference proteins were dissolved in 20 mM KPB, pH 7.8. The SAR of phytochrome samples was 1.14. [b] Analysis of the CD spectra with data taken in the range of 205–240 nm in 20 mM KPB with 1 mM EDTA (Chai et al., 1987a). [c] Analysis of phytochrome in 20 mM KPB and 1 mM EDTA, pH 7.8. [d] Analysis of phytochrome in 20 mM Tris, pH 7.8.

M = the mean residue weight, $A_L - A_R$ = the difference in absorbance of left and right circularly polarized light, l = the path length in decimeters, and c = the concentration of protein (grams per milliliter). The mean residue weight of 110.7 was calculated from a molecular weight of 124 870 on the basis of the known phytochrome sequence (Hershey et al., 1985). Phytochrome concentrations were calculated as described above. The method of Chang et al. (1978; Yang et al., 1986) was used to estimate the apparent α-helix, β-sheet, β-turn, and random-coil composition of the phytochrome samples. This method is derived from linear, least-squares analyses of CD spectra of 15 proteins with known X-ray crystallographic structures. Table I shows the secondary structure analyses of reference proteins from the CD measurements under the present experimental conditions.

Proteolytic Digestion of Phytochrome. The P_{fr} form of phytochrome, with and without the addition of $ZnCl_2$, was subjected to digestion by the following proteolytic enzymes: trypsin (bovine type XI), α-chymotrypsin (bovine pancreas, type II), and papaya crude protease. All digestions were performed in 20 mM Tris, pH 7.8, at 4 °C under green safety light. Stock solutions of the proteases (1 mg/mL) were prepared in 20 mM Tris, pH 7.8, and diluted to 0.01 mg/mL immediately before use. A phytochrome stock solution with A_{666} = 0.5 as P_r, prepared as described above, was converted to P_{fr} with a 5-min red light irradiation; 700 μL of this stock solution was pipetted into each of two test tubes; 2.7 μL of 1 mM $ZnCl_2$ (1:1 zinc to phytochrome) was added to one of the tubes, while 2.7 μL of 20 mM Tris, pH 7.8, was added to the other. The tubes were incubated for 1 h at 4 °C, with occasional gentle shaking. After verifying by absorbance measurements that the phytochrome to which the $ZnCl_2$ had been added was completely bleached, 200 μL from each phytochrome stock solution was pipetted into Eppendorf tubes, and 1.6 μL of 0.01 mg/mL trypsin, α-chymotrypsin, or crude papaya solutions was added. At 30-min or 4-h intervals during incubation with the proteases, 30-μL aliquots were removed and quenched by the addition of 0.8 μL of PMSF from a 200 mM stock solution of PMSF dissolved in 2-propanol; 7.5 μL of electrophoresis sample buffer containing 0.2 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 3% SDS, 0.7 M ME, and 0.01% (w/v) bromophenol blue was added to each aliquot, and they were immersed in boiling water for 5 min. The samples were stored at −80 °C until they were electrophoresed.

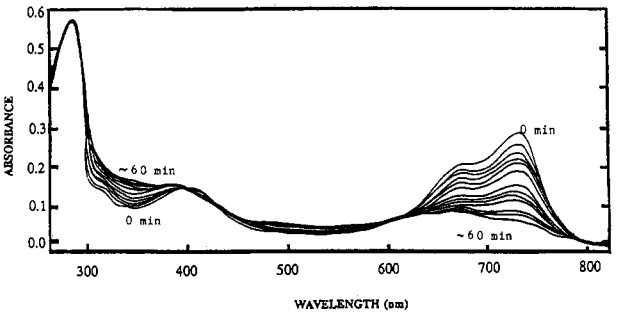

FIGURE 1: Absorption spectra of P_{fr} in 20 mM Tris, pH 7.8, with the addition of a 1:1 stoichiometric ratio of zinc ions to phytochrome molecules. 3.4 μL of 1 mM $ZnCl_2$ was added to 1 mL of 3.4 μM phytochrome. Measurements were taken at ca. 5-min intervals. The top curve at 730 nm was taken at 0 minutes, and the bleached spectrum (lowest) curve was taken at ca. 1 h after mixing with Zn^{2+} ions.

