Plant Physiol. (1975) 55, 212–217

Immunological and Physical Characterization of the Products of Phytochrome Proteolysis[1]

Received for publication July 16, 1974 and in revised form September 30, 1974

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ABSTRACT

The relationship between high molecular weight (large) and low molecular weight (small) forms of phytochrome has been shown earlier to be one of proteolysis. The products of such proteolysis are characterized here by chromatography through Bio-Gel P-200 using specific antiphytochrome sera as an assay system. Degradation of large oat (*Avena sativa* L. cv. Garry) phytochrome as phytochrome, red-absorbing form, phytochrome, far red-absorbing form, or under cycling conditions in crude preparations containing one or more proteases, always yields one fragment with the immunochemical, electrophoretic, spectroscopic, and size characteristics of small phytochrome. In addition, other fragments are detected which may account, in part, for the different molecular weight estimates reported by others for purified, photoreversible phytochrome. The small phytochrome produced by proteolysis with trypsin of a purified large phytochrome preparation is similar to that produced by the endogenously derived protease(s). A large (estimated molecular weight = 90,000), apparently nonphotoreversible peptide is also identified which is electrophoretically and immunochemically distinct from small phytochrome. Thus, it seems that small phytochrome may not represent more than approximately one-half of the native molecule.

Two sizes of phytochrome, each with different biochemical, immunochemical, and spectral properties, have been purified and characterized in recent years (5, 12, 14, 15). Small phytochrome was highly purified and characterized first by Mumford and Jenner (12) and has a mol wt of about 60,000. Large phytochrome has a mol wt of about 250,000 as first determined by Correll *et al.* (5). Sodium dodecyl sulfate gel electrophoresis of large phytochrome yields a subunit size of about 125,000 (6, 15).

The relationship between large and small phytochrome has recently been determined by Gardner *et al.* (9) and confirmed in our laboratory by subsequent immunochemical studies (7). Small phytochrome can arise either from incubation of crude preparations of large phytochrome which contain an endogenously derived protease (13), or from incubation in the presence of added commercial endopeptidases (7, 9). After incubation of large phytochrome with any one of a wide variety of endopeptidases with differing specificities, a common pattern of precipitin bands is observed upon immunoelectrophoretic analysis (8). The products include small phytochrome and at least two additional fragments recognized only by antiserum against large phytochrome (7, 8). Because preliminary analyses suggested that these latter fragments were not associated with a photoreversible chromophore, antilarge phytochrome serum would provide the only means at present for their assay.

The present investigation uses this unique ability of antilarge phytochrome serum to serve as an assay for nonphotoreversible peptides derived from large phytochrome. Fragments arising from proteolysis of a crude large oat phytochrome preparation in the Pr and Pfr forms, as well as under pigment cycling conditions, are compared. In addition, fragments derived from trypsin proteolysis of purified large phytochrome are compared to those derived from incubation of crude preparations without added protease.

MATERIALS AND METHODS

Phytochrome Preparation and Assay. Purified phytochrome preparations were obtained from Garry oat (*Avena sativa* L. cv. Garry) seedlings as described elsewhere (14) using three column chromatographic steps (Brushite, DEAE-cellulose, Bio-Gel P-300). "Crude" preparations were purified only through the Brushite step and then were precipitated with 20 g/100 ml of ammonium sulfate which selectively precipitates only large phytochrome (4). Purification and all subsequent experiments were conducted under dim green light (14).

One unit of phytochrome is defined as that quantity which, following saturating far red light and in a volume of 1 ml, yields $A^{1\,cm}_{667\,nm} = 1$. The specific absorbance ratio (A_{667}/A_{280} after saturating far red irradiation) is used as an estimate of purity. All single wavelength absorbance measurements were made with a Shimadzu MPS-50L recording spectrophotometer using a 1-cm cuvette cooled with circulating ice water. Phytochrome was also assayed by photoreversibility measurements with a custom-built dual wavelength spectrophotometer (11) at 666 *versus* 727 nm.

