Phytochrome from Green *Avena* Shoots Characterized with a Monoclonal Antibody to Phytochrome from Etiolated *Pisum* Shoots†

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ABSTRACT: A monoclonal antibody (pea-25) directed to phytochrome from etiolated pea (*Pisum sativum* L.) shoots detects by immunoblotting at least some of the immunochemically distinct green oat phytochrome that is present in green oat (*Avena sativa* L.) shoots. This antibody detects a pool of green oat phytochrome that is not recognized by oat-9, an antibody shown previously to detect about one-third of the immunochemically distinct photoreversible activity that is present in a green oat phytochrome sample. The native size of the green oat phytochrome that is recognized by pea-25 was determined by immunoblotting of sodium dodecyl sulfate–polyacrylamide gels following electrophoresis of sodium dodecyl sulfate sample buffer extracts of rapidly frozen, lyophilized green oat shoots. This size is near 124 kilodaltons (kDa), similar to that of phytochrome from etiolated oats (etiolated oat phytochrome). When appropriate precautions are taken, pea-25 detects only one size of green oat phytochrome. This phytochrome, however, is severalfold more labile to modification than is etiolated oat phytochrome. This modification, which is consistent with a size decrease of 8 kDa, occurs equally rapidly after saturating red or far-red irradiation. Spectral analysis of green oat phytochrome that is documented to have been largely unmodified confirms an earlier report that the maximum absorbance change in the red region occurs at about 10 nm shorter wavelength than it does for etiolated oat phytochrome. High-performance liquid chromatography through a TSK 3000 size-exclusion column reveals that unmodified green oat phytochrome has the same quaternary structure as does etiolated oat phytochrome, indicating that it too exists in solution as a dimer. Comparative mapping of peptides carrying the epitope recognized by pea-25, together with other data reported here, provides additional evidence consistent with the hypothesis that green and etiolated oat phytochromes are different gene products.

Phytochrome is a photoreceptor for photomorphogenic responses in plants (Shropshire & Mohr, 1983). This chromoprotein exists in two photointerconvertible forms: a biologically inactive, red-absorbing form (Pr)[1] and a biologically active, far-red-absorbing form (Pfr). While phytochrome is relatively abundant in etiolated plants, it is present in green plants in about 50-fold lower quantity (Jabben & Deitzer, 1978; Hunt & Pratt, 1979). In addition, the presence of chlorophyll makes its spectral assay impractical in green tissues (Pratt, 1983). Consequently, the overwhelming majority of work with phytochrome in vitro has been done with the chromoprotein as isolated from etiolated plants (Pratt, 1982; Lagarias, 1985). A tacit assumption had been that phytochrome in both green and etiolated plants would be the same molecule. Initial characterization of phytochrome from green oats, however, indicated that this assumption is invalid (Tokuhisa & Quail, 1983).

Tokuhisa and Quail (1983) reported that phytochrome from green oats was both spectrally and immunochemically different than that from etiolated oats. Independently, both Shimazaki et al. (1983) and Thomas et al. (1984) found that antibodies directed to phytochrome from etiolated oats failed to quantitate accurately spectrophotometrically detectable phytochrome in extracts of green oat shoots. Both suggested that phytochrome from green oats might be immunochemically distinct from phytochrome from etiolated oats.

These initial observations have recently been extended by Tokuhisa et al. (1985) and Shimazaki and Pratt (1985). Shimazaki and Pratt found that most monoclonal antibodies directed to phytochrome from etiolated oats failed to bind to any photoreversibly detectable phytochrome that was obtained from green oats. They concluded that all of the phytochrome from such plants was immunochemically distinct from the phytochrome isolated from etiolated oats. Two monoclonal antibodies (oat-9 and oat-16), however, were found to immunoprecipitate about 30% of the phytochrome from green oats, verifying that some of this immunochemically distinct chromoprotein shares at least one epitope with phytochrome from etiolated oats. Tokuhisa et al. (1985) probed partially purified extracts from green oats by immunoblotting of $NaDodSO_4$–polyacrylamide gels. Using polyclonal antibodies to phytochrome from etiolated oats, they detected two polypeptides, one yielding a minor band at 124 kDa, which is the size of phytochrome from etiolated oats, and the other a major band at 118 kDa. They attributed the 118-kDa band to the immunochemically distinct phytochrome obtained from green oats.

A major emphasis of the work reported by Tokuhisa et al. (1985) and Shimazaki and Pratt (1985) was to document that

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[1] Abbreviations: Pr and Pfr, red-absorbing and far-red-absorbing forms of phytochrome, respectively; kDa, kilodalton(s); $NaDodSO_4$, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography.

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the differences observed between phytochrome from etiolated and green oats were not artifactual. Limitations inherent to the tools that were available to them prevented unambiguous characterization of the immunochemically distinct phytochrome that is obtained from green oats. In particular, the experiments performed by Shimazaki and Pratt (1985) required the use of phytochrome that could have been modified during the relatively extensive purification protocol that they employed. Moreover, the antibodies with which they worked failed to detect phytochrome from green oats on immunoblots of $NaDodSO_4$–polyacrylamide gels. While Tokuhisa et al. (1985) were able to detect phytochrome from green oats on similar immunoblots, they could do so only after its enrichment following extraction. They could not document whether they were working with the chromoprotein in its native size, although they made every effort to maximize the probability that they were doing so. More significantly, much of the characterization that they reported involved electroblots of $NaDodSO_4$–polyacrylamide gels immunostained with polyclonal rabbit antibodies to phytochrome from etiolated oats. The possibility that those antibodies might be recognizing a polypeptide from green oats different from, or in addition to, phytochrome, albeit of about the same size, was not addressed.

Here we characterize phytochrome from green oat shoots with a newly produced monoclonal antibody (pea-25) that recognizes the most highly conserved epitope on phytochrome that has been identified to date (Cordonnier et al., 1986). The unique properties of pea-25, together with an enhanced signal to background ratio in the immunoblotting assay that we are using, permit us to obtain new information about this immunochemically distinct chromoprotein. We present data concerning its native monomer size, its quaternary structure, and its susceptibility to extremely rapid modification in crude extracts. The latter observation emphasizes the need to document carefully the size of phytochrome from green oats with which one is working, something that previously had not been possible. Therefore, we also reexamine some of the earlier observations made by Tokuhisa et al. (1985) and Shimazaki and Pratt (1985) with appropriately characterized phytochrome samples.

Materials and Methods

Plant Material. Etiolated oats (*Avena sativa* L., cv. Garry) were grown for 5 days in total darkness at 25 °C and harvested as before (Pratt, 1973). Green oats were grown in a greenhouse under an approximately 12:12-h light:dark cycle for 10 or 11 days. Shoots were harvested in daylight near the end of the light period. Immediately following harvest, plants were frozen in liquid nitrogen. Subsequently, tissue was ground with a mortar and pestle under liquid nitrogen and lyophilized, with care taken to ensure that it never thawed. In most cases, the lyophilized tissue was further milled to a fine powder.

Phytochrome Extraction. For convenience, a phytochrome preparation will be referred to as etiolated oat or green oat phytochrome, depending upon its source. Four types of preparations were examined: (1) immunopurified etiolated oat phytochrome; (2) hydroxyapatite-purified etiolated and green oat phytochromes; (3) clarified extracts; and (4) enriched extracts. They were prepared and handled under green light (Pratt, 1984) and kept near 0 °C, unless otherwise specified. Final preparations were stored at −80 °C.

