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Spectral Perturbations and Oligomer/Monomer Formation in 124-Kilodalton *Avena* Phytochrome†

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ABSTRACT: We have studied the effects of pH, ionic strength, and hydrophobic fluorescence probes, 8-anilinonaphthalene-1-sulfonate (ANS) and bis-ANS, on the structure of intact (124-kDa) *Avena* phytochrome. The Pfr form of phytochrome forms oligomers in solution to a greater extent than the Pr form. Hydrophobic forces play a major role in the oligomerization of phytochrome, as suggested by fluorescence and monomerization by bis-ANS. However, electrostatic charges also take part in the phytochrome oligomerization. The partial proteolytic digestion patterns for the Pr and Pfr species are different, but binding of bis-ANS to the phytochrome abolishes this difference and yields an identical proteolytic peptide mapping for both spectral forms of phytochrome. This appears to result from bis-ANS binding at the carboxy-terminal domain, which induces monomerization of phytochrome oligomers. A second bis-ANS binding at an amino-terminal site blocks cleavage sites of trypsin and α-chymotrypsin. Bis-ANS especially blocks access of the proteases to the amino-terminal cleavage site that produces an early proteolytic product (114/118 kDa) on SDS gels. The bis-ANS binding does not, however, affect the proteolytic cleavage site that occurs in the hinge region between the two structural domains of phytochrome, the chromophore domain and the C-terminal non-chromophore domain. A chromophore binding site in the Pfr form is apparently exposed for preferential binding of bis-ANS, causing cyclization of the chromophore and bleaching of its absorbance at 730 nm. These observations have been discussed in terms of a photoreversible topographic change of the chromophore/apoprotein during the phototransformation of phytochrome.

Phytochrome is a chromoprotein consisting of a tetrapyrrole chromophore covalently linked to a polypeptide of molecular mass in the range of 120–127 kDa, depending upon plant species [for review, see Lagarias (1985) and Furuya (1987)]. Phytochrome exists in two forms that are photoreversible, with the Pr form absorbing maximally at 666 nm and the Pfr form at 730 nm. Photoconversion from the Pr to the Pfr form in vivo induces a number of photomorphogenic and developmental responses, including gene expression, whereas reversion of the Pfr to the Pr form cancels the induction of those responses [for review, see Pratt (1979), Schmidt and Mohr (1982), Song (1983), Quail (1984), Lagarias (1985), and Furuya (1987)].

On the basis of spectroscopic and ANS[1]-induced spectral bleaching studies on degraded 118/114-kDa oat phytochrome, a working model for the phytochrome phototransformation has been proposed (Song et al., 1979; Hahn & Song, 1981). According to the proposed model, the Pr to Pfr phototransformation generates a specific hydrophobic surface on the Pfr protein as a result of chromophore reorientation. The difference in hydrophobicity between the Pr and Pfr forms of 118/114-kDa phytochrome has been examined in terms of affinity for ANS (Hahn & Song, 1981), liposomes (Kim & Song, 1981), Cibacron blue F3GA (Smith & Daniels, 1981), and alkyl groups (Yamamoto & Smith, 1981).

However, the applicability of this model to native 124-kDa oat phytochrome (Vierstra & Quail, 1982, 1983) has not been examined. In fact, preliminary observations (Eilfeld & Ruediger, 1985) indicated that the spectroscopic behaviors of phytochrome, i.e., with respect to chromophore bleaching by ANS and tetranitromethane (Hahn et al., 1984), are significantly modulated by the presence of the 6/10-kDa amino-terminal fragment.

In this report, we describe a follow-up study of the structural properties of 124-kDa phytochrome as probed by bis-ANS. ANS and bis-ANS are unique fluorescence probes for the structural and topographical studies of the phytochrome molecule, not only because of their hydrophobic property (Brand & Gohlke, 1972) but more importantly due to their

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[1] Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CTAB, cetyltrimethylammonium bromide; HA, hydroxylapatite; HPLC, high-performance liquid chromatography; KPB, potassium phosphate buffer; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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specific bleaching effect on the spectra and conformations of the phytochrome molecule (Hahn & Song, 1981). The effects of bis-ANS, pH, and ionic strength on the phytochrome conformation were monitored by steric exclusion HPLC, gel electrophoresis, suberimidate cross-linking, peptide mapping, and spectroscopic methods.

Materials and Methods

Chemicals. Trypsin (bovine pancreas, type XI), chymotrypsin (bovine pancreas, type I-S), and PMSF were obtained from Sigma Chemical Co. (St. Louis, MO). Sperm whale myoglobin (type II, 95–100%) and human hemoglobin (2× crystallized) for suberimidate cross-linking standards were also purchased from Sigma. Ammonium sulfate (ultrapure) was purchased from Schwarz/Mann (Orangeburg, NY), and poly(ethylenimine) was obtained from Eastman Kodak Co. (Rochester, NY). Protein molecular weight standards for SDS–PAGE were from Bio-Rad Laboratories, Richmond, CA, and Pharmacia, Piscataway, NJ. Protein molecular weight standards for HPLC and gel electrophoretic reagents were purchased from Bio-Rad. Bis-ANS, ANS, dimethyl suberimidate (dihydrochloride), and triethanolamine (free base and hydrochloride) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Bis-ANS and ANS were further purified as described previously (Hahn & Song, 1981).

Phytochrome Purification. All experiments dealing with phytochrome were performed under a green safelight at 4 °C, unless otherwise noted. Phytochrome was purified from the extract of 4-day-old etiolated oat seedlings by ammonium sulfate precipitation, HA chromatography, and ammonium sulfate back-extraction (Chai et al., 1987a). The supernatant fraction from the back-extraction was applied to a Bio-Gel A 1.5-m column (2.5 × 90 cm) equilibrated with 20 mM KPB, pH 7.8, containing 1 mM EDTA at 4 °C. Phytochrome was eluted with the same buffer at a flow rate of 20 mL/h. The specific absorbance ratio (SAR = A_{660}/A_{280}) of phytochrome used for the present study ranged from 0.9 to 1.09. Phytochrome was photoconverted by irradiating with a Bausch and Lomb microscope illuminator combined with a 666-nm interference filter (Oriel C572-6600) for red light, and with a far-red cutoff filter (Ealing 26-4457) for far-red light irradiation. Fluence rates were 7.5 and 160 W/m^2 for the red and far-red lights, respectively.