Actinic light was obtained by filtering the output of a Unitron Model LKR microscope lamp through either a Balzer B-40, 665 nm interference filter or 3.2 mm of far red-transmitting Plexiglas (FRF-700; Westlake Plastics, Lenni Mills, Pa.).

Antisera and Immunochemical Assays. Antismall and antilarge phytochrome sera specific for phytochrome are the same as those described and used earlier (7, 8, 14). Double diffusion and immunoelectrophoresis assays were performed with the LKB immunoelectrophoresis apparatus (LKB 6800A) as described previously (8, 14).

Molecular Weight Estimation with Bio-Gel P-200. The apparent mol wt of the fragments obtained from phytochrome degradation experiments were determined by their relative

[1] This research was supported by National Science Foundation Grant GB17057.

elution volumes from a calibrated Bio-Gel P-200 column (1.8 cm × 45 cm). Blue dextran 2000 (1 mg/ml; Pharmacia) and tryptophan (0.5 mg/ml) were added to phytochrome samples before placing them on the column so that accurate determinations of relative elution volumes (K_{av}) could be determined. $K_{av} = (V_t - V_e)/(V_t - V_o)$ where V_o is the elution volume of blue dextran, V_t the elution volume of tryptophan, and V_e is the elution volume of the protein of interest. Phytochrome samples were given saturating far red light before placing them on the column. The column was calibrated by determining K_{av} values for 7S γ-globulin (8 mg/ml; Calbiochem 345872), BSA (5 mg/ml; Sigma A-4503), ovalbumin (5 mg/ml; Sigma A-5503), and ribonuclease (5 mg/ml; Sigma R-5000). Mol wt were taken to be 150,000 for γ-globulin, 68,000 for BSA, 43,000 for ovalbumin, and 11,750 for ribonuclease.

Approximately 1-ml fractions from the Bio-Gel P-200 column were assayed spectrally by measuring their absorbance at 667 nm and 280 nm and by determining their $\Delta(\Delta A)$ values at 666 *versus* 727 nm. They were then assayed by both double diffusion and immunoelectrophoresis assays using both antilarge and antismall phytochrome sera.

RESULTS

Degradation by Endogenously Derived Protease(s). Freshly isolated crude oat phytochrome samples, which were shown to contain only undergraded large phytochrome by immunoelectrophoresis, were allowed to incubate at a concentration of approximately 1 unit/ml in the dark for 96 hr at 4 C. One sample was incubated as Pfr, a second as Pr, and a third as Pcyc[2].

Immunoelectrophoretic analysis of these three degraded samples gave similar precipitin patterns (Fig. 1). Each sample had one fragment recognized by both the antilarge and the antismall phytochrome sera and two additional fragments recognized only by the antilarge serum. The degradation products of these same samples were separated by chromatography through a calibrated Bio-Gel P-200 column. In order to distinguish between phytochrome and blue dextran, both of which absorb at 667 nm, the $\Delta(\Delta A)$ between 666 and 727 nm was measured. All fractions between $K_{av} = 0$ and $K_{av} = 1$ were assayed by double diffusion and, unless representative immunoelectrophoresis diagrams are presented, no precipitin reaction was detected. The large phytochrome originally present in the undegraded crude extracts elutes with the void volume of this column (14).

When phytochrome was degraded as Pr, four distinct products were detected (Fig. 2). The largest fragment eluted at the void volume (fraction 27), exhibited little or no photoreversibility, and was recognized with certainty (but weakly) only by antismall serum. A somewhat smaller fragment, that was slightly retarded by the gel (fraction 32), was recognized by both sera, and was photoreversible. A third fragment, detected immunologically in fraction 39, showed little or no photoreversibility and reacted only with antilarge serum. The majority of the photoreversibility was associated with the smallest detectable fragment (fraction 47) which was recognized by both antisera.