Immunopurified, etiolated oat phytochrome (A_{667}/A_{280} = 0.77, greater than 95% pure) was prepared as before (Cordonnier & Pratt, 1982). Hydroxyapatite-purified etiolated oat phytochrome (A_{667}/A_{280} = 0.054, *ca.* 5% pure) was prepared by the procedure of Vierstra & Quail (1983), with modifications described in Pratt (1984). Hydroxyapatite-purified green oat phytochrome was purified similarly, with modifications described by Shimazaki and Pratt (1985).

Clarified and enriched extracts were prepared as described by Tokuhisa et al. (1985). Homogenates were obtained by blending lyophilized etiolated or green oat shoots for 15 s in extraction buffer [25% ethylene glycol (v/v), 50 mM tris(hydroxymethyl)aminomethane (Tris), 75 mM $(NH_4)_2SO_4$, 5 mM Na_4EDTA, 25 mM $NaHSO_3$, and 2 mM phenylmethanesulfonyl fluoride (PMSF), pH adjusted to 8.5 at 4 °C with HCl]. Clarified extracts were obtained by centrifuging homogenates for 5 min at 44000*g*. When the extracts were tested for phytochrome stability (see Figure 4 below), PMSF was omitted from the extraction buffer. Enriched extracts were obtained from homogenates that were clarified by centrifugation after addition of poly(ethylenimine). The resulting supernatants received an additional 4 mM PMSF and were irradiated for 5 min with Sylvania Gro-lux lamps, which produce essentially the same photoequilibrium as monochromatic red light. The irradiated supernatants were fractionated by precipitation with 40% saturated $(NH_4)_2SO_4$. After centrifugation, phytochrome-containing pellets were dissolved in extraction buffer without $(NH_4)_2SO_4$. Except when the enriched samples were to be used for proteolysis experiments, the resuspension buffer was made 4 mM in PMSF. For spectral assay, the enriched extracts from green oats were prepared with 2 mM benzamidine and 10 mM ϵ-aminocaproic acid in both the extraction and resuspension buffers, in addition to PMSF.

Spectrophotometric Assays. Phytochrome was quantified in enriched extracts, clarified extracts of etiolated oats, and hydroxyapatite-purified green oat phytochrome preparations by the photoreversibility assay (Pratt, 1983) at 667 and 724 nm with a fully automated, custom-built dual-wavelength spectrophotometer (Pratt et al., 1985). For purer preparations, phytochrome quantities were estimated from absorbance spectra of the pigment in its Pr form. When practical, phytochrome purity was estimated by the ratio of A_{667} to A_{280} with the pigment as Pr. All spectral assay units were converted to protein quantities with the extinction coefficient of Litts et al. (1983) as described in Pratt (1983). Phytochrome quantities in $NaDodSO_4$ sample buffer extracts of lyophilized shoots and in clarified extracts of green shoots were extrapolated from amounts detected by the photoreversibility assay in enriched extracts. To permit estimation of green oat phytochrome amounts, it was assumed to have the same extinction coefficients as etiolated oat phytochrome.

Absorbance spectra were obtained with a split-beam spectrophotometer (Hitachi Model 320) interfaced with a microcomputer (IBM AT). Software both to control the spectrophotometer with the microcomputer and to manipulate data were provided by Dr. John E. Wampler, Department of Biochemistry, University of Georgia (Wampler et al., 1979; Rich & Wampler, 1982). Spectra were measured with samples in either a 1-cm or a 10-cm light-path cuvette. Both cuvettes were maintained near 2 °C by circulating ice-cold water through the cuvette holder. Dry air was used to avoid accumulation of moisture on cuvette surfaces. Standard, as opposed to light-scattering, optics were used to minimize any possible artifact arising from chlorophyll fluorescence (Pratt, 1983). Phytochrome was photoconverted back and forth between its two forms by irradiation with light filtered through either a 663-nm or a 739-nm Balzers B-40 interference filter.

Phytochrome Modification in Clarified Extracts. PMSF-free clarified extracts were incubated after saturating red or

far-red light in darkness for up to 8 h at 2 °C with continuous mixing. Clarified extracts of etiolated and green oat shoots were incubated independently, or as a mixture at a ratio of 1:10. At the indicated times, aliquots were taken and prepared for gel electrophoresis.

Phytochrome Proteolysis. Enriched extracts were denatured by addition of an equal volume of double-strength $NaDodSO_4$ sample buffer (Laemmli, 1970). After incubation at 100 °C for 5 min, samples were cooled to 22 °C, and SV8 protease from *Staphylococcus aureus* (Sigma P8400) was added to a concentration of 50 $\mu g\ mL^{-1}$. Enriched extracts of etiolated and green oats were digested both as independent components and as a mixture at a ratio of 1:50. This ratio was selected so that the mixture would contain equal amounts of spectrophotometrically detectable etiolated oat and green oat phytochromes. Digestion proceeded at 22 °C with continuous mixing in darkness. At the indicated times, proteolysis was stopped by rapidly heating aliquots to 100 °C and then freezing them immediately in powdered dry ice.

Size-Exclusion Chromatography. Samples of 400 μL were injected into a TSK 3000 column (7.5 × 600 mm) previously equilibrated with 0.2 M sodium phosphate and 1 mM ethylenediaminetetraacetic acid, pH 7.0. The column was operated at ambient temperature (20–25 °C) under white fluorescent lighting and at a flow rate of 1 mL min^{-1}. Fractions of 1 mL were collected and assayed for photoreversibility in a cuvette that provided a 2-cm light path. The column was calibrated with thyroglobulin, apoferritin, catalase, carbonic anhydrase, and adrenocorticotropic hormone.

Monoclonal Antibodies. The new monoclonal antibody (pea-25), which was obtained by immunization of mice with etiolated pea phytochrome, is characterized elsewhere (Cordonnier et al., 1986). Oat-22 and oat-9, which are directed to etiolated oat phytochrome, have also been characterized previously (Cordonnier et al., 1983, 1984, 1985; Shimazaki & Pratt, 1985; Shimazaki et al., 1986). All antibodies were immunopurified from spent hybridoma medium with a column of immobilized rabbit antibodies to mouse immunoglobulins (Cordonnier et al., 1983). All are of the immunoglobulin G1 isotype (Cordonnier et al., 1985, 1986; Shimazaki & Pratt, 1985).

ELISAs. Details of the buffers and experimental methods are presented elsewhere (Cordonnier et al., 1983; Shimazaki et al., 1983; Pratt et al., 1986). Only the outlines of the two ELISAs used here will be presented.

Wells were coated with hydroxyapatite-purified etiolated oat phytochrome at the indicated concentrations by incubation for 2 h at 4 °C. After remaining nonspecific binding sites were blocked, subsequent incubations were with 1 $\mu g\ mL^{-1}$ either monoclonal antibody or nonimmune mouse immunoglobulin G for 2 h at 37 °C, followed by a 1:500 dilution of alkaline phosphatase conjugated rabbit antibodies to mouse immunoglobulins (Sigma A-1902) for 2 h at 37 °C.

Alternatively, wells were coated with rabbit antibodies to mouse immunoglobulins at 5 $\mu g\ mL^{-1}$ by incubation overnight at 4 °C. After remaining nonspecific binding sites were blocked, subsequent incubations were with 1 $\mu g\ mL^{-1}$ either monoclonal antibody or nonimmune mouse immunoglobulin G for 2 h at 37 °C, hydroxyapatite-purified etiolated oat phytochrome at the indicated concentrations for 2 h at 2–4 °C, and a 1:500 dilution of alkaline phosphatase conjugated rabbit antibodies to etiolated oat phytochrome (Cordonnier et al., 1985) for 2 h at 37 °C.