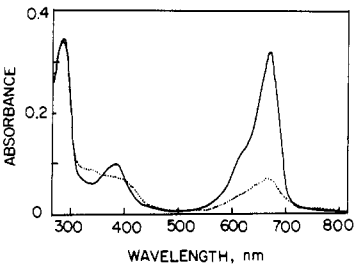

FIGURE 2: Absorption spectra of P_r (solid curve; 3.4 μM) and cycled ($P_r \rightarrow P_{fr} \rightarrow P_r$) phytochrome in the presence of Zn^{2+} ions (3.4 μM). The latter was obtained by converting P_r to P_{fr}, incubating with a 1:1 ratio of zinc ions and then incubating for 5-min with far-red light. The absorption spectrum of P_r in the presence of Zn^{2+} ions (1:1) is virtually identical with the solid curve for P_r (not shown).

Control digestions using BSA were also performed. A 3.8 μM BSA stock solution was prepared and divided into two 700-μL portions; 2.7 μL of 1 mM $ZnCl_2$ was added to one portion, while 2.7 μL of buffer was added to the other. The digestions were performed as described for phytochrome.

SDS–Polyacrylamide Gel Electrophoresis. After thawing to room temperature, the samples were applied to an 0.8-mm slab gel with a 4% stacking gel and a 5–20% linear gradient separating gel according to the method of Laemmli (1970). Ten microliters of sample was loaded per lane. Following electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue R-250.

Secondary Structure Calculations. The method of Chou and Fasman (1978) was used to calculate secondary structure predictions for oat phytochrome and C-phycocyanin. This method predicts the secondary structure of proteins based on their primary structure. The amino acid sequence of both subunits of C-phycocyanin from *Mastigocladus laminosus* (Frank et al., 1978) and the amino acid sequence obtained from cDNA of oat phytochrome (Hershey et al., 1985) were analyzed by using the MSEQ protein analysis program (Black & Glorioso, 1985).

RESULTS

UV–visible absorbance measurements of phytochrome solutions before and after the addition of a 1:1 ratio of $ZnCl_2$ show preferential bleaching of the P_{fr} form of phytochrome. At this concentration of zinc ions, the P_r spectrum remained unchanged, while P_{fr} was nearly completely bleached, particularly in the 730-nm region (Figure 1). The decrease in absorbance of the bleached P_{fr} spectrum at 730 nm is approximately 17% greater than the absorbance decrease at 666 nm. Far-red irradiation of the bleached P_{fr} produced a

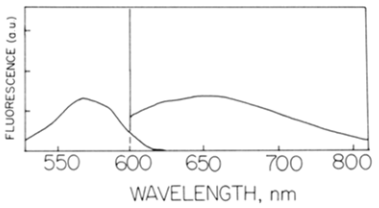

FIGURE 3: Fluorescence excitation (left panel) and emission (right panel) spectra of the phytochrome P_{fr}–Zn^{2+} complex with the monitoring wavelengths of 620 and 560 nm, respectively. The complex was prepared by incubating phytochrome (P_{fr}) with zinc ions (1:1) for more than 1 h; solution conditions are as described in the Figure 1 caption.