With phytochrome present initially as Pfr, degradation produced three distinct products (Fig. 3). The largest eluted with the void volume (fraction 23), reacted with certainty (as does the Pr sample) only with antismall serum, and showed little or no photoreversibility. A slightly smaller fragment (fraction 26) was clearly photoreversible and was recognized by both antisera. Most of the photoreversibility, however, was associated with a still smaller antigen (fraction 40), which was recognized by both antisera.

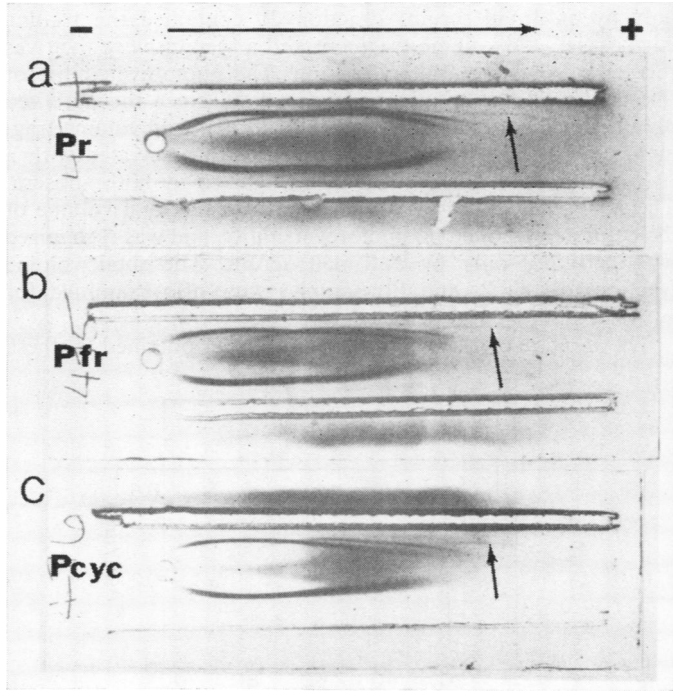

FIG. 1. Immunoelectrophoretic plates of a crude oat phytochrome preparation (1 unit/ml; specific absorbance ratio = 0.050) incubated for 96 hr at 4 C as Pr (a), Pfr (b), or Pcyc (c). The upper trough was filled with undiluted antilarge phytochrome serum; the lower trough with undiluted antismall phytochrome serum. Arrows identify minor bands.

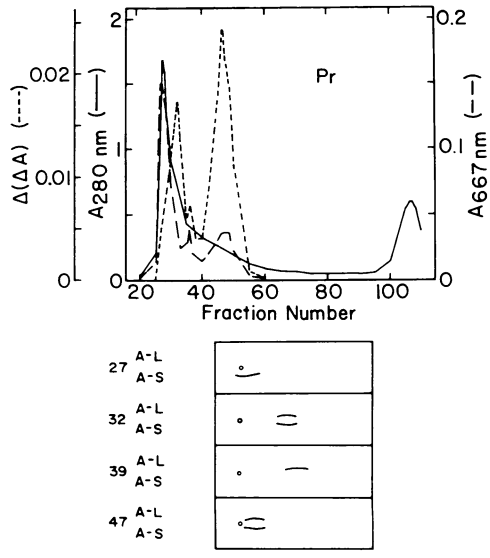

FIG. 2. Elution diagram from a Bio-Gel P-200 column of a 1-ml sample of the crude phytochrome preparation incubated as Pr. Absorbance at 280 nm (——) and 667 nm (– –) for a 1-cm pathlength and $\Delta(\Delta A)$ for an 0.8-cm pathlength (- - - -) are plotted as a function of fraction number. Approximately 1-ml fractions were collected. Diagrams of relevant immunoelectrophoretic patterns, shown with the fraction number used as antigen and obtained as in Figure 1, are also shown. The first peak is blue dextran; the last, tryptophan.

[2] Abbreviation: Pcyc: phytochrome in the presence of continuous 665 nm actinic light.