After a 30-min incubation with *p*-nitrophenyl phosphate, the reaction product was measured with a custom-built, automated ELISA reader as the absorbance difference between 400 and 500 nm.

Immunoprecipitation. The protocol used here is modified as follows from that of Shimazaki and Pratt (1985). Hydroxyapatite-purified green or etiolated oat phytochrome (1.5 μg) was incubated for 2 h at 2–4 °C with 30 μg of oat-9 or 30 μg of nonimmune mouse immunoglobulin G, or in the absence of added immunoglobulin. The final incubation volume was 120 μL, which included 25 μL of 10 mM sodium phosphate, 140 mM NaCl, 1% bovine serum albumin, and 0.02% NaN_3, pH 7.4, as well as 25 μL of 0.2 M sodium borate, and 75 mM NaCl, pH 8.5. Antibodies were precipitated by addition of 180 μL of a 10% suspension of washed *Staphylococcus aureus* cells, incubation for 15 min at 2–4 °C, and centrifugation for 15 min at 12000*g*. Pellets to be assayed for phytochrome photoreversibility were resuspended in 0.1 M sodium phosphate, pH 7.8. Both pellets and supernatants were assayed by dual-wavelength spectrophotometry in the presence of $CaCO_3$, which not only increases the photoreversibility signal by enhancing the measuring light path but also effectively eliminates turbidity differences between supernatants and resuspended pellets (Butler & Norris, 1960). Replicate pellets and supernatants were assayed by electrophoresis and immunoblotting.

Electrophoresis and Immunoblotting. Aliquots of clarified extracts and supernatants from immunoprecipitation experiments were mixed with half their volume of triple-strength $NaDodSO_4$ sample buffer (Laemmli, 1970) at 100 °C and incubated at that temperature for 2–10 min. Pellets from immunoprecipitation experiments were resuspended in 250 μL of $NaDodSO_4$ sample buffer, the composition of which was the same as that used with lyophilized tissue samples (Vierstra et al., 1984), incubated at 100 °C for 2 min, and clarified by centrifugation. Aliquots of enriched extracts, including those prepared after spectral assay, were mixed with an equal volume of double-strength sample buffer at 100 °C and incubated at that temperature for 10 min. Lyophilized powdered tissue was extracted directly into boiling $NaDodSO_4$ sample buffer as before (Vierstra et al., 1984; Cordonnier et al., 1985). Fractions of 1 mL obtained from the TSK 3000 column were precipitated with 50% saturated $(NH_4)_2SO_4$. Pellets were collected by centrifugation and were redissolved with 0.1 mL of 0.1 M sodium phosphate, pH 7.8, after which they were mixed with an equal volume of $NaDodSO_4$ sample buffer and incubated for 5 min at 100 °C. Hydroxyapatite-purified green oat phytochrome was mixed with single-strength $NaDodSO_4$ sample buffer and incubated at 100 °C for 5 min.

$NaDodSO_4$–polyacrylamide gradient gel electrophoresis was performed according to Laemmli (1970) and as described elsewhere (Cordonnier et al., 1985).

The electrotransfer of proteins to nitrocellulose and the immunostaining of blots were performed as described elsewhere (Cordonnier et al., 1985; Pratt et al., 1986). In some cases, however, the previously used substrate solution for the alkaline phosphatase label was replaced with that described by Blake et al. (1984), with minor changes. The 5-bromo-4-chloroindolyl phosphate stock solution was prepared in dimethyl sulfoxide, and the nitro blue tetrazolium stock solution was prepared in substrate buffer, which was 0.15 M sodium bicarbonate, pH 9.6, with 4 mM $MgCl_2$.

Molecular weight standards were obtained from Sigma (mixtures SDS-6H and SDS-7). While polypeptide size assignments were generally made with reference to these standards, size assignments near 120 kDa were made with reference to the mobility of etiolated oat phytochrome ex-

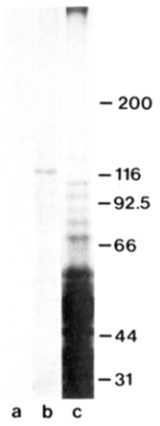

FIGURE 1: Specificity of pea-25 for a single polypeptide in a Na-DodSO$_4$ sample buffer extract of lyophilized green oat shoots. The extract was electrophoresed on a 5–10% linear gradient polyacrylamide gel in 20-μL aliquots, each estimated to contain 5 ng of phytochrome. One lane was stained in the gel with Coomassie blue (lane c). Other lanes were transferred to nitrocellulose and immunostained with 1 μg mL^{-1} nonimmune mouse immunoglobulin G (lane a) or with 1 μg mL^{-1} pea-25 (lane b). Positions of molecular weight standards, with sizes in kilodaltons, are shown on the right.

tracted from lyophilized tissue directly into NaDodSO$_4$ sample buffer preheated to 100 °C (Vierstra et al., 1984). This phytochrome was assumed to be 124 kDa (Vierstra & Quail, 1982a; Kerscher & Nowitzki, 1982; Litts et al., 1983).

RESULTS

Pea-25 Immunostains Phytochrome in Green Oat Extracts. Pea-25 immunostains a polypeptide approximately the size of etiolated oat phytochrome in NaDodSO$_4$ sample buffer extracts of lyophilized green oat shoots (Figure 1, lane b). Moreover, pea-25 immunostains this polypeptide specifically (Figure 1, compare lanes a and b) even though it represents an exceptionally minor component of the total protein applied to the polyacrylamide gel (Figure 1, lane c).

Oat-22 and pea-25 exhibit similar sensitivity when used to probe a dilution series of lyophilized etiolated oat shoots extracted with NaDodSO$_4$ sample buffer (Figure 2, lanes E). Each antibody detected the approximately 2 ng of phytochrome estimated to be present at the lowest sample load. Under the same conditions, however, only pea-25 stains a comparably sized polypeptide from similarly extracted green oat shoots (Figure 2, lanes G).

More detailed comparison of the sizes of the immunostained polypeptides obtained from etiolated and green oat shoots indicates that they are all approximately the same (Figure 3a). While the phytochrome detected in NaDodSO$_4$ sample buffer extracts of lyophilized green oats (Figure 1, lane b, and Figure 3a, lanes G) is homogeneous in size, that detected in enriched extracts (lanes G′) indicates the existence of two monomer sizes. Mixing experiments verify that the more abundant, higher molecular weight band observed in enriched extracts of green oats is identical in mobility with the phytochrome obtained by direct sample buffer extraction of lyophilized tissue (lane G′/G). In this immunoblot, which was prepared in Athens, GA, the highest molecular weight band seen for green oat phytochrome is slightly smaller than that observed for etiolated oat phytochrome (lane E/G). Routinely, however, green oat phytochrome detected in comparable immunoblots prepared in Geneva, Switzerland, always migrated at the same rate or slightly more slowly than did etiolated oat phytochrome (Figures 2, 4, and 8; other data not shown). These differing outcomes arose even though identical protocols were followed and even when identical samples (transported from Geneva to Athens in dry ice) were assayed in both locations. By reference to etiolated oat phytochrome as a size standard, size estimates for native green oat phytochrome range from 121 to 125 kDa.

