Monoclonal Antibodies with Differing Affinities to the Red-Absorbing and Far-Red-Absorbing Forms of Phytochrome†

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ABSTRACT: Three newly isolated monoclonal antibodies (oat-23, oat-24, and oat-25) directed to *Avena* phytochrome with a monomer size of 124 kilodaltons (kDa) discriminate between the red-absorbing and far-red-absorbing forms of this chromoprotein (Pr and Pfr, respectively). Quantitative enzyme-linked immunosorbent assays indicate that the antibodies recognize an inherent difference between biologically inactive Pr and biologically active Pfr. They exhibit by this assay a 4–5-fold greater affinity for Pr than for Pfr. Competitive enzyme-linked immunosorbent assays indicate that all three antibodies possibly bind to the same epitope. One of the three (oat-25), which was selected for further characterization, has effects on phytochrome spectral properties resembling those that occur as a consequence of phytochrome degradation from 124 to 118 kDa. These effects include induction of the ability of Pfr to revert nonphotochemically to Pr and a decrease in the wavelength of maximum Pfr absorbance. Immunoblotting of sodium dodecyl sulfate–polyacrylamide gels indicates that oat-23, oat-24, and oat-25 bind to 124-kDa but not to 118-kDa phytochrome. Immunoblotting experiments indicate further that oat-25 inhibits proteolytic cleavage of a 6-kDa fragment from phytochrome. On the basis of a comparison to the results of earlier work, it appears that this fragment derives from the amino terminus. The data reported here indicate (1) that the epitope recognized by these three antibodies is preferentially exposed to the environment when phytochrome is in its Pr form, consistent with earlier reports that a proteolytic cleavage site at or near this location is more accessible on Pr than on Pfr, and (2) that a plant cell could regulate important properties of phytochrome by making differentially available a binding partner that interacts with this same site.

Phytochrome is a chromoprotein that mediates a vast range of developmental responses of plants to light (Shropshire & Mohr, 1983). The undegraded protein moiety of phytochrome from etiolated oat shoots has a monomer size of about 124 kilodaltons (kDa)[1] (Kerscher & Nowitzki, 1982; Vierstra & Quail, 1982a). The pigment exists in two photointerconvertible forms: the biologically inactive red-absorbing form, Pr, and the biologically active far-red-absorbing form, Pfr. Considerable effort has been expended searching for differences between Pr and Pfr, since one or more such differences must be responsible for their differing biological activities [see Pratt (1982) and Smith (1983) for reviews]. Nevertheless, our understanding of how Pr and Pfr differ remains poor.

On the basis of observations that Pr and Pfr exhibited differential sensitivity to a variety of agents that interact with protein, Butler et al. (1964) suggested that a change in the phytochrome chromophore was accompanied by changes in the protein moiety. Hopkins & Butler (1970) subsequently demonstrated that polyclonal rabbit antibodies to a 60-kDa, photoreversible degradation product of native phytochrome would discriminate between Pr and Pfr in a micro complement fixation assay. Attempts to confirm their observations, however, failed (Pratt, 1973), even when polyclonal rabbit antibodies were raised to what was presumably slightly degraded phytochrome of approximately 120 kDa (Cundiff & Pratt, 1975). Rice & Briggs (1973) were unable to discriminate between Pr and Pfr by double immunodiffusion assays with polyclonal rabbit antisera.

Although utilizing polyclonal antibodies to phytochrome thus did not turn out to be a promising approach to investigating differences between Pr and Pfr, the more recent availability of monoclonal antibodies creates new possibilities. Nagatani et al. (1983, 1984) reported that six monoclonal antibodies to rye and eight to pea phytochrome bound to Pr and Pfr with similar affinities. An initial screening by ELISA of 23 monoclonal antibodies to oat and pea phytochrome indicated that none detected any difference between Pr and Pfr (Cordonnier et al., 1984). Thomas et al. (1984) did report that of eight monoclonal antibodies to oat phytochrome, two showed preferential binding to Pr while one reacted better with Pfr. As will be discussed below, however, their data do not indicate whether the antibodies recognized inherent, as opposed to induced, differences between Pr and Pfr, nor have they as yet characterized them in detail.

We describe here three new monoclonal antibodies to 124-kDa oat phytochrome, each of which binds with greater affinity to Pr. These antibodies are characterized with respect to their binding site on phytochrome and to their effects on spectral properties and reversion behavior of phytochrome.

Materials and Methods

Phytochrome. Phytochrome from etiolated oat (*Avena sativa* L., cv. Garry) shoots was partially purified by the procedure of Vierstra & Quail (1983a), with several modifications as described in detail elsewhere (Pratt, 1984a). In

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[1] Abbreviations: Pr and Pfr, red-absorbing and far-red-absorbing forms of phytochrome, respectively; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; kDa, kilodalton; $NaDodSO_4$, sodium dodecyl sulfate.

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brief, crude homogenates were clarified in the presence of poly(ethylenimine), fractionated by ammonium sulfate precipitation, chromatographed through hydroxyapatite, and concentrated by ammonium sulfate precipitation. Final preparations were dissolved in 0.1 M sodium phosphate and 1 mM ethylenediaminetetraacetate, pH 7.8, clarified by centrifugation, and stored at −80 °C. In some instances, not all phytochrome could be redissolved as evidenced by the presence of a blue pellet following the final clarification step. These blue pellets were dissolved in $NaDodSO_4$ sample buffer (Laemmli, 1970) at 100 °C. They yielded only one significant band following electrophoresis on 7.5% $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970) and staining with Coomassie Brilliant Blue R. They were therefore used for immunization of mice as described below. On the basis of the results of $NaDodSO_4$–polyacrylamide gel electrophoresis, the phytochrome thus obtained (both soluble and insoluble) was judged to be predominantly (>90%) 124 kDa in size. The major phytochrome band in these preparations exhibited the same mobility as phytochrome extracted from lyophilized, etiolated oat shoots following analysis by immunoblotting (Kerscher & Nowitzki, 1982; Vierstra et al., 1984; see below for methods). Spectral estimates of purity for the soluble preparations (Litts et al., 1983; Pratt, 1983; Vierstra & Quail, 1983) indicated that typically about 5% of the protein was phytochrome (A_{666}/A_{280} of about 0.05 with phytochrome as Pr). With one lot of hydroxyapatite, however, phytochrome samples of about 15% purity (A_{666}/A_{280} of about 0.15) were obtained. These purer samples were used here only for immunization of mice (see below).