Steric Exclusion HPLC. A Perkin-Elmer Series 4 liquid chromatograph equipped with a Perkin-Elmer LC-85 spectrophotometric detector was employed. Aliquots of phytochrome (A_{666} = 0.5) dissolved in 50 mM Tris-HCl buffer, pH 7.8, containing different KCl concentrations (0–0.8 M) were injected into a steric exclusion column (TSK 3000 SW, 0.75 × 30 cm with a guard column, 0.75 × 7.5 cm). The injection volume was 10–30 μL, depending upon the peak height of phytochrome. The TSK 3000 SW column was equilibrated with 50 mM Tris-HCl buffer containing the same concentration of KCl as in the phytochrome solution and operated at a flow rate of 0.8 mL/min at 25 °C. Molecular weight standards were run at different KCl concentrations as necessary. The apparent molecular mass of phytochrome was determined from a plot of retention time vs molecular weight of protein standards. The amount of oligomeric form of phytochrome was determined by integrating the HPLC peak for the oligomeric phytochrome, which appeared at the exclusion limit of a TSK 3000 SW column.

Proteolytic Digestion of Phytochrome. All proteolytic reactions were carried out under a green safelight in 1 mM EDTA and 20 mM KPB, pH 7.8, at 4 °C. Phytochrome was digested with trypsin at a 1:500 (w/w) protease to phytochrome ratio, whereas the reaction with chymotrypsin was carried out at a 1:100 (w/w) ratio. Bis-ANS was incubated with phytochrome either as Pr form or as Pfr form before adding proteases. Enzyme stock solutions (1 mg/mL) dissolved in the same buffer as above were added to phytochrome solutions to make the appropriate enzyme/substrate ratio. At various time intervals, an aliquot of digestion mixture was removed and the reaction was quenched by adding PMSF (2 mM). The hydrolysates were incubated at 4 °C for 5 min and then stored at −40 °C until the samples were applied to gel electrophoresis.

Native and Denaturing (SDS) PAGE. Native PAGE was carried out according to the manufacturer's instruction on a PhastSystem gel electrophoresis separation unit from Pharmacia (Piscataway, NJ), using a miniature gradient gel (10–25% gel, 3 × 4 cm) obtained from the company. The native gel buffer system used in the PhastSystem is 0.112 M acetate and 0.112 M Tris, pH 6.4, for the gel and 0.88 M L-alanine and 0.25 M Tris, pH 8.8, for the buffer strips (analogous to the running buffer in a reservoir-containing system). The gels were stained with Coomassie brilliant blue R-250 and scanned with a Hoefer Scientific Instruments GS 300 densitometer. SDS–PAGE was conducted by using Laemmli's discontinuous buffer system with an 8% gel (Laemmli, 1970). The frozen proteolytic digests were thawed and diluted with an equal volume of SDS–PAGE sample buffer containing 0.2 M Tris-HCl, pH 6.8, 15% (v/v) glycerol, 3% SDS, 0.7 M 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue. The samples were then heated at 100 °C for 2 min before being applied to the gel. The apparent molecular weight of phytochrome was estimated from the calibration curve of the molecular weight protein standards.

Suberimidate Cross-Linking. Suberimidate cross-linking of myoglobin, hemoglobin, and phytochrome was performed according to Davies and Stark (1970). EDTA (1 mM) was added to prevent bleaching of phytochrome. Cross-linking was carried out in 0.2 M triethanolamine, pH 8.5, and 0.0 or 0.5 M KCl. Forty microliters of 1 mg/mL stock solutions of each protein was added to a suberimidate solution for a final volume of 125 μL and a final suberimidate concentration of 1.5 mg/mL. Reactions were also carried out with 0.5 and 2.5 mg/mL suberimidate for myoglobin and hemoglobin. All suberimidate solutions were prepared immediately before use with a newly purchased stock. The reaction mixtures were incubated for 3 h at 25 °C. A 31.3-μL aliquot of SDS–PAGE sample buffer containing 0.1 M Tris-HCl, pH 6.75, 10% SDS, 50% glycerol, 25% 2-mercaptoethanol, and 0.2% (w/v) bromophenol blue was added to each solution after the 3-h incubation. These SDS-containing mixtures were then incubated at 37 °C for 2 h before application to gels. Molecular weight proteins standards as well as untreated phytochrome (no suberimidate treatment) were prepared by using the same SDS–PAGE sample buffer and 0.2 M triethanolamine reaction buffer as the suberimidate-treated samples. Additionally, untreated phytochrome and molecular weight standards used for phytochrome analysis also contained 1.5 mg/mL suberimidate, but cross-linking was prevented by addition of the suberimidate after addition of the SDS–PAGE sample buffer. Molecular weight standards and untreated phytochrome samples were incubated for 2 min at 100 °C before application to gels.

Suberimidate-treated myoglobin and hemoglobin were analyzed as previously described [native and denaturing (SDS) PAGE] except that a 5% acrylamide gel was used. Suberimidate-treated phytochrome and untreated phytochrome were

applied to a 3% acrylamide continuous gel (no stacking gel) using the buffer system of Laemmli (1970). Gels sized 10 × 8 × 0.1 cm were used, and a Bio-Rad Mini PROTEAN II electrophoresis unit was employed. Apparent molecular weights of suberimidate-treated phytochrome were estimated from a calibration curve of standards as well as untreated phytochrome. The specific absorbance ratio of the phytochrome used in these cross-linking studies was 0.99 as measured in the 0.2 M triethanolamine reaction buffer described above. Gels were then stained with Coomassie brilliant blue R-250 and photographs taken. The same gels were then silver stained by using a Sigma Silver Stain Kit AG-25 according to the instructions provided with the kit.

Spectroscopic Assays. Absorbance spectra of phytochrome were recorded on a Lambda-3B spectrophotometer (Perkin-Elmer Corp., Norwork, CT) or a HP 8451A diode-array spectrophotometer equipped with single-beam optics (Hewlett-Packard Co., Palo Alto, CA). The diode-array spectrophotometer was used with a 5% transmittance neutral density filter (Oriel GD-130) to reduce the intensity of the deuterium source light which affects phytochrome phototransformation. For fluorescence measurements, an Aminco-Bowman spectrofluorometer (American Instrument Co., Silver Spring, MD) was used with a 1-cm path length rectangular cuvette. The excitation wavelength for bis-ANS was 410 nm. The band path width for excitation was 6.4 nm and that for emission was 3 nm. CD spectra were recorded on a Jasco-20 CD-ORD spectropolarimeter. The instrument was modified by replacing a Pockels cell with a Morvue photoelastic modulator and adding a lock-in amplifier to enhance the signal to noise ratio (Jung et al., 1980). The spectropolarimeter was calibrated by using *cis*-anderosterone as a reference (Kirk & Klyne, 1976).