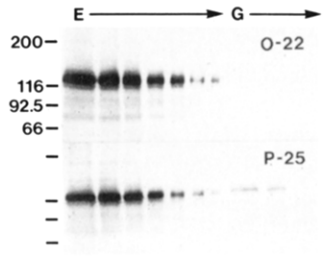

FIGURE 2: Immunoblotting of phytochrome from etiolated and green oat shoots. Lyophilized tissues were reconstituted with NaDodSO$_4$ sample buffer. Decreasing quantities of the extracts were electrophoresed on 5–10% linear gradient polyacrylamide gels. Lanes E contained 20, 10, 5, 2, 1, 0.5, and 0.2 μL of the extract of etiolated oat shoots while lanes G contained 20, 10, and 5 μL of the extract of green oat shoots. Extracts were diluted with sample buffer to facilitate addition of small quantities. Extracts of etiolated and green shoots contained an estimated 10 and 0.25 ng μL^{-1} phytochrome, respectively. The lane with 5 μL of green oat phytochrome is not visibly stained. Top and bottom panels, which had identical sample loads, were stained with oat-22 and pea-25, respectively. Positions of molecular weight standards, with sizes in kilodaltons, are indicated on the left. Only the central region of each immunoblot is shown.

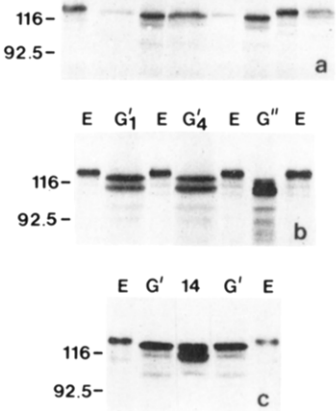

FIGURE 3: Immunoblot analyses of phytochrome. Panel a is a comparison of the mobilities of phytochrome from etiolated and green oat shoots, panel b of phytochrome used to obtain the spectral data presented in Figure 5, and panel c of phytochrome eluted from the HPLC column, the elution profile of which is presented in Figure 6. NaDodSO$_4$ sample buffer extracts of lyophilized etiolated oat shoots (E, 2-μL samples, estimated to contain 20 ng of phytochrome) and of lyophilized green oat shoots (G, 10-μL samples, estimated to contain 2.5 ng of phytochrome), enriched extracts of green oats (G′, 10-μL samples, estimated to contain 15 ng of phytochrome), hydroxyapatite-purified phytochrome from green oat shoots (G″, 1-μL sample, estimated to contain 20 ng of phytochrome), and a peak sample from an HPLC elution profile of green oat phytochrome (fraction 14, see Figure 6, 10-μL sample, estimated to contain 20 ng of phytochrome), were electrophoresed separately or in various combinations on 5–10% linear gradient polyacrylamide gels. The lane labeled G′$_1$ is of a sample prepared immediately after measuring the first far-red light-induced absorbance change while that labeled G′$_4$ is of a sample prepared after measuring four far-red light-induced absorbance changes with the same enriched extract from green oat shoots. Following electrotransfer to nitrocellulose, the blots were immunostained with pea-25. Positions of molecular weight standards, with sizes in kilodaltons, are indicated. Only the central region of each immunoblot is shown.

Specific Modification of Phytochrome in Clarified Extracts of Green Oat Shoots. During incubation of PMSF-free, clarified extracts of green oat shoots for 8 h at 2 °C, the

Table I: Spectral Properties of Phytochrome

phytochrome (source)[a]	wavelength (nm) A_{max}[b]	ΔR_{max}[c]	ΔFR_{max}[c]	isosbestic	$\Delta A_r/\Delta A_{fr}$[d]
etiolated shoots					
HA purified	666.3	665.2	732.2	687.5	1.04
green shoots					
enriched extract (1)	669.0	651.0	729.2	682.0	0.87
enriched extract (4)	669.0	654.9	726.7	679.5	1.08
HA purified	659.1	654.0	722.8	679.5	1.09
Tokuhisa et al.[e]		652	729	681	1.09

[a] Phytochrome samples were obtained from either etiolated or green oat shoots. Samples were partially purified through the hydroxyapatite (HA) step or were enriched by poly(ethylenimine) and ammonium sulfate fractionation. Data for enriched extracts from green oats were obtained either from the first far-red light-induced absorbance change (1) or from an average of four sequential far-red light-induced absorbance changes using the same sample (4). [b] A_{max} is the absorbance maximum of the sample, which in the case of extracts of green shoots is not indicative of phytochrome (see Figure 5a). [c] ΔR_{max} and ΔFR_{max} are the wavelengths of maximum absorbance change in the red and far-red spectral regions, respectively (see Figure 5b). [d] $\Delta A_r/\Delta A_{fr}$ is the ratio of the absorbance change at ΔR_{max} to that at ΔFR_{max}. [e] Data taken from Tokuhisa et al. (1985).

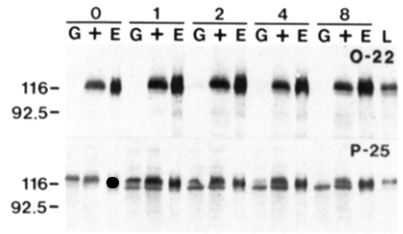

FIGURE 4: Comparative immunoblots of clarified extracts as a function of incubation time. Clarified extracts from green (G) and etiolated (E) oat shoots were incubated in darkness at 2 °C either separately or together at a ratio of 10:1 (+). After 0, 1, 2, 4, or 8 h of incubation, aliquots were mixed with half their volume of triple-strength NaDodSO$_4$ sample buffer. Two identical immunoblots were prepared from 5–10% linear gradient polyacrylamide gels. Sample loads were 20 μL (G, estimated phytochrome content of 4.5 ng), 2 μL (E, estimated phytochrome content of 22 ng), or 22 μL (+). One lane (L) contained for reference 1 μL of a NaDodSO$_4$ sample buffer extract of lyophilized etiolated oat shoots containing an estimated 10 ng of phytochrome. One blot was stained with oat-22 (upper panel) and the other with pea-25 (lower panel). Positions of molecular weight standards, with sizes in kilodaltons, are indicated on the left. Only the central region of each blot is shown.

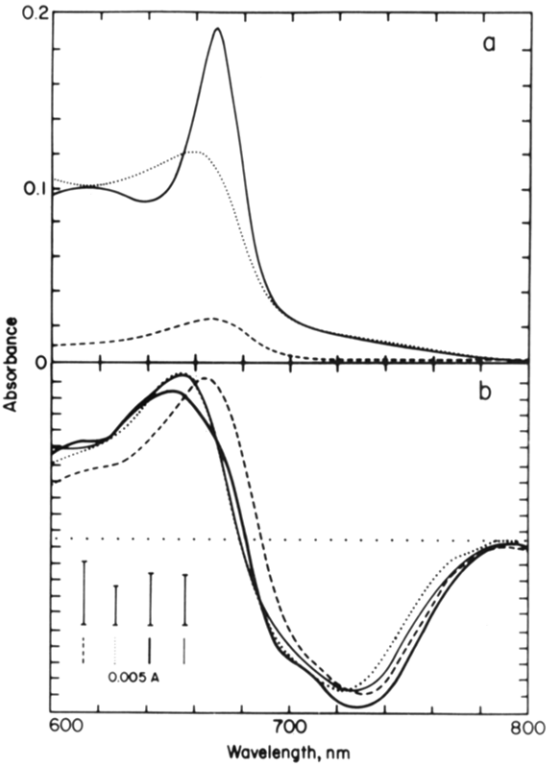

FIGURE 5: Absorbance (panel a) and absorbance difference spectra (panel b) for green and etiolated oat phytochrome samples. Etiolated oat phytochrome (---) was purified through a hydroxyapatite column. Green oat phytochrome was assayed either in an enriched extract (thin and thick solid curves) or after purification through a hydroxyapatite column (···). The spectrum for the enriched extract shown in panel a, which was measured with a 10-cm path-length cuvette, has been divided by 10 so that it may be compared directly to the other two spectra, which were measured with a 1-cm path-length cuvette. To facilitate comparison, the difference spectra in panel b have been normalized; calibrations for each curve are indicated at the lower left. Both the initial (thick solid curve) and an average of four (thin solid curve) far-red light-induced absorbance changes are shown for the enriched extract of green oat shoots. The difference spectra for the enriched extract of green oat shoots were measured in a 10-cm path-length cuvette; other difference spectra were measured in a 1-cm path-length cuvette. The signal to noise ratio was improved by digital smoothing (Wampler et al., 1979; Rich & Wampler, 1982) of all difference spectra and of the spectra for hydroxyapatite-purified green oat phytochrome and etiolated oat phytochrome.