Spectrophotometric Assays. Absorbance spectra were measured with an Hitachi Model 320 spectrophotometer. The curvette was maintained at 2–4 °C and was kept free of frost with a stream of dry air. Extinction coefficients determined by Litts et al. (1983) were used to estimate absolute amounts of phytochrome.

Actinic Irradiations. Pr and Pfr were produced by saturating irradiation with light derived from tungsten-filament lamps, the outputs of which were filtered through 729- or 737-nm and 666- or 667-nm interference filters (B-40, Balzers, nominal half-band width of 10 nm), respectively.

Polyclonal Antibodies. Immunopurified rabbit antibodies to oat phytochrome (Hunt & Pratt, 1979) were conjugated with alkaline phosphatase (type VII-S, Sigma) by the protocol in Voller et al. (1980) and stored as before (Shimazaki et al., 1983). Immunopurified rabbit antibodies to mouse IgG were prepared as described elsewhere (Cordonnier et al., 1983). Other polyclonal antibodies were obtained commercially.

Monoclonal Antibodies. The three new monoclonal antibodies to 124-kDa oat phytochrome, which are designated oat-23, oat-24, and oat-25 and which are described here for the first time, were obtained by modification of the procedure described in Cordonnier et al. (1983). Phytochrome that did not redissolve following ammonium sulfate precipitation of the hydroxyapatite pool (see above) was resuspended by drawing it repeatedly through a 23-gauge needle, after which it was used for immunization of mice (Cordonnier et al., 1983). Redissolved hydroxyapatite-purified phytochrome, which in this case was about 15% pure, was used for the final tail-vein injection. Initial growth of hybridomas was in liquid medium in 11 96-well culture plates rather than in the previously used semisolid, methylcellulose-containing medium. Cell lines were recloned until it was certain that they were both stable and monoclonal. Oat-23, oat-24, and oat-25 were immunopurified from culture medium as described by Cordonnier et al. (1983). Since fusion products were plated out immediately after the fusion, and since the three new cell lines described here were derived from different culture wells, these new cell lines must be derived from different fusion products. It is therefore highly probable that these three antibodies are different from one another. Other monoclonal antibodies (oat-13 and oat-22) have been characterized previously (Cordonnier et al., 1983, 1984).

ELISAs. Methodological details of the ELISAs as used here are available elsewhere (Cordonnier et al., 1983; Pratt, 1984b). Variations from these earlier procedures will be indicated.

The direct ELISA utilized the following protocol. (1) Wells in ethanol-washed assay plates were coated overnight at 4 °C with rabbit antibodies to mouse IgG at 5 $\mu g\ mL^{-1}$. (2) After the wells were blocked with bovine serum albumin, monoclonal antibody at 5 $\mu g\ mL^{-1}$ was added and incubated for 2 h at 37 °C. (3) Beginning with this step, the remainder of the ELISA was done under green light, with incubations in darkness. Phytochrome was added at the indicated concentrations after saturating irradiation with light of either 737 (to produce Pr) or 666 nm (to produce Pfr). Incubation was for 2 h at 4 °C. (4) Alkaline phosphatase conjugated rabbit antibodies to phytochrome at a dilution of 1:500 were added and left to incubate for 2 h at 37 °C. (5) Substrate (*p*-nitrophenyl phosphate) was added. After a 30-min incubation at room temperature, the reaction was stopped by addition of 3 N NaOH. Reaction product was monitored with a custom-built, automated spectrophotometer as the difference in absorbance between 400 and 500 nm.

The competitive ELISA incorporated the following steps. (1) Assay wells were coated with monoclonal antibody (MA-1) at the indicated concentrations for 2 h at room temperature. During this time, appropriate mixtures of phytochrome and monoclonal antibody (MA-2) were prepared in separate tubes and allowed to incubate for 2 h at 4 °C before being added to the assay wells. (2) After assay wells were blocked with bovine serum albumin, the preincubated mixtures of phytochrome and MA-2 were added and left to incubate for 2 h at 4 °C. (3) The remainder of the procedure was as described above for the direct ELISA (steps 4 and 5). Inhibition by MA-2 of binding of phytochrome to MA-1 was calculated as follows. When MA-2 was omitted, inhibition was set arbitrarily at 0%. When MA-1 was omitted, which resulted in absorbance values of less than 0.03, the outcome was set arbitrarily at 100% inhibition. MA-1 concentrations were chosen from preliminary titration curves so that absorbance values obtained when MA-2 was omitted would be close to 1 (0% inhibition).

Electrophoresis and Immunoblotting. Samples for electrophoresis were prepared as follows. Crude phytochrome extracts for proteolysis were prepared from 5-day-old, ice-cold etiolated oat shoots. Tissue (5 g) was minced with a razor blade and then ground with a mortar and pestle in 3.75 mL of ice-cold 0.1 M 3-(*N*-morpholino)propanesulfonic acid, 5 mM ethylenediaminetetraacetate, and 28 mM 2-mercaptoethanol, pH 7.6 (Vierstra & Quail, 1982b). The homogenate was clarified at 2 °C for 5 min at 44000*g*. An aliquot was mixed immediately with an equal amount of double-strength $NaDodSO_4$ sample buffer (Laemmli, 1970) at 100 °C and incubated at that temperature for 5 min. The remainder of the extract was divided into three aliquots, each of which contained about 12 μg of phytochrome as judged from photoreversibility assays (Pratt, 1983). Two aliquots were kept in darkness (Pr), and the third was given 2 min of 666-nm light