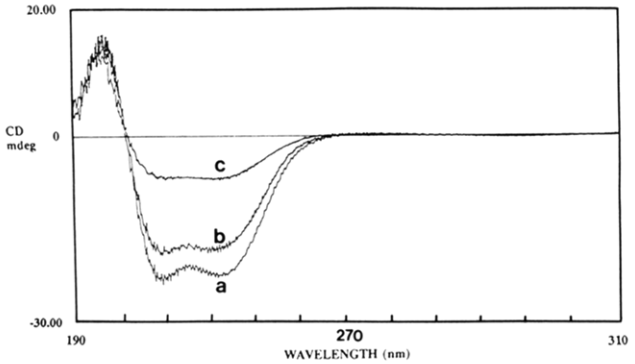

FIGURE 4: Far-UV CD spectra of 124-kDa phytochrome in 20 mM Tris, pH 7.8, with and without the addition of a 1:1 ratio of zinc ions. Spectra were obtained with a 2-mm path-length cell at room temperature. The phytochrome concentration was 0.09 mg/mL. (a) P_{fr}; (b) P_r; (c) P_{fr} after the addition of 0.73 μL of 1 mM $ZnCl_2$ to a 1-mL sample.

spectrum in which the absorbance at 666 nm decreased by over 80% (Figure 2). These observations reflect the fact that the P_{fr} form of phytochrome is a photostationary mixture of ca. 87% P_{fr} and 13% Pr (Lagarias et al., 1986; Holdsworth & Whitelam, 1987).

Fluorescence measurements of P_{fr} and P_{fr}–zinc complexes indicate that P_{fr} is nonfluorescent, while chelated P_{fr} emits broad fluorescence at 650 nm (Figure 3). The $P_r + Zn^{2+}$ (1:1) showed no fluorescence corresponding to the same excitation wavelength. These observations are consistent with data reported by Berkelman and Lagarias (1986). No differences were seen between fluorescence scans of BSA solutions with or without added zinc ions.

CD spectra of 124-kDa oat phytochrome in 20 mM Tris buffer and 20 mM KPB display a clear increase in ellipticity upon P_r-to-P_{fr} phototransformation. Photocycling of P_{fr} back to P_r produces a CD spectrum that is identical with the spectrum of the original P_r form.

A dramatic decrease in CD is observed in the P_{fr} spectrum following bleaching by zinc ions (Figure 4). Photocycling of bleached P_{fr} did not occur upon irradiation with far-red light. In contrast to the P_{fr} spectrum, the CD spectrum of P_r is relatively unaffected by the addition of a 1:1 ratio of zinc ions. Upon red light irradiation of the P_r solution containing zinc ions, the bleached P_{fr} CD spectrum is obtained. Addition of zinc ions to BSA and myoglobin in a 1000:1 stoichiometric ratio produced no change in the CD spectra.

The analysis of the secondary structure of phytochrome by the method of Chang et al. (1978), using CD data obtained in this study, differs slightly from that previously reported by Chai et al. (1987a) (Table I). The previous calculations were based on CD data obtained in the range of 205–240 nm, while our analyses utilized data in the range of 190–240 nm. Extending the data used in the calculations to the lower wavelengths has increased the accuracy of the analyses (Johnson, 1988). More importantly, the secondary structure of phytochrome in 20 mM Tris appears to differ somewhat from the structure of phytochrome in 20 mM KPB. For example, 54.5% α-helix is predicted for P_{fr} in 20 mM KPB, while the predicted α-helical content is only 50.5% in 20 mM Tris. This variation is consistent with other reports (Cantor & Schimmel, 1980), suggesting the conformationally flexible nature of proteins, depending upon the characteristics of the solvent environment.