mobility of the polypeptide stained by pea-25 increased by an amount consistent with a size decrease of 8 kDa (Figure 4, lower panel, lanes G). This polypeptide is not stained by oat-22 (Figure 4, upper panel, lanes G). During the same time, no change in mobility of etiolated oat phytochrome is observed, whether incubated alone (Figure 4, upper and lower panels, lanes E) or in the presence of the green oat extract (Figure 4, upper panel, lanes +). Probing of a mixed sample by pea-25 (Figure 4, lower panel, lanes +) gave the results expected if only the green oat phytochrome was being modified. These data were obtained with samples irradiated at zero time with far-red light. The same results were obtained with extracts irradiated at zero time with red light (data not shown). The presence of 4 mM PMSF, 2 mM benzamidine, and 10 mM ϵ-aminocaproic acid, all of which were freshly prepared immediately prior to use, also had no perceptible effect on the outcome (data not shown).

Spectral Characterization. The absorbance spectrum of an enriched extract of green oats (Figure 5a) is similar to that reported by Tokuhisa et al. (1985). Spectra for samples containing hydroxyapatite-purified green and etiolated oat phytochromes are superimposed for reference. As also reported by Tokuhisa et al. (1985), green oat phytochrome in an enriched extract is similar to etiolated oat phytochrome in the far-red spectral region but differs markedly in the red region (Figure 5b, Table I). This similarity in the far-red region is closest, however, only when the first far-red-induced absorbance change is monitored. When four sequential changes for the same sample are averaged, the position of the far-red difference peak shifts significantly toward shorter wavelength (Table I). As before (Shimazaki & Pratt, 1985), hydroxyapatite-purified green oat phytochrome also has a far-red difference maximum at a much shorter wavelength (Figure 5b, Table I).

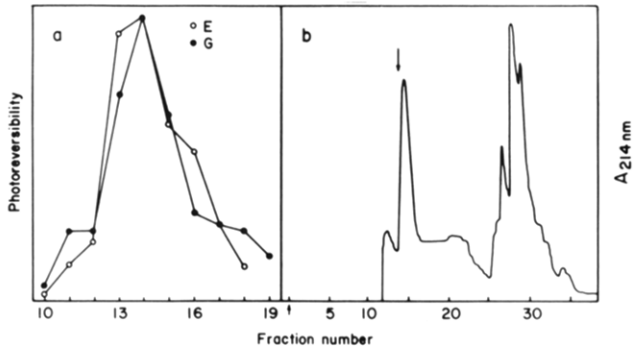

FIGURE 6: Elution profiles from a TSK 3000 column for etiolated and green oat phytochromes. (Panel a) Photoreversibility assay was used to follow phytochrome elution [(○) 124-kDa etiolated oat phytochrome, average of 3 elution profiles; (●) enriched extract of green oat shoots, average of 19 elution profiles]. Each elution profile of green oat phytochrome was obtained with an independently prepared extract. Data were averaged and are presented in arbitrary units. Peak photoreversibility values ($\Delta\Delta A$) were about 0.0005–0.001A for green oat phytochrome and 0.0015A for etiolated oat phytochrome. (Panel b) A diluted sample of an enriched extract from green oats was monitored at 214 nm to illustrate the total protein profile. Sample injection is indicated by the arrow below the x axis. The position of fraction 14 is indicated by an arrow above the profile. Fraction volume was 1.0 mL.

Only far-red light-induced absorbance changes are shown because red light not only photoconverted Pr to Pfr but also irreversibly bleached some of the chlorophyll that is still present in the enriched green oat extracts (data not shown). The red-light-induced change was thus the sum of two photochemical events. The far-red actinic light, however, was found empirically not to bleach any chlorophyll.

Immunoblot analyses of aliquots taken from these samples immediately after the corresponding spectra were recorded indicate that the position of the far-red absorbance maximum correlates at least partially with the extent to which the sample is modified (Figure 3b, compare lanes G'_1 and G''), as predicted by Tokuhisa et al. (1985).

Size-Exclusion Chromatography. Green oat phytochrome in enriched extracts elutes from a TSK 3000 column at the same position as does 124-kDa etiolated oat phytochrome. The peak fraction with respect to photoreversibly detectable phytochrome (Figure 6a) is just prior to the first major protein peak (Figure 6b). Even though the photoreversibility values being measured for green oat phytochrome are low (peak $\Delta\Delta A$ = 0.0005–0.001), they are highly reproducible and statistically significant. No photoreversibility was detected in fractions collected subsequent to those shown in Figure 6a. Calibration of the column with molecular weight standards revealed that phytochrome eluted with an apparent size of about 400 kDa.

Immunoblot analyses indicated that immunochemically detectable green oat phytochrome was associated with its photoreversibility (data not shown). Furthermore, an appreciable quantity of the green oat phytochrome eluting from the column is still of native size (Figure 3c).

Pea-25 Immunostains Green Oat Phytochrome That Is Not Detected by Oat-9. As observed previously (Shimazaki & Pratt, 1985), spectrophotometric assay revealed that oat-9 immunoprecipitates most of the photoreversibility from samples of hydroxyapatite-purified etiolated oat phytochrome. Nevertheless, it precipitates no more than about one-third of the phytochrome from hydroxyapatite-purified green oat phytochrome preparations, even when antigen binding sites are in 33-fold molar excess (Table II). Insignificant quantities of phytochrome were precipitated either when no antibody or when nonimmune mouse immunoglobulins were added.

Table II: Immunoprecipitation of Green and Etiolated Oat Phytochromes by Oat-9

	phytochrome[a]			
	green oat		etiolated oat	
immunoglobulins	supernatant	pellet	supernatant	pellet
oat-9	67	29	8	94
nonimmune mouse	97	2	90	2
none	99	1	98	2

[a] The sums of the photoreversibility values obtained with the supernatants and the pellets for the samples to which no immunoglobulins were added are set arbitrarily to 100%. Other values are presented relative to these in order to facilitate comparisons. The total photoreversibility ($\Delta\Delta A$) for each supernatant/pellet pair was about 0.02A.