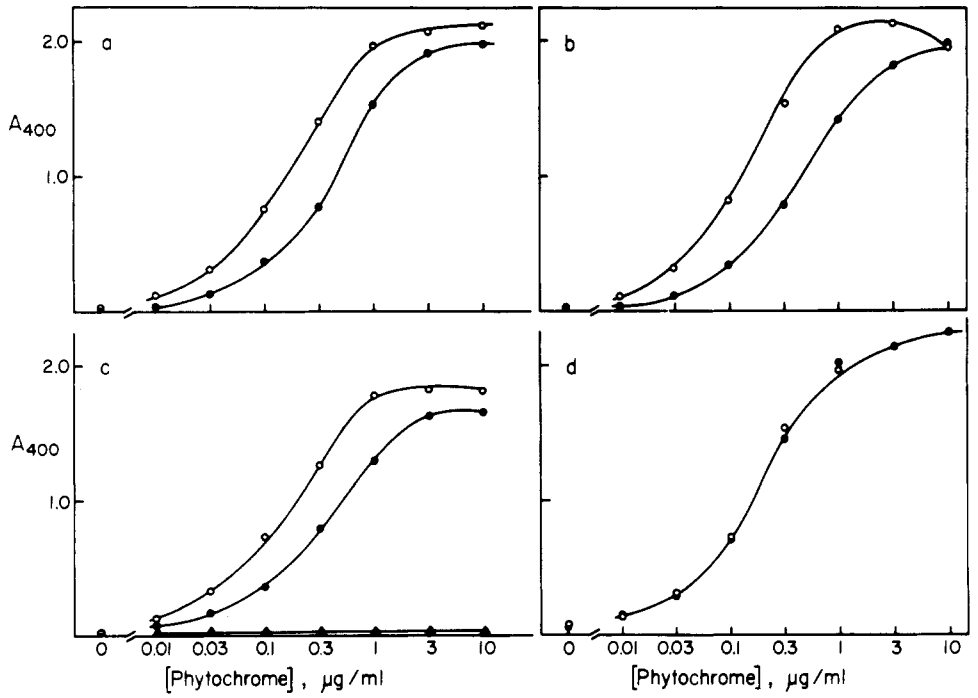

FIGURE 1: Relative affinity of monoclonal antibodies to Pr and Pfr as assayed by ELISA. Phytochrome was added to assay wells at the indicated concentrations following either saturating 667- (●) or 729-nm (○) irradiation. ELISA activity was assayed with oat-23 (a), oat-24 (b), oat-25 (c), and oat-13 (d). Background activity was evaluated by adding diluent without monoclonal antibody (c, ▲). Each entry is the average of two or three replicate values from a single experiment.

(Pfr). Oat-25 (24 μg, which provides about 3.3 antigen binding sites per phytochrome monomer) was added to one of the aliquots kept in darkness. The three aliquots were then warmed to 22 °C and allowed to incubate for 7 h, after which they were prepared for electrophoresis as already described.

Etiolated shoots (grown at 25 °C) of oat (5 days old), maize (*Zea mays* L., 5 days old), barley (*Hordeum vulgare* L., 6 days old), rye (*Secale cereale* L., 6 days old), pea (*Pisum sativum* L., 4 days old), soybean (*Glycine max* L., 4 days old), zucchini (*Cucurbita pepo* L., 5 days old), and spinach (*Spinacia oleracea* L., 5 days old) were frozen in liquid nitrogen, ground to a powder under liquid nitrogen in a mortar and pestle, and lyophilized without being permitted to thaw at any time. Lyophilized powder was added to boiling $NaDodSO_4$ sample buffer (45 mg of powder/mL of buffer) and incubated for 5 min at 100 °C. For this application only, sample buffer was 0.125 M tris(hydroxymethyl)aminomethane hydrochloride, pH 6.8 at 22 °C, 1.43 M 2-mercaptoethanol, 4% $NaDodSO_4$, 20% glycerol, and 0.0025% bromophenol blue (Vierstra et al., 1984).

Standards consisted of the high molecular weight series from Sigma (MW-SDS-200) dissolved directly into $NaDodSO_4$ sample buffer.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) with 1.5-mm-thick, 5–10% linear gradient gels. For electrophoresis of extracts of lyophilized plant tissue only (see Figure 6), electrophoresis buffer was 50 mM tris(hydroxymethyl)aminomethane, 0.38 M glycine, and 0.1% $NaDodSO_4$ (Studier, 1973). These are double the usual concentrations. With this reservoir buffer, stacking of phytochrome in the presence of large quantities of other protein was improved, and phytochrome was observed to migrate slightly more slowly relative to the β-galactosidase standard, whether in the presence or absence of other protein. After electrophoresis, protein was electrotransferred to nitrocellulose (Bio-Rad) at 150 mA for 3 h followed by overnight diffusion in transfer buffer [192 mM glycine, 25 mM tris(hydroxymethyl)aminomethane, and 20% methanol, pH 8.3] (Towbin et al., 1979). Details of the immunostaining protocol are given elsewhere (Pratt, 1984b). Nitrocellulose was first marked with waterproof ink to permit precise reconstitution of immunoblots after immunostaining, then stained with Ponceau S, photographed, and destained in blocking solution. Phytochrome was then visualized by sequential application of monoclonal antibody (1 μg mL^{-1}), immunopurified rabbit antibodies to mouse IgG (1 μg mL^{-1}), 500-fold diluted, alkaline phosphatase conjugated goat antibodies to rabbit immunoglobulins (Sigma A-8025), and 0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate (Knecht & Dimond, 1984).