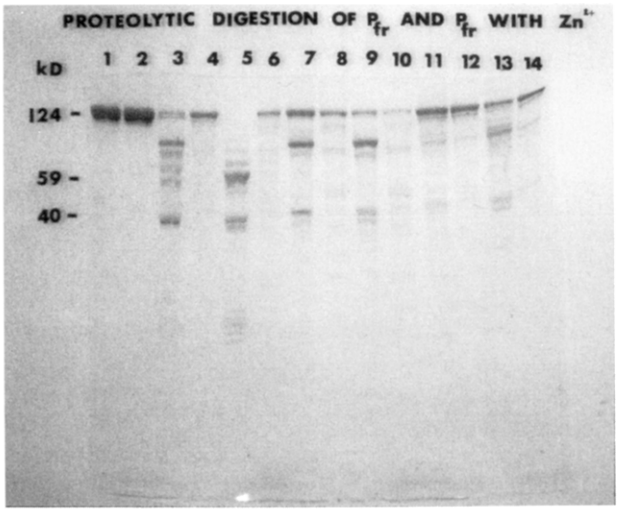

FIGURE 5: Proteolytic digestion of P_{fr} and P_{fr}–Zn^{2+} complex. Each lane represents 5.0 μg of protein prepared as described under Materials and Methods. Lane 1, P_{fr}; lane 2, $P_{fr} + Zn^{2+}$; lane 3, P_{fr} + trypsin, T = 30 min; lane 4, $P_{fr} + Zn^{2+}$ + trypsin, T = 30 min; lane 5, P_{fr} + trypsin, T = 4 h; lane 6, $P_{fr} + Zn^{2+}$ + trypsin, T = 4 h; lane 7, P_{fr} + α-chymotrypsin, T = 30 min; lane 8, $P_{fr} + Zn^{2+}$ + α-chymotrypsin, T = 30 min; lane 9, P_{fr} + α-chymotrypsin, T = 4 h; lane 10, $P_{fr} + Zn^{2+}$ + α-chymotrypsin, T = 4 h; lane 11, P_{fr} + crude protease, T = 30 min; lane 12, $P_{fr} + Zn^{2+}$ + crude protease, T = 30 min; lane 13, P_{fr} + crude protease, T = 4 h; lane 14, $P_{fr} + Zn^{2+}$ + crude protease, T = 4 h.

Comparative proteolytic digestion of P_{fr} with and without the addition of zinc ions produced differences in cleavage patterns for the proteases employed, not only with respect to the type of protease but also with respect to their activity on P_{fr} vs the P_{fr}–zinc complexes. That is, each protease produced a unique band pattern, and the pattern of bands differed between the chelated and nonchelated phytochrome incubated with a particular protease. The 30-min trypsin digestion of P_{fr} produced an equal amount of 124- and 114-kDa polypeptides, a large 84-kDa band, and 69-, 59-, and 40-kDa species (Figure 5). Similar results were reported by Lagarias and Mercurio (1985) in a study which demonstrated that the P_{fr} form of phytochrome is digested more rapidly than the P_r form. The predominant band in the α-chymotrypsin digestions were at 84 kDa. In addition, the rate of proteolysis also differed between the P_{fr} and P_{fr}–zinc samples. Among the samples incubated with trypsin, proteolysis was more rapid in the samples that did not contain zinc, as compared to the zinc-containing phytochrome samples. At the end of 4 h, the 124-kDa native phytochrome was completely cleaved, while approximately 20% of the zinc-chelated phytochrome remained. On the other hand, the phytochrome chelated by zinc ions was degraded more rapidly by chymotrypsin than was the nonchelated phytochrome. Although the rate of cleavage was approximately the same for samples incubated with crude papaya protease, the pattern of cleavage differed. No differences were observed in either the cleavage pattern or the rate of proteolysis between BSA samples incubated with or without the addition of a 1:1 molar ratio of zinc ions. The

Table II: Analysis of the Secondary Structure of Oat Phytochrome and C-Phycocyanin According to the Method of Chou and Fasman (1978)

protein	α-helix (%)	β-sheet (%)	β-turn (%)	random coil (%)
Avena phytochrome[a]	29.9	22.9	33.9	13.3
C-phycocyanin[b]				
α-subunit	27.7	27.7	34.6	10.0
β-subunit	30.2	24.3	34.9	10.7

[a] Analysis based on the amino acid sequence of Hershey et al. (1985). [b] Analysis based on the amino acid sequence of C-phycocyanin isolated from *Mastigocladus laminosus* (Frank et al., 1978).

tryptic patterns for the P_r and $P_r + Zn^{2+}$ were also indistinguishable (data not shown).