Results

Effects of pH and Ionic Strength on Phytochrome Conformations. The solution behavior of phytochrome in the absence of bis-ANS has been examined in detail. It has been conclusively shown that native 124-kDa *Avena* phytochrome exists as a dimer in aqueous solution (Lagarias & Mercurio, 1985; Jones & Quail, 1986). Phytochrome gave two major peaks on a size exclusion HPLC chromatogram in the range of KCl concentrations from 0 to 0.8 M, as seen in Figure 1. From apparent molecular weights for the peak fractions determined by using molecular weight standards, the first peak (peak a) corresponded to the exclusion limit of the column and the second peak (peak b) was considered to be a dimeric form (MW 508 000 and 572 000 for Pr and Pfr at 0.0 M KCl, respectively, and MW 331 000 and 318 000 for Pr and Pfr at 0.8 M KCl, respectively) of phytochrome. To describe the solution behavior, particularly hydrophobicity, migratory tendencies of the phytochrome molecule were examined as a function of pH and ionic strength of the mobile phase. The apparent MW of the dimeric phytochrome (peak b in Figure 1) was dependent on the pH of the mobile phase. The apparent MW of the Pfr dimer gradually increased as the pH of the mobile phase increased. In the case of the Pr dimer, the apparent MW increased as the pH was raised from 6.9 to 7.5 but remained approximately constant from pH 7.5 to 8.1. At lower pH's, the difference in apparent MW between the Pr and Pfr forms was greater than at higher pH's. The apparent MW of the Pfr form was greater than that of the Pr form in the whole range of pH selected except at pH 7.5. At pH 7.5, Pr showed a slightly higher apparent MW than did Pfr, but the small difference was not greater than experimental error.

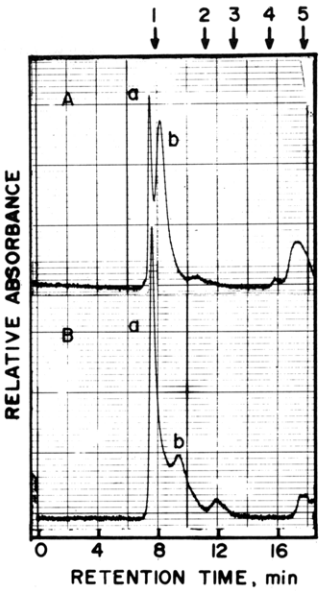

FIGURE 1: A typical chromatogram of steric exclusion HPLC for phytochrome (SAR = 0.98). The column was a Micropac TSK 3000 SW column (7.5 × 300 mm), and the mobile phase was 50 mM Tris-HCl buffer, pH 7.8. The column was operated at a flow rate of 0.8 mL/min at 25 °C, and the effluent was monitored by the absorbance at 280 nm. The mobile phase contained no KCl (A) and 0.8 M KCl (B); peak a represents an oligomeric form of phytochrome and peak b a dimeric form of phytochrome. The numbers on the top indicate the positions of molecular weight standards run in a mobile phase of 50 mM Tris buffer, pH 7.8, with 0.1 M KCl: (1) thyroglobulin (bovine; MW 670 000), (2) γ-globulin (MW 158 000), (3) ovalbumin (MW 44 000), (4) myoglobin (MW 17 000), and (5) vitamin B_{12} (MW 1355).

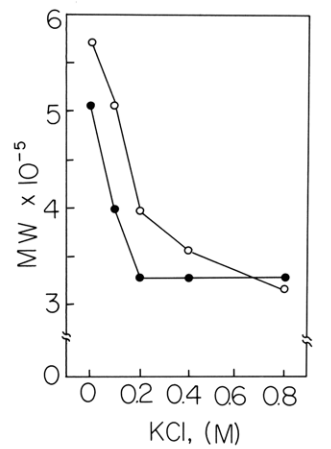

FIGURE 2: Apparent molecular weights of dimeric phytochrome (SAR = 0.98) in the Pr and Pfr forms as a function of ionic strength in mobile phase: Pr (●); Pfr (○). The mobile phase was 50 mM Tris-HCl, pH 7.8, with different concentrations of KCl. Standards (same as in Figure 1) for MW approximation were run at each concentration of KCl used.

The apparent MW of the dimeric species was studied as a function of KCl concentrations of the mobile phase (Figure 2). Without added KCl, the apparent MW of the dimeric phytochrome was over 500 000 for the both forms of phytochrome. As the KCl concentration of the mobile phase increased up to 0.2 M, the apparent MW sharply decreased. In the range of KCl concentrations from 0.2 to 0.8 M, the apparent MW became constant (from 350K to 320K). At lower ionic strengths, the Pfr form behaved as a larger molecule than the Pr form.

The phytochrome peak appearing at the exclusion limit of a TSK 3000 SW column (peak a in Figure 1; approximately 670 kDa) can be considered an oligomeric species with its size

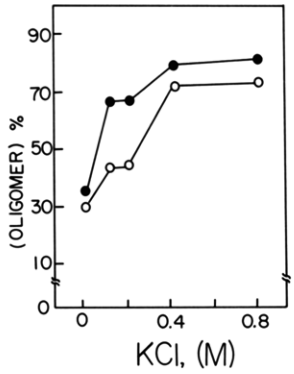

FIGURE 3: Content of an oligomeric form of phytochrome (SAR = 0.98) as a function of ionic strength of the mobile phase: Pr (○); Pfr (●). The mobile phase was 50 mM Tris-HCl buffer, pH 7.8, containing different KCl concentrations. Standards (same as in Figure 1) for MW approximation were run at each concentration of KCl used.

larger than the dimeric form. The content of oligomeric species increased with increasing ionic strength of the mobile phase (Figure 1 and 3). The Pfr form shows a higher percentage of oligomeric species than the Pr form over the entire KCl concentrations examined, especially in the 0.1–0.2 M KCl range. The relative oligomer ratio in Pfr/Pr varied as follows: 1.206 at 0.0 M KCl, 1.528 at 0.1 M KCl, 1.532 at 0.2 M KCl, 1.066 at 0.4 M KCl, and 1.111 at 0.8 M KCl.

The quaternary structure of phytochrome was characterized by suberimidate cross-linking (Davies & Stark, 1970). Figure 4 shows the results of cross-linking of phytochrome for both Pr and Pfr in 0.0 and 0.5 M KCl. Without KCl present, two bands were stained with Coomassie blue, an apparent dimer (broad band, average apparent MW 282000, calculated MW 248000) and an oligomer which is observed at the top of the gel (apparent MW greater than 580000). Upon silver staining of the same gel an apparent monomer (apparent MW 113000, calculated MW 124000) became visible in both the Pr and Pfr reactions (0.0 M KCl). A somewhat fainter band was also visible in both Pr and Pfr reactions (apparent trimer of MW 388000, calculated MW 372000), and a second faint band was visible in the Pfr reaction only (apparent tetramer of MW 515000, calculated MW 496000) containing 0.0 M KCl.