RESULTS

Comparative Affinities of Monoclonal Antibodies to Pr and Pfr. Screening by ELISA of newly produced monoclonal antibodies to oat phytochrome indicated that three (oat-23, oat-24, and oat-25) react better with Pr than with Pfr (Figure 1a–c). Each of the three antibodies exhibits the same relative affinity to Pr. By comparison, oat-13, which had previously been found to react equally well with the two forms by a different ELISA (Cordonnier et al., 1984), does not discriminate between Pr and Pfr by the assay used here (Figure 1d). Given that a Pfr sample for phytochrome of the size used here is actually 86% Pfr and 14% Pr (Vierstra & Quail, 1983b), it is possible to calculate from the displacement in the ELISA curves that oat-23 and oat-25 have about 4 times lower affinity for Pfr than for Pr while oat-24 exhibits 5-fold lower affinity. Time courses for incubation of phytochrome with monoclonal antibody indicated that the observed differences were not a function of binding kinetics (data not shown).

Control experiments indicated that the observed activity differences (Figure 1a–c) did not arise from differential modification of Pr and Pfr during incubation in the ELISA wells but did in fact arise from inherent differences between Pr and Pfr (Figure 2). The difference in ELISA activity was the same regardless of whether the phytochrome used was preincubated as Pr or Pfr for 2 h in ELISA diluent (to mimic

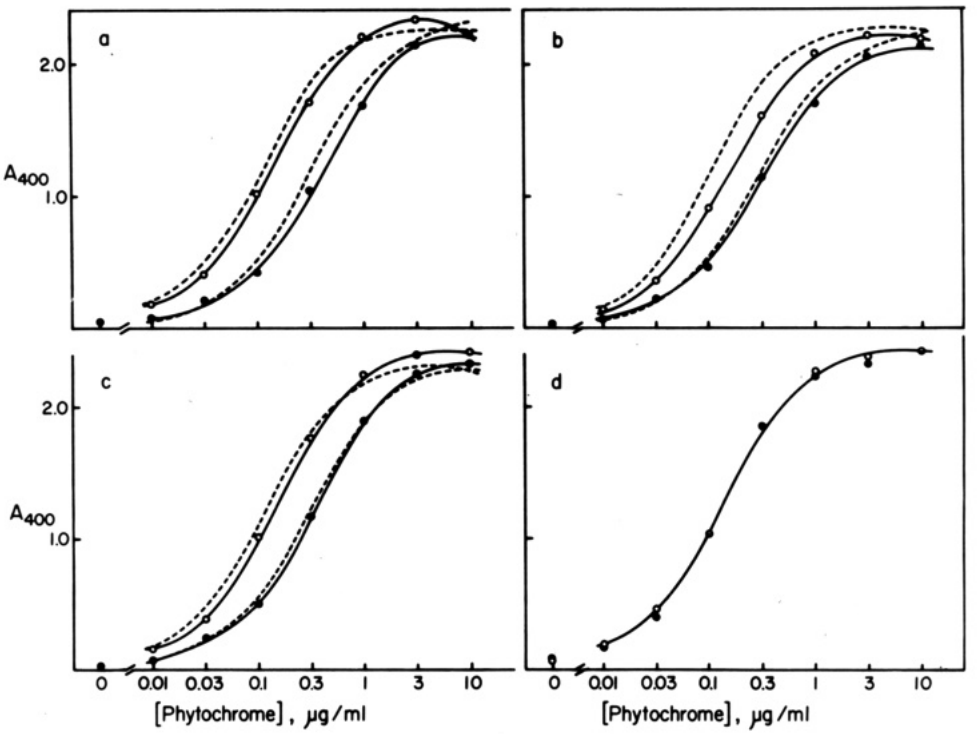

FIGURE 2: Relative affinity of monoclonal antibodies to Pr and Pfr as assayed by ELISA. Phytochrome in diluent was prepared at the indicated concentrations, given a saturating irradiation with 667- (○) or 729-nm (●) light, and incubated in darkness for 2 h at 4 °C. After this 2-h preincubation, samples were irradiated with the opposite wavelength of light and then added to the assay wells. Activity was assayed with oat-23 (a), oat-24 (b), oat-25 (c), and oat-13 (d). Dotted lines indicate assay results (minus points for clarity) measured at the same time when phytochrome was added to assay wells without prior conversion to the opposite form by the second irradiation. For oat-23, oat-24, and oat-25, left-hand curves are for Pr and right-hand curves for Pfr. Each point represents the average of two replicate values from a single experiment.

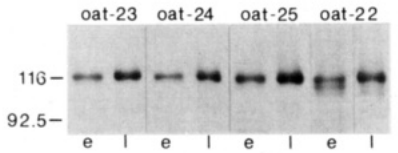

FIGURE 3: Comparative immunoblots of protein from lyophilized, etiolated oat shoots (l; 1 μL of prepared extract containing about 20 ng phytochrome) and of a crude extract of etiolated oat shoots that was first irradiated with 666-nm light and then incubated for 7 h at 22 °C prior to addition of $NaDodSO_4$ sample buffer (e; 10 μL of prepared sample containing about 100 ng of phytochrome prior to the 7-h incubation). Phytochrome on the nitrocellulose was immunostained with oat-22, oat-23, oat-24, and oat-25. Only the region around the 124-kDa phytochrome band is shown. The positions of β-galactosidase and phosphorylase *b*, with sizes in kilodaltons, are indicated to the left. The strips have been reconstituted as they were before the nitrocellulose was cut.

Table I: Competition among Monoclonal Antibodies for Binding to Phytochrome

MA-2[b]	IgG subclass[c]	MA-1[a] oat-13	oat-22	oat-23	oat-24	oat-25
oat-13	2a	56	−6[d]	−16	−5	−7
oat-22	1	29	96	19	22	21
oat-23	1	14	11	98	98	94
oat-24	1	−2	10	95	97	91
oat-25	1	−22	−8	87	91	83

[a] Assay wells were coated with the following concentrations of monoclonal antibody 1 (in μg mL^{-1}): oat-13, 6; oat-22, 3; oat-23, 1; oat-24, 2; oat-25, 3. [b] Monoclonal antibody 2 was added in excess such that 16 antigen binding sites were added per phytochrome monomer (10 μg mL^{-1} antibody to 1 μg mL^{-1} phytochrome). [c] Ig subclasses were determined by an ELISA using subclass-specific, alkaline phosphatase conjugated antibodies to mouse immunoglobulins (Southern Biotechnology Associates, Inc., 5010-AP). [d] A negative entry means that ELISA activity was greater in the presence of competitor antibody (MA-2) than in its absence.

the incubation in the ELISA wells).