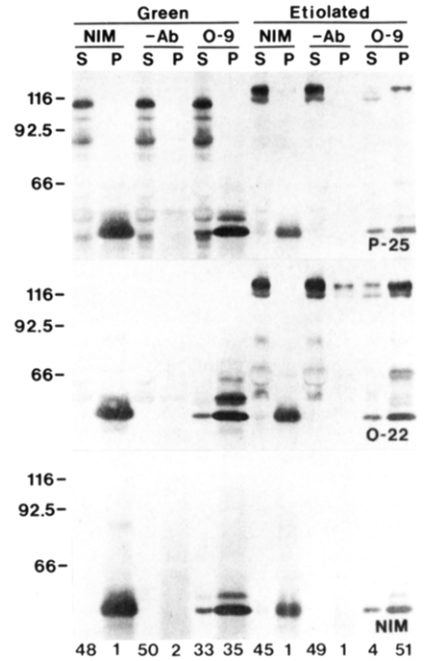

FIGURE 7: Immunoblot analyses of samples from immunoprecipitation experiments. Green and etiolated oat phytochromes were incubated with oat-9 or nonimmune mouse immunoglobulin G (NIM) or in the absence of added antibody (−Ab). After addition of *S. aureus*, samples were divided into supernatants (lanes S) and pellets (lanes P) by centrifugation, prepared for electrophoresis, and separated on 5–10% linear gradient polyacrylamide gels. Sample volumes were 15 μL for all supernatant samples and either 20 μL (green oat phytochrome) or 9 μL (etiolated oat phytochrome) for precipitate samples. Estimated quantities of phytochrome in each lane are indicated in nanograms at the bottom. Three replicate blots were prepared. One was immunostained with pea-25 (upper), one with oat-22 (middle), and one with nonimmune mouse immunoglobulin G (lower). Only the central region of each blot is shown. Positions of molecular weight standards, with sizes in kilodaltons, are indicated on the left.

Immunoblot analyses of replicate samples indicate that pea-25 detects the green oat phytochrome that oat-9 fails to precipitate but does not detect the phytochrome that was immunoprecipitated (Figure 7). Parallel immunoblot analyses of etiolated oat phytochrome indicate that the protocol does permit pea-25 to detect precipitated phytochrome, although apparently with reduced sensitivity (Figure 7). In replicate immunoblots, oat-22 detected etiolated oat, but not green oat, phytochrome as expected, while nonimmune mouse immunoglobulins failed to detect any polypeptides the size of undegraded phytochrome (Figure 7). The replica blot immunostained with nonimmune mouse immunoglobulins does, however, exhibit bands near 50 kDa, which correspond to the immunoglobulins that were used for the immunoprecipitation assay. No polypeptides immunostained specifically by oat-22

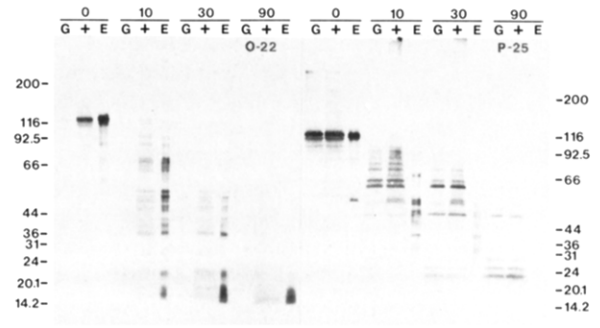

FIGURE 8: Immunoblots of etiolated and green oat phytochromes denatured in $NaDodSO_4$ sample buffer and digested with *S. aureus* V8 protease. Enriched extracts of etiolated (E) and green (G) oat shoots were digested separately or together (+) at a ratio of 1:50. At 0, 10, 30, and 90 min, aliquots were heated to 100 °C to stop proteolysis and frozen rapidly in powdered dry ice. Frozen samples were thawed rapidly and electrophoresed immediately on 5–15% linear gradient polyacrylamide gels. Sample loads were 20 μL (lanes G, estimated phytochrome content of 30 ng), 0.4 μL (lanes E, estimated phytochrome content of 30 ng), and 20.4 μL (lanes +). After electrotransfer, one blot was immunostained with oat-22 (left panel) and a replicate blot with pea-25 (right panel). Positions of molecular weight standards, with sizes in kilodaltons, are shown on either side. Since the two blots are from different gels, the mobilities of the standards are not identical.

or pea-25 were visible in areas of the immunoblots that are not shown here.

Peptide Mapping. Digestion of green and etiolated oat phytochromes with SV8 protease in the presence of $NaDodSO_4$ sample buffer produces different peptide patterns (Figure 8, right panel). The multiple peptides above 60 kDa, which derive from enriched extracts of green oat shoots and are immunostained by pea-25, are not observed with enriched extracts of etiolated oat shoots (Figure 8, right panel). Immunoblotting with oat-22 of aliquots from the same digests gives the same peptide pattern regardless of whether the etiolated oat phytochrome was digested alone or together with the enriched green oat extract (Figure 8, left panel).

Pea-25 Exhibits Reduced Affinity for Phytochrome in Solution. Although pea-25 immunostains phytochrome with high sensitivity when it is present on an immunoblot of a $NaDodSO_4$–polyacrylamide gel, it functions effectively neither for immunopurification of phytochrome nor for its immunoprecipitation from solution (data not presented). Similarly, while it recognizes well phytochrome bound to the surface of a plastic ELISA plate (Figure 9a), it exhibits approximately 10-fold lower activity for the same phytochrome when it is made available to the antibody in solution (Figure 9b).

Discussion

The recent identification of pea-25, a monoclonal antibody that is directed to etiolated pea phytochrome and that recognizes a highly conserved epitope (Cordonnier et al., 1986), together with the enhanced signal to background ratio of the immunostaining protocol used here (e.g., Figure 1), creates new possibilities for the characterization of green oat phytochrome. Of major concern in any immunochemical characterization, however, is the question of whether the antigen being probed is in fact the molecule of interest. Although the polyclonal antibodies with which Shimazaki and Pratt (1985) and Tokuhisa et al. (1985) were working immunoprecipitated some of the photoreversibility from extracts containing green oat phytochrome, that observation alone does not establish that polypeptides visualized on immunoblots by these antibodies are also phytochrome. Even when probing with a monoclonal antibody, care must be taken to identify with certainty the antigen that is detected.

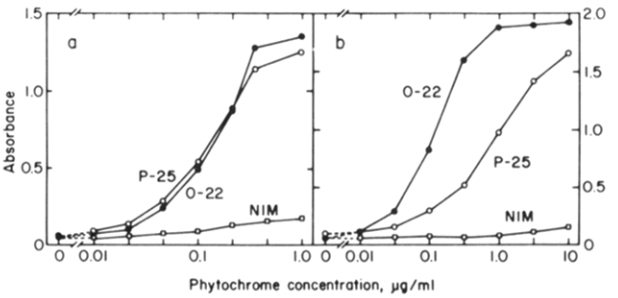

FIGURE 9: ELISAs indicating a reduced activity of pea-25 for etiolated oat phytochrome in solution. (a) The ELISA plate was coated with hydroxyapatite-purified, etiolated oat phytochrome at the concentrations indicated, after which the chromoprotein was detected by sequential incubation with pea-25 (○), oat-22 (●), or nonimmune mouse immunoglobulin G (□), and alkaline phosphatase conjugated rabbit antibodies to mouse immunoglobulins. (b) The ELISA plate was coated with rabbit antibodies to mouse immunoglobulins followed by pea-25 (○), oat-22 (●), or nonimmune mouse immunoglobulins (□). After incubation with phytochrome at the indicated concentrations, the amount of chromoprotein bound to the plate was assayed with alkaline phosphatase conjugated rabbit antibodies to etiolated oat phytochrome. Alkaline phosphatase reaction product was quantitated by the absorbance difference between 400 and 500 nm.