Only one clear band was visible on the Coomassie-stained gel of cross-linked Pr and Pfr in the presence of 0.5 M KCl (top of gel with apparent MW greater that 580000). A second broad band was only partially visible but became clear upon silver staining (apparent dimer of MW 240000). This broad band (also present in the 0.0 M KCl reaction) has previously been observed by Jones and Quail (1986) using cross-linking studies and resolved into three components (apparent MW 225000, 245000, and 265000) by using 5% acrylamide gels. Differences in the apparent MW of this band depending on conditions (0.0 or 0.5 M KCl) used may result from effects of salts on the mobility of the protein or actual differences in cross-linking which would result in differences in denatured conformations [discussed by Jones and Quail (1986)]. Two other bands were visible in the 0.5 M KCl silver-stained gel, an apparent trimer (apparent MW 370000) and an apparent tetramer of MW 530000 (only visible in the Pfr reaction). Some precipitation does occur upon adding SDS–PAGE sample buffer to samples that contain 0.5 M KCl. For this reason, quantitative comparisons cannot be made between reactions with and without 0.5 M KCl. There is, however, an increase in high molecular weight oligomer which only slightly entered the gel with respect to cross-linked dimer in the reaction containing 0.5 M KCl. This is apparent in the Coomassie-stained gel (Figure 4), but the region at the top of the gels is too optically dense for comparison in the silver-stained gels. An apparent dimer is also present in the un-cross-linked (no suberimidate reaction) sample. This has previously been observed and discussed by Jones and Quail (1986).

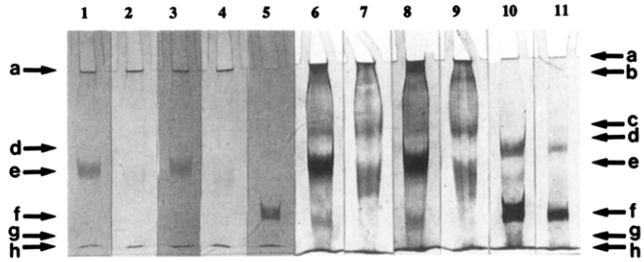

FIGURE 4: SDS–polyacrylamide gel electrophoresis of the dimethyl suberimidate cross-linked reaction mixtures. Lanes 1–5 are Coomassie stained, and lanes 6–11 are silver stained. Two gels were run, Coomassie stained and silver stained, simultaneously. No differences between the two gels in mobility or staining of standards were observed. Slight expansion of the gels occurs upon silver staining. Arrows on the left refer to lanes 1–5. Arrows on the right refer to lanes 6–11. Lanes 1–10 were loaded with 6 μg of protein each. Approximately 2 μg of protein was loaded on lane 11. Standards are as follows: (c) thyroglobulin (MW 335000), (f) un-cross-linked phytochrome (MW 124000), (g) β-amylase (MW 50000), and (h) tracking dye; standards less than MW 38000 were not sieved by the gel. Approximate location of bands is indicated by arrows as follows: (a) top of gel (apparent MW greater than 580000), (b) apparent tetramer, (d) apparent trimer, (e) apparent dimer, and (f) monomer. The exact positions of the apparent tetramers, trimers, and especially dimers vary depending on conditions. (See text for discussion.) Lanes 1 and 6 are Pr, 0.0 M KCl; lanes 2 and 7 are Pr, 0.5 M KCl; lanes 3 and 8 are Pfr, 0.0 M KCl; lanes 4 and 9 are Pfr, 0.5 M KCl; lanes 5, 10, and 11 are un-cross-linked phytochrome.

Hemoglobin and myoglobin controls showed the expected gel patterns, with tetramer being the highest cross-linked form of hemoglobin (for both 0.0 and 0.5 M KCl) and no observable cross-linking in the myoglobin reaction. This held true for reactions using 0.5, 1.5, and 2.5 mg/mL suberimidate. Although total amounts of cross-linked products increased with increasing suberimidate concentration, the ratios of cross-linked products remained the same and no higher aggregate forms appeared. This same reactivity toward suberimidate (no higher aggregate forms even at 2.5 mg/mL suberimidate) has also been observed for several other proteins (Pfeiffer et al., 1986) with known quaternary structure.

Effect of Bis-ANS on the Solution Behaviors of Phytochrome. The effects of bis-ANS on the spectral and conformational/oligomeric properties of phytochrome were studied. Figure 5 shows the fluorescence emission spectra of bis-ANS bound to the Pr and Pfr forms of phytochrome. The fluorescence intensity of the probe was 30% higher with the Pfr form than with the Pr form under the same conditions. However, it was not possible to determine whether the enhanced fluorescence of bis-ANS in the Pfr form was due to an increased binding affinity or binding at an additional/different binding site(s) on the protein (relative to the binding to the Pr form). Attempts to determine these binding parameters were unsuccessful because of incomplete saturation of the binding site(s), the heterogeneity of phytochrome subunit equilibria (monomer, dimer, and oligomer) induced by bis-ANS (vide infra), and an apparent energy transfer from the bound probe to the chromophore of the proteins (data not shown).

Absorbance changes of phytochrome upon addition of 0.4 mM bis-ANS were recorded. The spectral changes of 124-kDa phytochrome by bis-ANS were similar to the ANS effects on the degraded large (118-kDa) phytochrome (Hahn & Song,

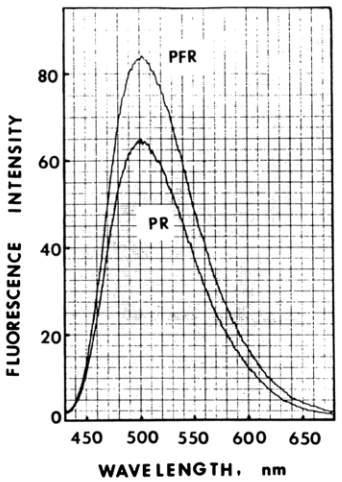

FIGURE 5: Fluorescence enhancement of bis-ANS by binding to phytochrome (SAR = 1.09) upon Pr to Pfr phototransformation. Phytochrome concentration was 1.0 μM in 20 mM phosphate buffer, pH 7.8, and bis-ANS concentration used was 10 μM. The fluorescence was measured at 20 °C with an excitation wavelength of 410 nm. The band paths of excitation and emission were 6.4 and 3 nm, respectively.

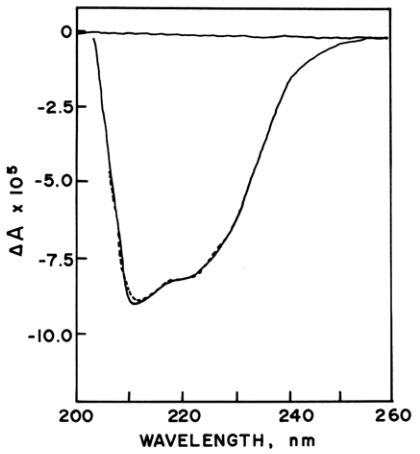

FIGURE 6: Far-ultraviolet circular dichroic spectra of phytochrome (A_{667} = 0.023; SAR = 1.09) in the presence of 0.3 mM bis-ANS: Pr (—); Pfr (——). The spectra were measured in 20 mM KPB, pH 7.8, with 1 mM EDTA at 20 °C, with a 2-mm path length cuvette.