Epitope Location for Oat-23, Oat-24, and Oat-25. All three antibodies that discriminate between Pr and Pfr bind to phytochrome of 124 kDa on immunoblots. While oat-22 also binds to proteolytically derived 114- and 118-kDa polypeptides, oat-23, oat-24, and oat-25 do not (Figure 3). Oat-23, oat-24, and oat-25 have never been observed to bind to phytochrome fragments in the range of 110–118 kDa, whether they were fragments produced by endogenous proteases or by trypsin.

Epitope Competition. An ELISA that was designed to determine whether one antibody competed for binding of a second to phytochrome indicated that oat-23, oat-24, and oat-25 compete with one another about as well as they compete with themselves (Table I). For comparison, two other antibodies (oat-13 and oat-22) that did not discriminate between Pr and Pfr (Figure 1; Cordonnier et al., 1984) and that immunostained a unique set of peptides derived from phytochrome by proteolysis (Figure 3; unpublished data) both competed poorly, if at all, with the other antibodies tested here (Table I). Since the three antibodies of interest (oat-23, oat-24, and oat-25) exhibited indistinguishable properties in these initial assays, only one (oat-25) was selected as a representative of this group for further characterization.

Oat-25 Changes Pfr Absorbance Spectrum and Induces Nonphotochemical Reversion of Pfr to Pr. Incubation of oat-25 with Pfr caused a time-dependent shift in absorbance maximum from 728 to about 720 nm, as well as an apparent decrease in extinction in this spectral region (Figure 4a). Conversion of this Pfr back to Pr resulted in an apparently unaltered Pr spectrum (Figure 4a). The small decrease in overall absorbance by Pr is accounted for by the dilution resulting from the addition of oat-25. When oat-25 was incubated initially with Pr, except for dilution there was no effect on its absorbance properties in the red spectral region. When Pr that had been preincubated with oat-25 was photoconverted to Pfr, the Pfr that was produced exhibited maximum ab-

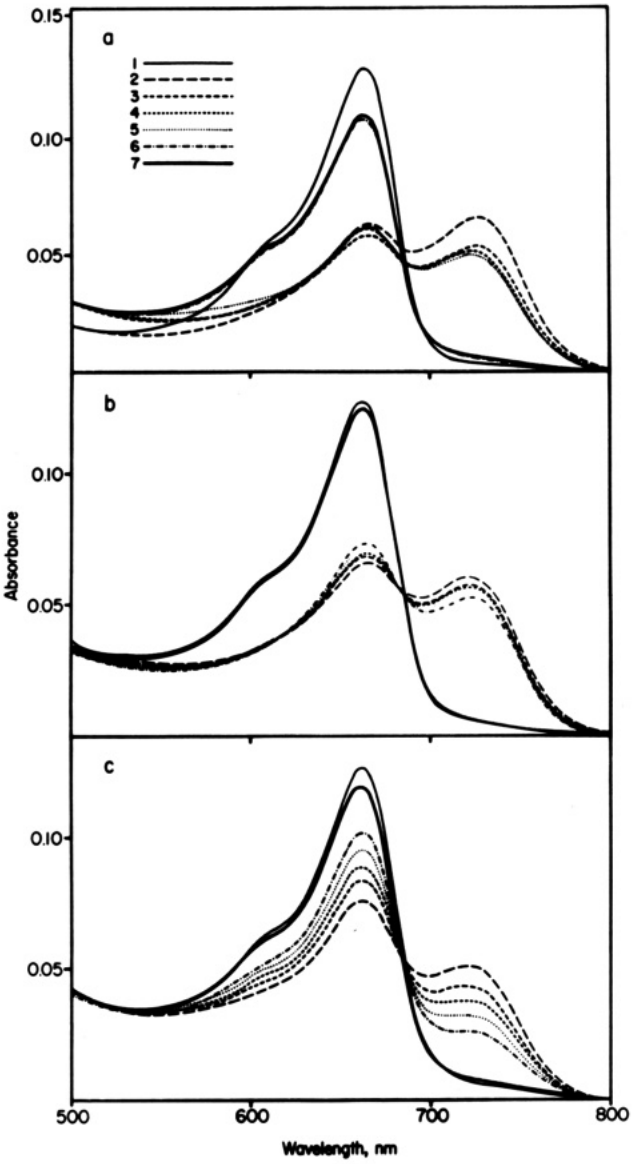

FIGURE 4: Phytochrome absorbance spectra and reversion behavior as influenced by oat-25. Within each panel, numbers refer to the sequence in which spectra were measured. Each sample consisted of 100 μg of phytochrome in 500 μL of 0.1 M sodium phosphate and 1 mM ethylenediaminetetraacetate, pH 7.8, prior to antibody addition. Samples were kept at 2–4 °C. All actinic irradiations were empirically determined to be saturating. (Panel a) (1) Spectrum after 737-nm irradiation; (2) after 666-nm irradiation; (3) 200 μg of oat-25 (3.3 antigen binding sites per phytochrome monomer) was added, mixed with phytochrome in the cuvette, and the spectrum recorded immediately thereafter; (4) after 20-min dark incubation; (5) after an additional 20-min dark incubation; (6) after 737-nm irradiation; (7) after 20-min dark incubation. (Panel b) Antibody (200 μg of oat-25) was added to the sample as Pr, after which the sample was incubated in darkness for 90 min prior to beginning spectral measurements. (1) Initial spectrum; (2) immediately after 666-nm irradiation; (3) after 10-min dark incubation; (4) after an additional 10-min dark incubation; (5) after an additional 20-min dark incubation; (6) after an additional 40-min dark incubation (80 min total); (7) after 737-nm irradiation. (Panel c) Immediately after scanning the last spectrum shown in (b), dithionite was added to 5 mM from a freshly prepared solution of 0.5 M sodium dithionite in water. Spectra were then measured following exactly the same protocol as in (b).

sorbance near 720 nm as soon as it could be assayed (Figure 4b,c). This Pfr exhibited appreciable dark reversion to Pr in the absence of added reductant (Figure 4b) and also exhibited an enhanced rate of reversion in the presence of 5 mM dithionite (Figure 4c). Control phytochrome samples, including in one experiment an aliquot of the same preparation used to obtain the spectra shown in Figure 4, exhibited no spectral changes in the presence of a quantity of nonimmune mouse IgG equal to the amount of oat-25 added (data not shown). These control samples also exhibited no measurable reversion in the absence of dithionite and only 7% reversion in its presence during a 70-min incubation (data not shown). For comparison, phytochrome in the presence of both dithionite and oat-25 exhibited 59% reversion during the same period (Figure 4c).