Several observations establish that pea-25 not only is directed to phytochrome but also, with respect to other polypeptides that might be present in crude plant extracts, is specific for this chromoprotein. (i) Pea-25 recognizes a single antigen in immunoblots of $NaDodSO_4$ sample buffer extracts of both etiolated (Cordonnier et al., 1986) and green (Figure 1) oat shoots. (ii) In both cases, this antigen is of a size appropriate to phytochrome. (iii) In the case of etiolated oat shoots, it was possible to document unequivocally that the antigen recognized by pea-25 was phytochrome (Cordonnier et al., 1986). (iv) The abundance of the antigen that is recognized by pea-25 in green oat phytochrome preparations correlates well with phytochrome photoreversibility in both clarified and enriched extracts and in elution profiles of hydroxyapatite columns (data not shown), (diethylaminoethyl)cellulose columns (Y. Shimazaki and L. H. Pratt, unpublished results), and a TSK 3000 size-exclusion column (Figures 3c and 6). (v) A change in the spectral properties of green oat phytochrome is correlated with a change in the apparent size of the polypeptide that is immunostained by pea-25 (Figures 3b and 5b and Table I).

Unfortunately, pea-25 is not useful as an adsorbent for an immunoaffinity column nor does it immunoprecipitate effectively phytochrome from dilute solutions of the chromoprotein. It appears that pea-25 recognizes a determinant that is best exposed when phytochrome is denatured, as evidenced by its ability to immunostain phytochrome with high sensitivity on an electroblot of a $NaDodSO_4$–polyacrylamide gel (e.g., Figures 1 and 2). This suggestion is consistent with the observation that while pea-25 recognizes phytochrome well when it is adsorbed to the plastic surface of a 96-well plate (Figure 9a), it recognizes the same chromoprotein poorly when it is made available to pea-25 in solution (Figure 9b).

Results from comparative immunostaining of both etiolated and green oat phytochromes by oat-22 and pea-25 indicate that the polypeptide in extracts of green oat shoots that is stained by pea-25 is not residual phytochrome like that from etiolated oats. While both antibodies exhibit approximately equal sensitivities for etiolated oat phytochrome in this assay (Figure 2, lanes E), only pea-25 immunostains green oat phytochrome (Figure 2, lanes G, and Figures 4, 7, and 8). As before (Shimazaki & Pratt, 1985), we were also unable to immunostain green oat phytochrome with a variety of other

monoclonal and polyclonal antibodies to etiolated oat phytochrome.

Oat-9 had been shown previously to recognize no more than about one-third of the phytochrome in a hydroxyapatite-purified green oat preparation (Shimazaki & Pratt, 1985). It is evident that pea-25 immunostains the phytochrome that is not precipitated by oat-9, but not that which is (Figure 7). Since pea-25 immunostains etiolated oat phytochrome that is immunoprecipitated in the same fashion (Figure 7), the inability to stain precipitated green oat phytochrome is not an inherent methodological limitation. Dual-wavelength spectrophotometric assays verify that the immunoprecipitates do contain the photoreversibility that was lost from the supernatants (Table II).

It is possible, as suggested previously (Shimazaki & Pratt, 1985), that green oat phytochrome consists of more than one immunochemically distinct pool of this chromoprotein, each of which is distinct from etiolated oat phytochrome. It is also possible, however, that the epitope for pea-25 has been artifactually modified or deleted from the photoreversible phytochrome that is detected by oat-9. One possible explanation might derive from the fact that pea-25 detects an epitope on the carboxyl-terminal half of phytochrome (Cordonnier et al., 1986) while oat-9 detects an epitope on the chromophore-bearing, amino-terminal half (Shimazaki et al., 1986). Since the chromophore-bearing half of etiolated oat phytochrome is still photoreversible in the absence of the carboxyl-terminal half (Pratt, 1982), it could be that proteolytic cleavage of green oat phytochrome into two halves not only could eliminate the epitope for pea-25 but also might simultaneously reveal the epitope that is detected by oat-9, thereby allowing this fraction to be immunoprecipitated selectively. The observations reported here (Figure 7) could thus be explained without invoking the existence of two pools of green oat phytochrome. Unfortunately, we have no antibodies that immunostain the phytochrome that is precipitated together with oat-9 (data not shown) and are therefore unable to determine its size by direct assay. In particular, oat-9 itself does not function effectively for immunostaining blots of $NaDodSO_4$–polyacrylamide gels.

Assuming that the phytochrome present in a $NaDodSO_4$ sample buffer extract of lyophilized green oats is native with respect to size, it is evident that green oat phytochrome is approximately the same size as etiolated oat phytochrome. Results obtained in Geneva (e.g., Figures 2 and 4) indicate that it is about 1 kDa larger than the 124-kDa phytochrome present in extracts of lyophilized, etiolated oats. Results obtained in Athens (e.g., Figure 3a) indicate that it is 2–3 kDa smaller. The reason for this discrepancy is unclear, since the protocols are the same and since in some cases identical samples were assayed in both laboratories. Regardless of this discrepancy, however, we have never detected two sizes of green oat phytochrome (Figures 1, 2, 3a, and 4), in contrast to the results of Tokuhisa et al. (1985), except when the samples are likely to have been degraded (Figure 4). Moreover, the size that we obtain for green oat phytochrome (121–125 kDa) is closer to that for the minor 124-kDa polypeptide detected by Tokuhisa et al. (1985) than it is to the more abundant 118-kDa polypeptide that they also reported.

The observation that green oat phytochrome is more susceptible than etiolated oat phytochrome to a modification that is not perceptibly inhibited by PMSF, benzamidine, and ϵ-aminocaproic acid and that results in an apparent size decrease of 8 kDa (Figure 4) provides the most likely explanation for the differences between our observations and those of Tokuhisa et al. (1985). Since they indicated that their procedure permitted one to obtain extracts within 4 h, and since our results indicate that most of the green oat phytochrome is degraded within this period of time (Figure 4), it appears that the minor, 124-kDa band that they reported might represent undegraded green oat phytochrome, while the major band that they observed at lower molecular weight might be the degradation product described here. This explanation is also consistent with the fact that, as already noted, we have never detected two bands on immunoblots of green oat phytochrome when the possibility of in vitro modification has been excluded. Furthermore, enriched extracts of green oat phytochrome that were prepared beginning with larger quantities of tissue as described by Tokuhisa et al. (1985) invariably indicated the presence of substantial degradation product even when (i) they were prepared in less time (2 vs. 4 h), (ii) two additional protease inhibitors were employed, and (iii) the extraction buffer was at −20 °C at the time of initial use (the ethylene glycol prevents it from freezing) (Figure 3b, lane G'_1).

Alternatively, if one were to assume that the major band that they observed is native green oat phytochrome, which is documented here to be about 124 kDa (Figures 2–4), then the minor band that they detected would have to have been about 130 kDa, that is, 6 kDa larger. Since they conclude that this larger polypeptide is like etiolated oat phytochrome, in part because it is immunoprecipitated by the polyclonal antibodies with which they worked, and since it would then be too large to be etiolated oat phytochrome, this alternative explanation would imply that the polyclonal antibodies with which they worked might be recognizing an antigen other than phytochrome. This alternative explanation is inconsistent with their size assignments, however, and therefore seems unlikely. Regardless of whether other reconciliations of our data with those of Tokuhisa et al. (1985) are possible, the inescapable conclusion is that extra care must be taken in characterizing each green oat phytochrome preparation after it has been used. It is evident, for example, that the hydroxyapatite-purified green oat phytochrome used earlier by Shimazaki and Pratt (1985, 1986) was almost certainly modified (Figure 3b, lane G″).