1981); bis-ANS rapidly bleached the Pfr absorbance of 124-kDa phytochrome, whereas its Pr form remained unbleached for 27 min of bis-ANS incubation. Approximately 54% of the Pfr absorbance was lost (730 nm) after an 18-min incubation. The Pr species also bleached after a prolonged incubation. ANS effects on the absorbance of 124-kDa phytochrome were similar to the bis-ANS effects. However, the effective concentration of ANS for the spectral bleaching was 10-fold higher than that of bis-ANS.

Phytochrome is known to exhibit a photoreversible change in its far-ultraviolet CD spectrum (Chai et al., 1987b). However, no detectable far-ultraviolet CD signal changes in both forms of phytochrome were observed when bis-ANS was bound to phytochrome, as shown in Figure 6. By assuming that the bound bis-ANS contributes a negligible induced activity to the far-ultraviolet CD signals, the α-helix content of both forms of phytochrome in the presence of 0.3 mM bis-ANS was calculated to be identical. In the absence of bound bis-ANS, however, the helix content was reported to be 52.2 and 55.3% for Pr and Pfr, respectively (Chai et al., 1987b). Thus, the bound bis-ANS abolished a photoreversible CD change in phytochrome.

Steric exclusion HPLC of phytochrome in the presence of bis-ANS was run, as seen in Figure 7. The hydrophobic probe

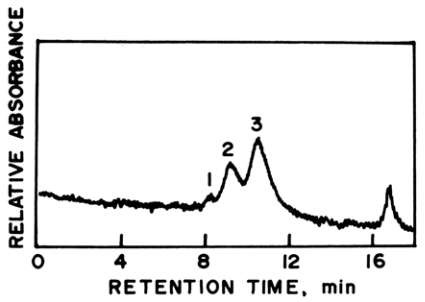

FIGURE 7: Steric exclusion HPLC chromatographic profiles of phytochrome (3.8 μM) in the presence of 0.52 mM bis-ANS. Peaks 1, 2, and 3 correspond to oligomer (ca. 670 kDa) dimer (384 kDa) and monomer (194 kDa) species of phytochrome, respectively.

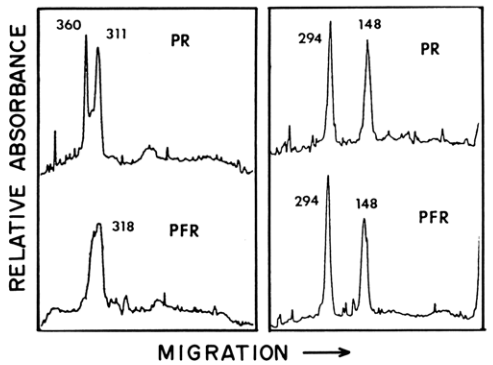

FIGURE 8: Densitometric patterns of electrophoresis of native (left panel) and bis-ANS-treated phytochrome (right panel) under nondenaturing conditions. Electrophoresis was carried out with a Pharmacia PhastSystem electrophoresis gel (8–25% linear gradient, 0.112 M acetate, 0.112 M Tris, pH 6.4). All operations were done under a green safelight at 8 °C. For treatment of phytochrome with bis-ANS, either form of phytochrome (6 μM; SAR = 0.9) was incubated with 0.4 mM bis-ANS for 20 min before being applied to the Pharmacia PhastSystem gel. The figures on the top of each peak indicate the molecular weights estimated by the instructions of Pharmacia PhastSystem.

markedly reduced the oligomer peak of both forms of phytochrome (670-kDa peak). At concentrations of 0.52 mM or less bis-ANS, the oligomer peak was barely discernible and a dimer peak at ca. 390 kDa also decreased in amplitude, whereas a new peak corresponding to 194 kDa appeared as a predominant component. This form appears to be a monomeric species of phytochrome.

On native gel electrophoresis, Pr and Pfr forms of phytochrome showed different band patterns (Figure 8). Although the phytochrome preparation was electrophoretically pure on SDS–PAGE, both forms of phytochrome had more than one band on native PAGE, in agreement with the observation by Schendel and Rudiger (1989). The Pr form of phytochrome showed two bands having apparent MW of 360 000 and 311 000. The Pfr form showed one broad band which could be resolved into two bands with apparent molecular masses of 318 and 290 kDa. The native gel electrophoretic pattern of phytochrome in the presence of 0.4 mM bis-ANS showed both forms of phytochrome having an identical band pattern (Figure 8). The two bands corresponded to apparent molecular masses of 294 and 148 kDa, which may be assigned to dimer and monomer, respectively.

Effects of ANS and Bis-ANS on the Proteolytic Patterns of Phytochrome. To probe the binding site(s) of the hydrophobic fluorescence probe, bis-ANS, proteolytic digestions of the probe-bound protein were analyzed electrophoretically on a gradient SDS–PAGE. Limited proteolytic digests of the Pr and Pfr forms of phytochrome confirm the previous results that the two forms of phytochrome had different cleavage