Analysis of the amino acid sequences of oat phytochrome by the method of Chou and Fasman (1978) resulted in predictions for the secondary structures of apophytochrome (Table II). Approximately 30% α-helix and 23% β-turn were predicted for oat apophytochrome. A similar prediction resulted from the analysis of the α- and β-subunits of C-phycocyanin. These predictions clearly contrast with the secondary structures of their holoproteins, as determined experimentally (Schirmer et al., 1985, 1987).

Discussion

Although cysteine and histidine residues are known metal-binding sites in proteins (Berg, 1986; Hamer, 1986) and phytochrome contains several of these residues (Hershey et al., 1985), chelation with zinc ions at the phytochrome chromophore appears to be preferred. The low concentration of zinc ions required to produce a spectrophotometrically detectable change in phytochrome, the differential bleaching of P_{fr} relative to P_r, and the emission of fluorescence by chelated phytochrome support this assumption. At a 1:1 stoichiometric ratio of zinc ions to phytochrome molecules, nonspecific chelation at amino acid residues is expected to have similar effects on the two forms of phytochrome. However, the present data, as well as previous studies involving the addition of zinc ions to phytochrome (Lisansky & Galston, 1974; Furuya et al., 1965), clearly demonstrate that zinc ions preferentially bleach the P_{fr} form of phytochrome. These observations are consistent with data obtained in studies using tetranitromethane (TNM) (Hahn et al., 1984b; Thümmler et al., 1985), ANS (Song & Yamazaki, 1987), sodium borohydride (Chai et al., 1987a), and bilirubin oxidase (Singh et al., 1989) to probe the topography of the P_r and P_{fr} chromophores. The results of these experiments, as well as the present data, support the proposed model for the phototransformation of phytochrome, in which the P_{fr} chromophore is exposed to the environment and is accessible to chemical agents, while the P_r chromophore lies within a hydrophobic crevice and is therefore less accessible.

The absence of a zinc-induced change in the fluorescence, CD, and proteolytic digestion of BSA under these experimental conditions also supports site-specific chelation at the phytochrome chromophore.

The fluorescence emitted by zinc–P_{fr} chelation is attributed to cyclization of the semiextended P_{fr} chromophore (Thümmler & Rüdiger, 1983), possibly through cis–trans isomerization of the D ring of the chromophore. Crystallographic studies of zinc–biliverdin complexes demonstrate that the four nitrogen atoms of the tetrapyrrole bind to one zinc ion, resulting in a slightly distorted square planar configuration around the zinc atom (Thewalt & Führhop, 1975). Since biliverdin and the phytochrome chromophore are structurally very similar, a similar configuration may result upon chromophore–zinc chelation.

Upon phototransformation of native 124-kDa phytochrome from P_r to P_{fr} in 20 mM KPB, a photoreversible increase in ellipticity is reproducibly observed, corresponding to a 3% increase in α-helical folding of the apoprotein (Vierstra et al., 1987). This increase in α-helicity is attributed to increased chromophore–apoprotein interaction at the N-terminus sequence, possibly through hydrogen bonding, hydrophobic forces, and/or electrostatic forces (Chai et al., 1987a). Cyclizing the chromophore through chelation with zinc ions perturbs this interaction, attenuates the interactive forces, and frees the apoprotein from many of the conformational restraints imposed upon it by the chromophore. Thus, in effect, chelation with zinc ions "removes" the chromophore from the protein pocket and allows the examination of the conformation of phytochrome's apoprotein-like species.

Analysis of the CD spectrum of chelated phytochrome yields a secondary structure prediction of 28% α-helix, 39.5% β-sheet, 7.5% β-turn, and 25% random coil. This prediction deviates significantly from the structural analysis for native 124-kDa phytochrome in 20 mM Tris buffer of 50.5% α-helix, 17.5% β-turn, 32% random coil, and no β-sheet. Apparently, once the interactive forces between the chromophore and apoprotein are reduced/eliminated, the phytochrome molecule undergoes a conformational rearrangement. This change in secondary structure is analogous to the conformational change produced by the removal of the tetrapyrrolic heme prosthetic group of myoglobin (Beychok, 1966). Removal of the heme destabilizes the α-helical conformation of the protein, producing an altered secondary structure, which is detectable by CD. Addition of hemin to the apomyoglobin restores the α-helical content.