Etiolated oat phytochrome is not degraded even in the presence of the green oat extract (Figure 4, upper panel, lanes +). This observation confirms that the more rapid modification of green oat phytochrome is unique to this chromoprotein. It does not occur as a consequence of some difference between other components present in green and etiolated oat extracts, in which case the etiolated oat phytochrome would have degraded more rapidly in the mixed sample. Even though etiolated oat phytochrome has previously been shown to undergo a rapid proteolytic cleavage near its amino terminus, resulting in a size decrease from 124 to 118 kDa (Kerscher & Nowitzki, 1982; Vierstra & Quail, 1982a), we do not observe it here. The reason for this difference is unclear, although it is evident that even the rate of degradation reported by Vierstra and Quail (1982a) for etiolated oat phytochrome under similar conditions is still much slower than that observed here for green oat phytochrome. Furthermore, not only is green oat phytochrome more susceptible to modification but also the modification occurs equally rapidly after both actinic red and far-red irradiation (data not shown). In contrast, the initial proteolytic cleavage of etiolated oat phytochrome occurs preferentially after far-red irradiation (Kerscher & Nowitzki, 1982; Vierstra & Quail, 1982a,b).

On the basis of its monomer size and its behavior during size-exclusion chromatography, 124-kDa, etiolated oat phytochrome appears to be a nonglobular dimer in solution (La-

garias & Mercurio, 1985), a conclusion that has recently been confirmed by Jones and Quail (1986). Since the monomer size of the green oat phytochrome that is detected by pea-25 is almost the same as that of etiolated oat phytochrome, and since green and etiolated oat phytochromes exhibit indistinguishable behavior by size-exclusion chromatography (Figure 6), it appears that the full size green oat phytochrome that is recognized by pea-25 is also a nonglobular dimer in solution. Even though much of the green oat phytochrome was modified during the separation, a significant quantity of green oat phytochrome of native size eluted with the peak of photoreversibility (Figure 3c).

It is evident that predominantly native size green oat phytochrome could be obtained when enriched extracts were prepared on an analytical scale (Figure 3a), which meant beginning with 900 mg of lyophilized tissue. To obtain enough sample to permit spectral assay, however, it was necessary to begin with 9 g of lyophilized tissue, as described by Tokuhisa et al. (1985). As already noted above, we could never obtain samples that had not been degraded significantly under these circumstances, even though we took even greater precautions to preclude postextraction modification (Figure 3b, lanes G'_1 and G'_4). Even so, the first far-red-induced difference spectrum for an enriched extract of green oats provided a difference spectrum (Figure 5b; Table I) indistinguishable from that reported by Tokuhisa et al. (1985).

As predicted by Tokuhisa et al. (1985), the position of the far-red absorbance peak correlates at least in part with the extent of apparent proteolysis (Table I and Figure 3b; compare especially lanes G'_1 and G''), as has already been well established for etiolated oat phytochrome (Vierstra & Quail, 1982b). Even though it has not yet been possible to obtain a difference spectrum for a green oat phytochrome sample that is documented to be unmodified, it is likely that the difference spectrum reported by Tokuhisa et al. (1985), which indicates an absorbance difference maximum in the red at about 14 nm shorter wavelength than for etiolated oat phytochrome, is indicative of unmodified green oat phytochrome in this region. Even when green oat phytochrome is modified completely to the apparently smaller size, its difference spectrum in the red region remains essentially unchanged, even though there is a marked change in the far-red region (Figures 3b and 5b, compare hydroxyapatite-purified green oat phytochrome to that present in an enriched extract).

Although peptide maps for green and etiolated oat phytochromes have already led to the conclusion that the two proteins are different (Tokuhisa et al., 1985), the data used to reach this conclusion suffer from two unavoidable limitations. (i) The peptide maps were probed with polyclonal antibodies, which means that the peptides being compared were likely not homologous. This conclusion is based on the assumption, supported by the data presented by both Tokuhisa et al. (1985) and Shimazaki and Pratt (1985, 1986), that such a polyclonal preparation should contain a large proportion of antibodies that bind to etiolated oat, but not green oat, phytochrome. Thus, it is probable that while most proteolytically derived peptides from etiolated oat phytochrome were being immunostained, only those containing a limited number of epitopes (perhaps only one) were being immunostained when beginning with green oat phytochrome. (ii) The possibility that the polyclonal antibodies used for this application were immunostaining one or more unrelated polypeptides was not addressed. The data presented here (Figure 8), which were obtained with an epitope-specific monoclonal antibody, suffer from neither limitation. As already discussed, considerable empirical evidence is available to reinforce the assumption that pea-25 not only is directed to phytochrome but also, with respect to other polypeptides present in extracts of oat shoots, is specific to phytochrome. Moreover, only those peptides containing the epitope for pea-25 will be visualized in the resultant maps. Independent epitope mapping experiments (unpublished observations), as well as consideration of primary sequence data for etiolated oat phytochrome (Hershey et al., 1985), are consistent with the conclusion that the epitope for pea-25 is not duplicated, at least on the etiolated oat phytochrome monomer.

Immunoblot analyses of V8 protease digests of green and etiolated oat phytochromes verify that the two yield different peptide maps (Figure 8, right panel, compare lanes G and E). A replicate blot was immunostained by oat-22 (Figure 8, left panel), which does not detect green oat phytochrome (e.g., Figure 2) and which detects an epitope on the other (i.e., amino terminal) half of etiolated oat phytochrome (Cordonnier et al., 1985, 1986). This blot documents that the same peptides are generated regardless of whether etiolated oat phytochrome is digested alone (lanes E) or together with the green oat phytochrome (lanes +). The bands in the mixed digest appear fainter, primarily because they are wider due to the much higher protein load in these lanes. The bands immunostained with pea-25 in the mixed digest (right panel, lanes +) represent the sum of the lanes on either side as anticipated.

While different peptide maps generated under denaturing conditions can be interpreted to reflect differences in the primary structure of two proteins, this explanation is not the only one possible. Alternatively, the two proteins may possess identical amino acid sequences, but one may be modified relative to the other. The following argument, however, suggests that at least etiolated oat phytochrome is not a modified form of green oat phytochrome. Apart from the presence of one tetrapyrrole chromophore, and an average of one phosphate residue per monomer, etiolated oat phytochrome is not known to possess any other covalent addition to its primary amino acid sequence (Pratt, 1982; Lagarias, 1985). While an initial report did claim that etiolated oat phytochrome is glycosylated (Roux et al., 1975), attempts to confirm it have failed [see Pratt (1982) for a discussion]. Moreover, the mature size of etiolated oat phytochrome is indistinguishable from that of its in vitro translation product (Bolton & Quail, 1982). Thus, even though etiolated oat phytochrome has perhaps not been examined for all possible modifications, it seems improbable that it could have been modified from green oat phytochrome enough to give rise to the immunochemical differences and different peptide maps that are observed. While not yet conclusive, therefore, the data are nevertheless consistent with the hypothesis that green and etiolated oat phytochromes are products of different, albeit related, genes. The recent observation that the four protein moieties of another photosensory system, namely, human color vision, are all of about the same size and derive from a single gene family (Nathans et al., 1986) serves as an intriguing precedent for this suggestion.

Conclusions

Evidence presented here indicates that green oat phytochrome is significantly more labile to modification in crude extracts than is etiolated oat phytochrome. A monoclonal antibody that is both directed to and specific for phytochrome has been used to document the native size of green oat phytochrome, which is closer to that of etiolated oat phytochrome than previously reported (Tokuhisa et al., 1985). Characterization of green oat phytochrome with this antibody yields

data that disagree in part with those presented earlier (Tokuhisa et al., 1985). This disagreement most likely results from modification of green oat phytochrome prior to its assay and/or from the immunovisualization of polypeptides other than phytochrome in that earlier work, although other explanations might be possible. Nevertheless, data presented here are consistent with the earlier conclusions (Tokuhisa et al., 1985; Shimazaki & Pratt, 1985) that green and etiolated oat phytochromes are inherently different proteins, which are possibly different gene products.

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