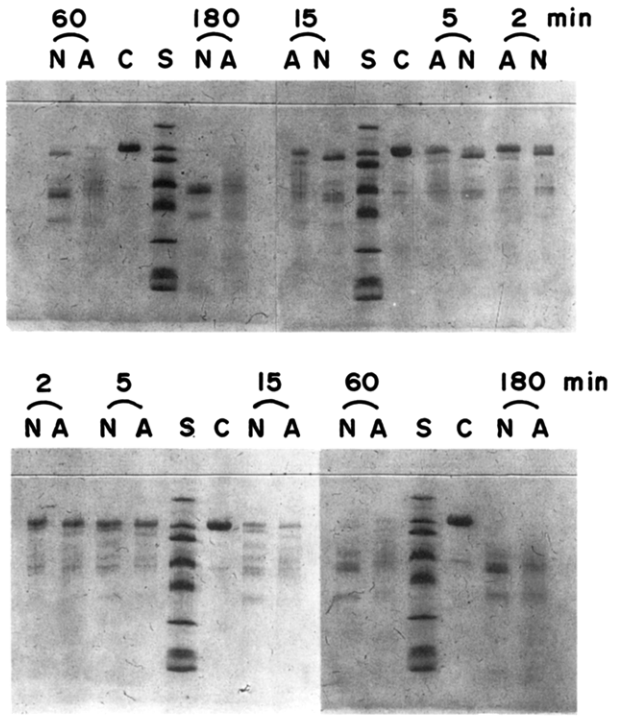

FIGURE 9: SDS–polyacrylamide gel electrophoresis of the tryptic digests of phytochrome (SAR = 0.97) with or without bis-ANS treatment. Upper panel: Digested as Pr form. Lower panel: Digested as Pfr form. The incubation time of phytochrome with trypsin is indicated on the top of each figure. Lane N: Phytochrome digested with the enzyme without bis-ANS treatment. Lane A: Phytochrome digested with the enzyme after treatment with 400 μM bis-ANS. Lane C: A control (not treated with enzyme and bis-ANS) phytochrome. Lane S: molecular weight standards. The molecular weight standards used were myosin (MW 200 000), β-galactosidase (MW 116 300), phosphorylase (MW 92 500), bovine serum albumin (MW 66 200), ovalbumin (MW 45 000), carbonic anhydrase (MW 31 000), soybean trypsin inhibitor (MW 21 500), and egg white lysozyme (MW 14 400) from Pharmacia.

patterns for both proteases of trypsin and α-chymotrypsin (Lagarias & Mercurio, 1985; Kim & Song, 1987). Time courses of the trypsin digestion patterns of the Pr vs the Pfr are shown in Figure 9. After 2 min of incubation, most of the intact 124-kDa band in the Pr form hydrolyzed into the 114-kDa band. On the other hand, the Pfr form showed a trace of 114-kDa band, which might have originated from a photostationary presence of the Pr form that was preferentially hydrolyzed. After 15 min, hydrolysis of the 124-kDa species to 114-kDa was nearly complete in the Pr form, but the former remained as a distinct band in the Pfr digest. After 15-min incubation, the Pfr form revealed two additional cleavage sites, one yielding 85- and 39-kDa species and another yielding 69- and 55-kDa species. These two sites appeared to have similar susceptibility toward trypsin digestion. Main species for both forms of phytochrome after 180-min incubation were 59-, 55-, and 39-kDa fragments, which were thus stable toward tryptic proteolysis. Thus, a prominent difference in trypsin digestion between the two forms of phytochrome was the susceptibility at a site that yields 118/114-kDa species. It is well-known that the 118-kDa species arises from a cleavage of 6-kDa N-terminal fragment of the 124-kDa full-length phytochrome, whereas the 114-kDa species comes from the same cleavage site (6 kDa) plus a cleavage at the C-terminus fragment (Lagarias & Mercurio, 1985; Grim et al., 1988).

In the presence of 400 μM bis-ANS the tryptic cleavage patterns for the Pr and Pfr forms of phytochrome became identical as seen in Figure 9. These patterns, however, were

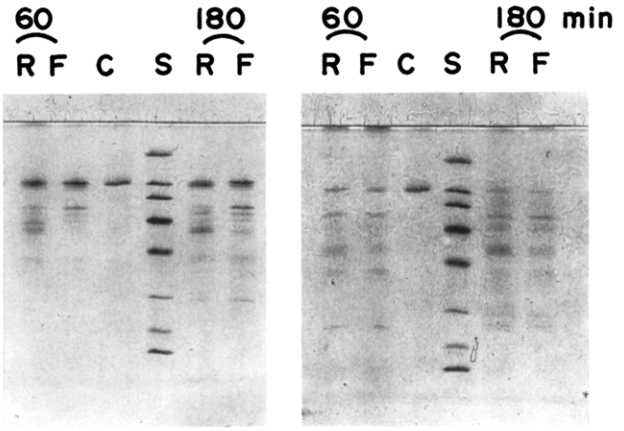

FIGURE 10: SDS–PAGE patterns of the chymotryptic digests of phytochrome with (right) or without (left) treatment of 100 μM bis-ANS. Lanes R, F, C, and S represent the Pr form, the Pfr form, a control (not treated with enzyme and bis-ANS) phytochrome, and molecular weight standards, respectively. Molecular weight standards were the same as those used in Figure 9.

different from those without added bis-ANS. This indicates that the binding of bis-ANS protected the terminal cleavage site in the Pr form that yielded a 118/114-kDa species. Both forms of phytochrome had a new cleavage site producing a 95-kDa species. After 15-min incubation, 75-, 66-, and 59-kDa species intensified, and after 180 min, a major band at 59 kDa and a minor band at 39 kDa remained.

Figure 10 shows the α-chymotryptic digestion patterns of both forms of phytochrome. α-Chymotrypsin, whose cleavage site is at hydrophobic sequences, yielded more complicated proteolytic digestion patterns for the Pfr form of 124-kDa phytochrome than for the Pr form, as was the case of degraded large (118-kDa) phytochrome (Kim & Song, 1987). An N-terminal proteolytic site that produces 117-kDa species was proteolytically more susceptible in the Pr form than in the Pfr form. Upon prolonged digestion, the resulting major fragments of proteolysis were identified as 70- and 54-kDa species in the Pr form and 83- and 70-kDa species in the Pfr form. The site producing 70 kDa appeared to have similar susceptibility toward the Pr and Pfr forms.

The α-chymotryptic digestion patterns for the Pr and Pfr forms also appeared indistinguishable in the presence of 100 μM bis-ANS. However, they were also different from those of the Pr and Pfr forms without bis-ANS. The site that produces a 117-kDa species was no longer susceptible to α-chymotryptic cleavage. The cleavage sites at 83 and 70 kDa could be seen, as is the case without bis-ANS. Instead of a 54-kDa species appearing in the absence of bis-ANS, a new band of 50 kDa was observed.

Discussion

Phytochrome preparations containing components with higher aggregate forms than dimer have been observed for some time. Pratt (1973) and Grombein and Rudiger (1976) noticed a small peak or shoulder of photoreversibility preceding the main phytochrome species on molecular sieve columns. This higher molecular weight shoulder has been recorded more recently (Kim et al., 1989) using intact (undegraded) phytochrome. Size exclusion HPLC has been performed on phytochrome previously (Lagarias & Mercurio, 1985) in order to study differences between the Pr and Pfr dimers. Actual chromatographs were not published, however, and the presence of an oligomeric form of phytochrome was not discussed. This study of phytochrome performed by Lagarias and Mercurio (1985) yielded a "markedly lower" estimate (Lagarias, 1985)

of the Stokes radius of the phytochrome dimer (56 Å) than was obtained for phytochrome in solution (81 Å) by quasi-elastic light scattering (Sarker et al., 1984). Jones and Quail (1986) also determined a Stokes radius of 56 Å for the dimer. This "inconsistency" is likely due to the presence of phytochrome oligomers which are evident from SEC-HPLC studies (Figure 1) as well as suberimidate cross-linking analysis (Figure 4). Both of these studies were carried out with highly purified phytochrome (SAR = 0.9–1.09) and spectral characteristics (photoreversibility and chromophore absorbance) which indicate that these studies were performed on native phytochrome and not a denatured molecule. Additionally, standard proteins run on SEC-HPLC under the conditions described above produce artifact-free chromatographs (single protein peaks) that generate the proper linear relationship between log molecular weight and retention time, indicating the SEC-HPLC analysis system is valid. The quaternary structure standards for suberimidate cross-linking used in this study (hemoglobin and myoglobin) showed no artifactual subunit cross-linking (intermolecular cross-linking), even with suberimidate concentrations 67% higher than those used for phytochrome. Aldolase, β-lactoglobins A and B, and carbonic anhydrase have also previously been (Pfeiffer et al., 1986) shown to demonstrate no artifactual cross-linking in conditions identical (except 1 mM EDTA was not used) with the ones for our controls (67% greater suberimidate concentration than was used in the phytochrome reaction).