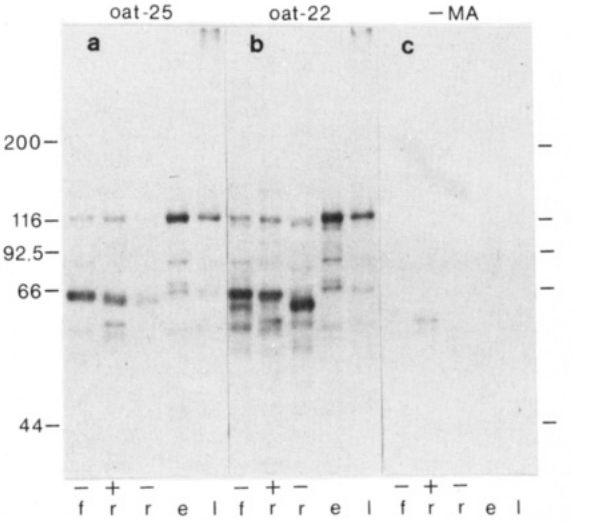

FIGURE 5: Comparative immunoblots of phytochrome as a function of proteolysis in the presence (+) or absence (−) of oat-25 and as Pr (r) or Pfr (f). A crude extract of etiolated oats was divided into aliquots, oat-25 was added as indicated, and the aliquots were incubated for 7 h at 22 °C. Aliquots were mixed with $NaDodSO_4$ sample buffer; 5-μL samples (each containing about 50 ng of phytochrome at zero time) were electrophoresed, transferred to nitrocellulose, and immunostained with oat-25 (panel a), oat-22 (panel b), or as a control 1 μg mL^{-1} nonimmune mouse IgG (panel c). For reference, 1 μL of lyophilized, etiolated oat shoots (containing about 20 ng of phytochrome) after reconstitution with $NaDodSO_4$ sample buffer (l) and 5 μL of phytochrome in sample buffer prepared prior to the 7-h incubation (e) were electrophoresed with the other samples. Positions of molecular weight standards are indicated on each side together with sizes in kilodaltons. The three pieces of nitrocellulose have been rejoined precisely as they were before being cut from the original immunoblot.

Oat-25 Alters the Pattern of Phytochrome Proteolysis. Phytochrome in a crude extract of etiolated oat shoots exhibits electrophoretic mobility indistinguishable from that observed for phytochrome obtained from lyophilized, etiolated oat shoots (Figure 5, lanes e and l). Only relatively minor contamination by apparent degradation products is observed. Following a 7-h incubation of a crude extract at 22 °C with phytochrome as Pr, little phytochrome of 124 kDa remains [Figure 5, panels a and b, lanes (−), r]. Oat-22 stains most strongly a degradation fragment at 66 kDa and more weakly a 118-kDa peptide [panel b, lane (−), r]. (Peptides of lower molecular weight will not be enumerated here since they are not relevant to the subject of this paper. In addition, the sizes given here for degradation fragments are derived from calibration curves. Consequently, they do not necessarily correspond to individual marker proteins. Note especially that the band identified as being at 72 kDa is in fact at about the position of bovine serum albumin. Regardless of the absolute accuracy of these individual size assignments, which is not an important issue here, the size differences noted below are accurate.) Following similar incubation as Pfr, oat-22 stains residual 124-kDa phytochrome as well as major peptides at 66 and 72 kDa (panel b, lane f). When Pr is incubated in the presence of oat-25, residual 124-kDa phytochrome is detected by oat-22,