Manabe and Furuya (1971) reported that incubating coleoptile tissues in buffer containing EDTA retards the loss of photodetectable phytochrome, while addition of metal ions to the buffer promotes phytochrome decay in vivo. Therefore, we tested the hypothesis that addition of EDTA to P_{fr} solutions bleached by zinc ions would restore the absorbance at 730 nm. Incubation of the bleached phytochrome with up to 3 mM EDTA for several hours produced only a very slight restoration of absorbance, however. This suggests that Zn^{2+} ions isomerized the configuration of the Pfr chromophore to that of the Pr form, i.e., dark reverted from Pfr to Pr.

Analysis of oat phytochrome by the method of Chou and Fasman (1978) yields a substantial amount of β-pleated sheet. On the basis of the present analysis of chelated oat phytochrome and preliminary studies of pea phytochrome apoprotein produced by *Escherichia coli*,[2] we propose that the absence of β-sheet as detected by CD in native phytochrome is the result of the interactive forces between the phytochrome chromophore and its binding domain. Once these forces are attenuated by the addition of zinc ions, β-sheet appears in the analysis, as predicted by the Chou–Fasman method. A similar result was found for C-phycocyanin. Although the Chou–Fasman method predicted that approximately one-fourth of the secondary structure of C-phycocyanin from *Mastigocladus laminosus* is comprised of β-sheet structures, X-ray crystallography reveals an absence of these structures (Schirmer et al., 1985, 1987). In addition, while the Chou–Fasman method predicts ca. 28% α-helix in the α-subunit and 30% α-helix in the β-subunit of C-phycocyanin, X-ray crystallography reveals that 65% and 62% of the structure of the α- and β-subunits

[2] D. Sommer, P.-S. Song, K. Tomizawa, and M. Furuya, unpublished results.

are composed of α-helix, respectively. Therefore, it appears that chromophore–protein interactions in both phytochrome and C-phycocyanin, not taken into account by the Chou–Fasman method, substantially alter the apoprotein conformation, specifically by increasing the α-helicity of the polypeptide at the expense of β-sheet structures.

The comparative proteolytic digestions of P_{fr} and zinc ion bleached P_{fr} provide further evidence of zinc-induced conformational changes in phytochrome. Trypsin acts on arginine and lysine residues, while α-chymotrypsin shows cleavage specificity toward the aromatic residues and leucine. The differences in the cleavage patterns between the chelated and nonchelated phytochrome (Figure 5) indicate that a change in the accessibility of the protease to these residues has occurred, presumably as the result of differences in protein folding.

Conclusions

The relative extent of chromophore exposure in the P_r and P_{fr} forms of phytochrome and the secondary structure of the phytochrome apoprotein have been investigated using zinc-induced modification of the phytochrome chromophore. The absence of bleaching of P_r in the presence of a 1:1 molar ratio of zinc ions, in contrast to the extensive spectral bleaching of the P_{fr} form, confirms previous reports of differential exposure of the P_{fr} chromophore relative to the P_r chromophore. Modification of the P_{fr} chromophore by chelation with zinc ions from its native extended/semiextended conformation to a cyclohelical conformation (i.e., spectral bleaching of the P_{fr} absorbance band at 730 nm) induces a marked change in the secondary structure of the protein as detected by CD. Phytochrome chelation with zinc ions apparently reduces/eliminates chromophore–protein interactions, which in effect stimulates the conformation of apophytochrome. The rate and pattern of proteolytic degradation of the P_{fr}–zinc complex differ from those of native P_{fr}, reflecting a change in the protein structure and accessibility of proteases to cleavage sites, which may have a biological significance.

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