Jones and Quail (1986) have previously performed suberimidate (other cross-linking reagents were also used) cross-linking of phytochrome. The buffer used (0.1 M borate, pH 10) was different from the buffer we employed (0.2 M triethanolamine, pH 8.5). the pH of the reaction carried out by Jones and Quail (pH 9.2) was approximately 1 pH unit higher than we used. This may account for the observation that bands corresponding to trimers and tetramers were "rarely observed". Additionally, trimer and tetramer bands are not visible (Figure 4) on Coomassie-stained gels whereas dimer bands are clear under these conditions (0.0 M KCl only; the dimer band is not as clear in the reaction containing 0.5 M KCl). This indicates that trimer and tetramer bands that are observable on silver-stained gels (Figure 4) may be 2 orders of magnitude less concentrated than the dimer band, which may account for the observation that "the major product [of cross-linking with various reagents and analysis by SDS–PAGE] has an apparent molecular mass of approximately 225 kDa" made by Jones and Quail (1986). We have determined that the high molecular weight cross-linked oligomer (Figure 4) does not enter 5% acrylamide stacking gels well and will not enter a 5% acrylamide separating gel using Laemmli's (1970) buffer system. The cross-linked oligomer is trapped at the top of the stacking gel, in much the same way that it is trapped at the top of the 3% acrylamide gel in Figure 4. Thus, any high molecular weight species (if present) may have not been observable under the conditions used by Jones and Quail (1986). It must be noted that the present work does not refute any of the conclusions drawn by Jones and Quail (1986) concerning the existence of phytochrome as predominately dimer at in vivo concentrations (about 0.1 mg/mL cytoplasm) or the presence of phytochrome as predominately dimer under equilibrium sedimentation conditions (or monomer in the case of phytochrome in 1 M NaCl). Sedimentation conditions are, of course, drastically different from conditions employed in the present study, and thus results cannot be expected to be identical for the two experiments. In fact, the latter observation (monomerization of phytochrome in 1 M NaCl) may shed light on the present study since increased oligomerization resulting from higher salt concentrations may also result in loss of some dimer interactions within the oligomer. This idea is also supported by the appearance of a significant apparent monomer peak in high salt concentrations (Figure 1B) at about 12.9-min retention time using SEC-HPLC.

We have observed that oligomerization of phytochrome is concentration dependent, since the oligomer content of phytochrome may be increased significantly (analysis by SEC-HPLC) by increasing phytochrome concentration (data not shown). Also, oligomer peaks are lost upon application to low-performance columns where the phytochrome becomes dilute for extended periods of time (i.e., in large high molecular weight sieve columns). This agrees qualitatively with Jones and Quail (1986), who observed no oligomer peak in low-concentration SEC (fraction analysis by immunoblotting).

Hydrophobicity seems to be a major force in oligomerization of phytochrome, since higher ionic strength enhanced the oligomer formation of both forms (Pr and Pfr) of phytochrome. However, the pH effects observed suggest that the oligomerization is not entirely governed by hydrophobicity but also by electrostatic interactions between the subunits. ANS and bis-ANS emit intense blue fluorescence when bound to hydrophobic sites of proteins and membranes (Brand & Cohlke, 1972). The enhanced bis-ANS fluorescence in the Pfr complex over the Pr complex is qualitatively consistent with a change in the hydrophobicity of phytochrome (Hahn & Song, 1981) and its oligomerization upon Pr → Pfr phototransformation. The enhanced fluorescence effect may be attributable partly to the binding of the probe at specific site(s) (Choi, 1987). In the present study, the proteolytic peptide mapping of phytochrome treated with bis-ANS also indicates bis-ANS binding at specific sites on the phytochrome surface.

Phytochrome has a p*I* of 5.5 and 5.7 for the Pfr and Pr forms, respectively (Choi, 1987). On native polyacrylamide gel electrophoresis at pH 7.8, the two forms of phytochrome have different mobility and each of the Pr and Pfr species showed two bands exhibiting different mobility (Figure 8). Schendel and Rudiger (1989) also observed the mobility differences between the Pr and Pfr forms by native gel electrophoresis. In their results, the single broad Pfr band migrated faster than the single broad Pr band in electrophoresis at pH 8.8, in contrast to the discrete two bands for both forms of phytochrome, at pH 7.8, in the present results. The reason for this difference is not clear, but it may be partly due to the profound pH effects on the solution behavior of phytochrome. These effects are evidenced by apparent MW shifts of phytochrome on SEC-HPLC discussed under Results. These results, along with HPLC data, reflect surface charge/conformational differences between the two forms. The different conformations and/or surface charge distributions as well as net charge differences may play a role in determining oligomerization and/or apparent MW or the Pr and Pfr forms of phytochrome. This is in agreement with the report that the Pfr form has a higher apparent MW than the Pr form (Lagarias & Mercurio, 1985).

Phytochrome exists as dimeric and oligomeric forms in solution (Figures 1 and 4). The nonchromophore C-terminal domain is involved in the phytochrome dimer structure (Jones & Quail, 1986; Jones & Erickson, 1989). Hydrophobic agents, ANS and bis-ANS, induce monomerization of the phytochrome oligomers (Figure 8). These results reflect an important role of hydrophobic force in the quaternary/dimer structure of phytochrome in solution. However, neutral and zwitterionic detergents, such as Mega-8, Tween 80, and

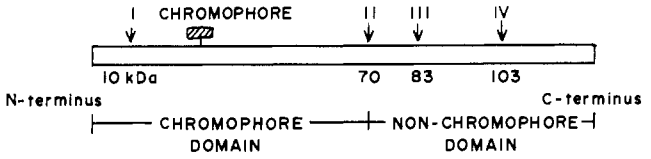

FIGURE 11: Proteolytic peptide map for 124-kDa *Avena* phytochrome proposed by Lagarias and Mercurio (1985); Roman numeral sites generate the corresponding molecular mass species (in kDa) when cleaved proteolytically.