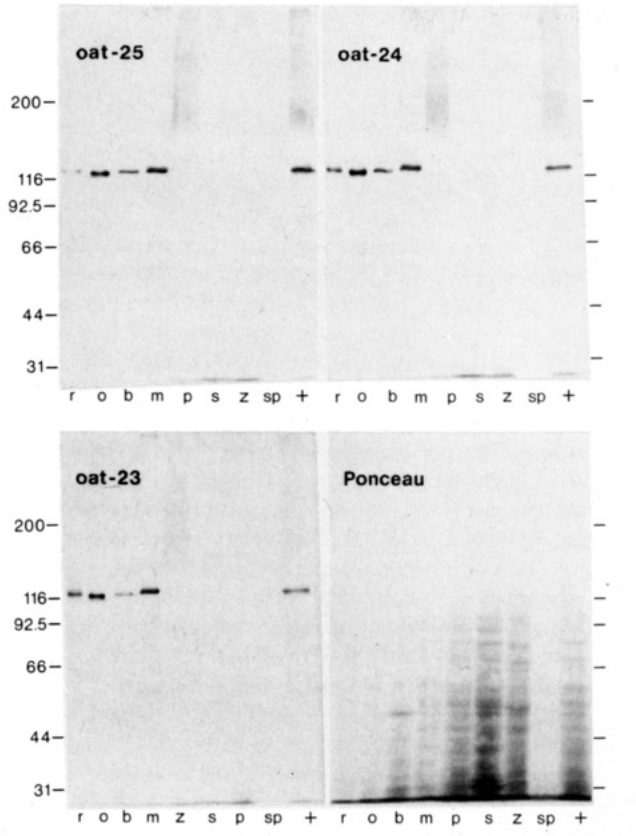

FIGURE 6: Immunostaining of phytochrome from different sources by oat-23, oat-24, and oat-25. Lyophilized, etiolated tissue was reconstituted in $NaDodSO_4$ sample buffer, and aliquots were electrophoresed as follows: rye, 3 μL (r); oat, 3 μL (o; about 60 ng of phytochrome); barley, 10 μL (b); maize, 10 μL (m); pea, 20 μL (p; about 200 ng of phytochrome); soybean, 20 μL (s); zucchini, 20 μL (z); spinach; 20 μL (sp); pea + oat (+; 23 μL of a mixture of the two at a ratio of 20:3). Following electrophoresis, polypeptides were electrotransferred to nitrocellulose and immunostained. Positions of molecular weight standards are indicated on each side together with sizes in kilodaltons. All of the patterns were photographed immediately after staining with Ponceau. One of them is shown to indicate the total protein profiles.

as well as a major band at 72 kDa [panel b, lane (+), r]. No 118-kDa phytochrome is detected, and the band at 66 kDa is exceedingly weak. Ignoring the minor bands not referred to specifically, oat-25 stains only the 124- and 72-kDa peptides (panel a). The control strip, which was stained with 1 μg mL^{-1} nonimmune mouse IgG, indicates that nonspecific staining of peptides is negligible. Those bands that do appear [panel c, lane (+), r] are presumably derived from the monoclonal antibody that was added to the crude extract prior to incubation.

Cross-Reactivity of Oat-23, Oat-24, and Oat-25 with Phytochrome from Different Species. Each of the three new antibodies stains phytochrome from all four monocotyledonous species tested, although relative intensities vary somewhat (rye, oat, barley, and maize; see Figure 6). For example, while oat-23 and oat-25 bind about equally well to oat phytochrome, oat-23 also binds well to rye phytochrome, while oat-25 binds only weakly. None of the three antibodies significantly stains phytochrome from the four dicotyledonous species tested (pea, soybean, zucchini, and spinach), however, even though all four are known to contain appreciable quantities of phytochrome (Cordonnier & Pratt, 1982; unpublished data). When extracts of oats and peas are combined, it is evident that the oat phytochrome still forms a sharp band in the immunoblot [Figure 6, lane (+)], indicating that the inability to immunostain dicotyledonous phytochrome is not a consequence of some artifact resulting from the application of larger sample volumes. The Ponceau stain (Figure 6) indicates that as anticipated the vast majority of protein is at much lower molecular weight than phytochrome and that the major immunostained band in each case represents an exceptionally minor component of what was applied. Controls immunostained with nonimmune mouse IgG exhibited no bands near 120 kDa while those immunostained with a mixture of polyclonal rabbit antibodies to oat and pea phytochrome, as well as with a monoclonal antibody to pea phytochrome that cross-reacts well with phytochrome from different species, exhibited bands near 120 kDa in every case (data not shown). Since the Ponceau stain indicates that little protein was present in the spinach extract, the negative outcome shown in Figure 6 (lane sp) cannot be considered significant in this case.

Discussion

A set of three monoclonal antibodies to phytochrome, which are described here for the first time, react by ELISA 4–5 times better with the biologically inactive Pr form than with the biologically active Pfr form (Figure 1). Moreover, it is evident that these three antibodies, oat-23, oat-24, and oat-25, recognize an inherent difference between Pr and Pfr, rather than a difference that might arise from differential modification of one or both forms during the assay (Figure 2).

With one exception, previous attempts to identify monoclonal antibodies that discriminate between Pr and Pfr have failed. Of 6 monoclonal antibodies to rye phytochrome, 17 to pea phytochrome, and 16 to oat phytochrome, none was found to react differentially with the two forms (Nagatani et al., 1983, 1984; Cordonnier et al., 1984). Thomas et al. (1984), however, reported that three out of eight monoclonal antibodies to oat phytochrome reacted by ELISA better with either Pr or Pfr. There is no clear explanation as to why Thomas et al. found such a high frequency of discriminating antibodies, as compared to the results from other laboratories. Thomas et al., however, did not report any control designed to determine whether the antibodies recognized inherent differences between Pr and Pfr, as opposed to differences that might have resulted from differential modification of the two forms during the assay. Such a control would have been especially important in their case since they incubated phytochrome in the ELISA plate as a crude preparation and at a relatively high (20 °C) temperature. Under these conditions, it is anticipated that Pr and Pfr might be differentially modified by other components, for example, proteases (Kerscher & Nowitzki, 1982; Vierstra & Quail, 1982b), of crude extracts. In addition, their data indicate the existence of unresolved methodological problems. Titration curves for antibodies that appear to react differentially with Pr and Pfr are not parallel as would normally be expected. Moreover, in those cases where the antibody reacts more strongly with Pr, most notably in the case of MAC 49, the antibody should eventually exhibit equivalent saturating activity with Pfr as well. This is so since a Pfr preparation is unavoidably at least 14% Pr (Vierstra & Quail, 1983b). Thus, even if the antibody did not bind Pfr at all, it should still bind the residual Pr, thereby producing a titration curve shifted to 7-fold higher phytochrome concentrations but otherwise saturating at the same absorbance level. Instead, they found that maximal activity with Pfr preparations was markedly reduced. Consequently, because of these unresolved technical problems, and because there was no control to determine whether the outcome might be a consequence of differential modification of Pr and/or Pfr, it is not yet possible to conclude with certainty that the antibodies they described

do in fact recognize inherent differences between Pr and Pfr. Regardless, it is unlikely that any of the antibodies that they obtained are comparable to those described here. Thomas et al. reported that they used the slightly degraded, 118-kDa size of phytochrome as immunogen, which does not bear the epitope recognized by oat-23, oat-24, and oat-25 (Figure 3).

Epitope competition experiments (Table I) indicate that oat-23, oat-24, and oat-25 may recognize the same epitope. Nevertheless, two alternative possibilities must be considered. (1) These antibodies may recognize different epitopes that are close enough together, either in terms of primary sequence of phytochrome or as a result of its tertiary structure, such that binding of one antibody provides steric hindrance to binding of a second. (2) Binding of one antibody may reduce accessibility of a second antibody to its epitope via an induced conformational change. Given, however, that all three antibodies immunostain 124-kDa phytochrome but not 118- or 114-kDa phytochrome (Figure 3), that they all exhibit the same species specificity (Figure 6), and that they discriminate in the same way between Pr and Pfr (Figure 1), the simplest interpretation would be that they all bind to the same epitope or to epitopes that are spatially close together.

Oat-23, oat-24, and oat-25 are equivalent to the type 1 antibodies reported by Daniels & Quail (1984), who concluded that they bind to the amino-terminus region of phytochrome. Recent peptide mapping and site-specific gel electrophoresis derived ELISA data of Lagarias & Mercurio (1985) confirm the conclusion of Daniels and Quail that their type 1 antibodies bind to the chromophore end of the phytochrome peptide. Since sequence analysis of a phytochrome cDNA clone confirms that the chromophore is on the amino-terminus end of the phytochrome peptide (Hershey et al., 1985), it appears that the three new antibodies described here bind near the amino terminus of phytochrome.

Oat-25 inhibits proteolytic cleavage of phytochrome at a site 6 kDa from its amino terminus (Figure 5). This conclusion is based on three observations [Figure 5, panels a and b, lanes (+), r and (−), r]. (1) A polypeptide of 118 kDa is not obtained when phytochrome is degraded in the presence of oat-25 as it is in its absence. (2) In the presence of oat-25, the major degradation product is 6 kDa larger than in its absence (72 vs. 66 kDa). Moreover, the 72-kDa fragment still possesses the epitope for oat-25 while the 66-kDa fragment does not. (3) As already noted, by comparison to peptide (Lagarias & Mercurio, 1985) and epitope (Daniels & Quail, 1984) mapping data from other laboratories and to sequence analysis of a phytochrome cDNA clone (Hershey et al., 1985), it is possible to conclude that the 6-kDa cleavage observed here occurs at the amino-terminal end.

Cleavage at this site near the amino terminus has previously been shown to occur more readily with Pr than with Pfr, implying that this region of primary structure is more accessible when phytochrome is in its Pr conformation (Kerscher & Nowitzky, 1982; Vierstra & Quail, 1982a,b; Lagarias & Mercurio, 1985). The ability of oat-23, oat-24, and oat-25 to react better by ELISA with Pr than with Pfr (Figure 1), as well as the ability of oat-25 to prevent cleavage at this site (Figure 5), leads to the suggestions (1) that these antibodies, or at least oat-25 as a representative of this group, bind an epitope that is located about 6 kDa from the amino terminus, (2) that this epitope is more accessible in Pr than in Pfr, and (3) that the site of proteolytic cleavage is thus likely the same as that of the epitope. The possibility that oat-25 binds elsewhere on phytochrome and has an indirect effect on proteolysis at this site cannot be excluded by the data presented here, although the inability of this antibody to bind to 118-kDa phytochrome does indicate that its epitope most likely includes at least some primary structure between the site of cleavage and the amino terminus.

Interaction between phytochrome and oat-25 leads to at least two significant changes in phytochrome properties. (1) Maximum Pfr extinction is shifted to a shorter wavelength and apparently reduced in magnitude (Figure 4a). (2) The ability of Pfr to revert nonphotochemically to Pr is induced (Figure 4b,c). Both of these changes have previously been reported to accompany degradation of apparently native phytochrome of 124 to 118 kDa (Vierstra & Quail, 1982b, 1983a; Litts et al., 1983). It is thus clear, especially since oat-25 inhibits proteolytic cleavage near the amino terminus of phytochrome as discussed above, that 124-kDa phytochrome can exhibit properties that are essentially indistinguishable from those of proteolytically degraded phytochrome. Consequently, it is not possible to conclude a priori that earlier work with oat phytochrome was done with proteolytically degraded samples solely because of altered spectral properties or its ability to undergo reversion. Since that earlier work was typically done with phytochrome not characterized well enough to discriminate between 124- and 118-kDa sizes, one must instead accept an inherent ambiguity in interpretation of earlier work.

The three new antibodies reported here bind to phytochrome from all four grass species tested, albeit with varying relative affinities, as noted above (Figure 6). None of them, however, binds significantly to phytochrome from at least three of the four dicotyledonous species examined, at least by the immunoblotting procedure used here (Figure 6). It thus appears that while the region of phytochrome to which these antibodies bind is important with respect to at least some of its properties, as already suggested by Vierstra & Quail (1982b) and Litts et al. (1983) and as further emphasized by the data reported here (Figure 4), the epitope that they recognize is apparently not well conserved.

The three new antibodies described here have, of course, the potential to be used as differential probes for the two forms of phytochrome such as in immunoquantitation applications (Shimazaki et al., 1983). Of special interest, however, is the observation that having been bound by oat-25, possibly at a site 6 kDa from its amino terminus, the active Pfr conformation of phytochrome is induced to undergo nonphotochemical reversion to the inactive Pr form. Although no candidate for a binding partner for phytochrome that could mimic the effects of oat-25 has been identified, the possibility that such a binding partner might exist, which is created by the observations reported here, has potentially far-reaching ramifications. Such a binding partner could provide the plant with a means to regulate the disappearance of the active Pfr from during the night. Variable availability of an appropriate binding partner could also explain why reversion, when it is observed in situ, occurs in a limited and variable population of the phytochrome that is present (Schäfer & Schmidt, 1974; Schmidt & Schäfer, 1974). While this suggestion appears superficially to be inconsistent with the observation that oat phytochrome does not exhibit nonphotochemical reversion of Pfr to Pr in vivo [e.g., see Pike & Briggs (1972)], attempts to detect such reversion under a wide variety of conditions have not yet been made. The possibility that, for example, a light-grown plant might be able to regulate the extent of reversion during the night remains untested.

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