CHAPS, at critical micelle concentrations (Fendler & Fendler, 1975) were not effective in dissociating the dimeric and oligomeric species (data not shown). On the other hand, a cationic detergent, CTAB, at its critical micelle concentration (4 mM) dissociated the phytochrome dimers and oligomers (Choi, 1987). These observations suggest that specific electrostatic interactions between the subunits are also involved in the dimer structure and the oligomerization of phytochrome. It should be noted that bis-ANS caused dissociation of the dimers at 0.4 mM, which is below its critical micelle concentration. Furthermore, bis-ANS preferentially dissociated the Pfr dimers over the Pr dimers, indicating that hydrophobic force is more important in the Pfr association than in the Pr association.

Several proteolytic cleavage sites on the phytochrome polypeptide chain have been proposed (Lagarias & Mercurio, 1985). Site I is of special interest because it exhibits "conformation-dependent" sensitivity toward proteolytic digestion (Vierstra & Quail, 1982, 1983; Lagarias & Mercurio, 1985). The N-terminal chain preferentially interacts with the chromophore in the Pfr form (Hahn et al., 1984; Quail et al., 1986; Chai et al., 1987b; Jones & Quail, 1989). Thus, the proteolytic susceptibility of the NH_2-terminal site (site I) is strongly dependent on the spectral forms of phytochrome (Figure 11). The major effect of bis-ANS on the proteolytic maps of phytochrome was to abolish the difference in proteolytic patterns between the Pr and Pfr forms. The most prominent alteration in cleavage site by bis-ANS occurred in the 6-kDa region from the NH_2 terminus of the phytochrome polypeptide chain (site I). Site I on the NH_2 terminus was no longer attacked by trypsin and chymotrypsin when phytochrome was preincubated with bis-ANS. Instead, a 29-kDa fragment was cleaved from the protein in the presence of bis-ANS for both Pr and Pfr. The results suggest that the bound bis-ANS directly or indirectly interacts with the NH_2-terminus region and induces a conformational or topographic change in the region. Site I is, thus, protected from proteolytic attack by bis-ANS.

Site II divides the phytochrome polypeptide into two structural domains of 72 kDa (NH_2-terminal or chromophore domain) and 52 kDa (C-terminal domain). Site II is located at the hinge, close to the middle of the polypeptide chain (Jones & Quail, 1986), and appears to be easily hydrolyzed with the same susceptibility for both Pr and Pfr forms. This domain-dividing site is not differentially affected by bis-ANS in Pr and Pfr. This is explained by the monomerization of phytochrome dimers due to the disruption of both hydrophobic and electrostatic interactions between the dimerization contacts in the C-terminal domains by the negatively charged hydrophobic agent. Bis-ANS is known to bind similarly to a single tubulin site and to inhibit microtubule assembly (Horowitz et al., 1984).

The chromophore domain is known to be more stable toward proteolytic cleavage than the carboxy domain (Lagarias & Mercurio, 1985). Site I near the NH_2 terminus is labile to proteases in the chromophore domain, but the carboxy domain can be further proteolytically cleaved at sites III and IV with almost the same susceptibility as at site II. The 75- and 65-kDa species were seen in the tryptic digests obtained in the presence of bis-ANS; in the absence of bis-ANS, 85- and 69-kDa species were observed. These results indicate that bis-ANS also affects the carboxy domain (i.e., by monomerization) as well as the chromophore domain. As mentioned earlier, the differences in the proteolytic digestion patterns between the Pr and Pfr forms are negated by bis-ANS. A possible interpretation for these results is that (i) bis-ANS induces monomerization in both Pr and Pfr forms and results in similar monomer conformations, which are indistinguishable by the endoproteases used, and/or (ii) bis-ANS protects the species (Pr or Pfr) dependent cleavage site(s) from proteolysis, possibly by binding along the N-terminal chain (site I).

Conclusions

Hydrophobic forces appear to play an important role in dimer and oligomer formation in phytochrome. However, specific electrostatic interactions are also involved in the maintenance of the chromophore topography and the quaternary structure of 124-kDa phytochrome. These interactions and the chromophore topography can be specifically perturbed by bis-ANS and other negatively charged hydrophobic substances, preferentially in the Pfr form of phytochrome. The spectral bleaching (absorbance decrease at 730 nm) of the Pfr chromophore by ANS and bis-ANS is largely attributable to a disruption of the bondings between the chromophore and the binding sites in the apoprotein, resulting in a cyclohelical conformation of the chromophore (Hahn & Song, 1981; Song, 1988).

In large phytochrome (118/114 kDa), there is more than one binding site for ANS per phytochrome monomer (Hahn & Song, 1981). Although the precise binding site(s) of the hydrophobic fluorescence probes on the 124-kDa protein are unknown, it is possible that an additional binding inferred from the enhanced fluorescence of bis-ANS in the Pfr form of phytochrome is located at a site near the chromophore crevice. The chromophore carries two anionic groups and three or more hydrogen-bonding functions, and its tetrapyrrole core is hydrophobic. ANS and bis-ANS with their hydrophobic moiety, hydrogen-bonding functions, and anionic sulfonate can easily disrupt or compete for the ionic binding sites on the apoprotein. To explain the lack of spectral bleaching and the alteration of proteolytic maps in the Pr form of phytochrome by ANS or bis-ANS, we propose the following hypothesis. First, the lack of spectral bleaching in the Pr form by *submillimolar* ANS or bis-ANS is readily explained by the fact that the Pr chromophore is deeply buried inside its binding crevice (Hahn et al., 1984). Second, the fluorescence probe may bind at a site along the amino-terminal sequence which is then proteolytically protected in the Pr form.

One-third of the first 50 amino acid (N-terminal) sequence with hydroxyl-containing amino acids and Lys-45 are potential binding sites for bis-ANS (and ANS). It is possible that this site is still inaccessible to trypsin and chymotrypsin in the Pfr form. In the presence of bis-ANS, the proteolytic pattern of the Pr form becomes essentially indistinguishable from that of the Pfr form.

In summary, we deduce that bis-ANS binds (a) at the N-terminal chain [2 per dimer (Pr·Pr) or (Pfr·Pfr)], (b) at the Pfr chromophore vicinity [2 per (Pfr·Pfr)], and (c) additionally at the C-terminal domain for monomerization. It was not possible to examine quantitatively the effect of bis-ANS binding on the conformation of the phytochrome molecule by far-ultraviolet CD spectroscopy because of a strong

absorbance and the possible induced CD signal contributed by the bound probe. Since a major loss of the secondary structure of the protein molecule occurs within a 2-min incubation period (Figure 6), a study is in progress to elucidate the binding of ANS and bis-ANS to phytochrome by fluorescence-detected CD.

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