If phytochrome is degraded under continuous red light so that Pr, Pfr, and various intermediates are present, four degradation products can be detected (Fig. 4). The largest antigen was associated with the void volume, had little photoreversibility, and the Pr sample was recognized with certainty only by antismall serum. Fraction 29 contained the trailing edge of the void volume fragment and a second piece that was recognized only by antilarge serum. The photoreversibility of this fragment was extremely low, at best, since the observed photoreversibility may be associated with the void volume fragment. Almost all the photoreversibility was associated with a fragment in fraction 45 that was recognized by both antisera. The smallest fragment was associated with the total volume of the column, exhibited no photoreversibility, and was recognized with certainty only by antismall serum. The total volume fractions of the Pr and Pfr samples were also examined for activity, but none was detected.

The mol wt of the degradation products obtained in these three experiments were determined from their elution volumes relative to the standards. The calibration curve was extrapolated to 150,000 at $K_{av} = 0$ since γ-globulin eluted with the blue dextran. These mol wt values, as well as the spectral and immunological properties determined for the degradation products, are summarized in Table I.

Trypsin Degradation of Phytochrome. Purified large phytochrome samples at a concentration of approximately 1 unit/ml and with a specific absorbance ratio of about 0.23 were incubated in the dark for approximately 20 hr at 4 C with 10 μg/ml trypsin. As in the previous experiment, one sample was given 2 min of 665-nm light initially to convert it to Pfr, another was given 2 min of far red light initially to convert it to Pr, and a third sample was placed under continuous 665-nm light to establish conditions of pigment cycling. Immunoelectrophoresis of these samples (Fig. 5, d–f) demonstrates that the degradation products obtained in this manner are similar

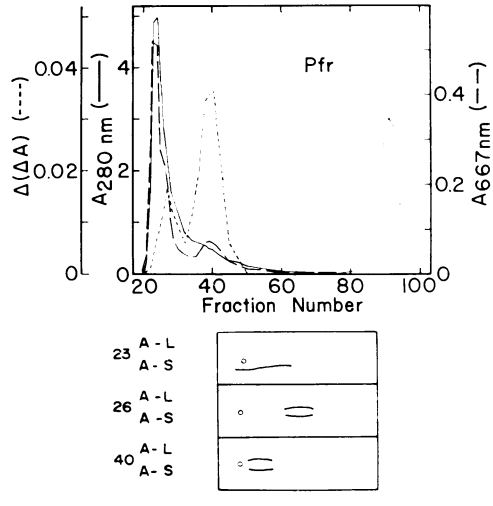

FIG. 3. As in Figure 2, except that a 2-ml sample of the crude phytochrome preparation incubated as Pfr is chromatographed.

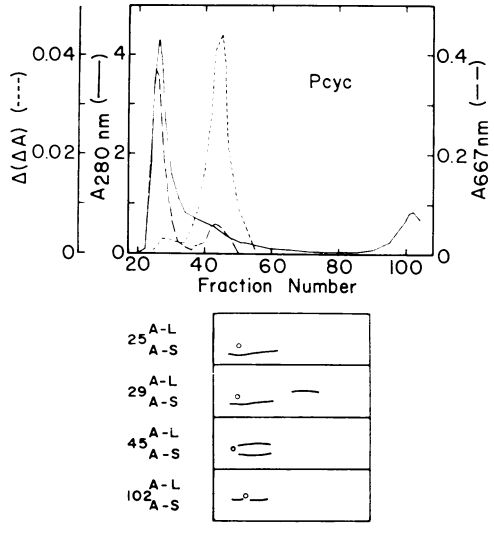

FIG. 4. As in Figure 2, except that a 2-ml sample of the crude phytochrome preparation incubation as Pcyc is chromatographed.

Table I. *Summary of Degradation Products*

Properties of fragments obtained when crude Garry oat phytochrome was degraded as Pr, Pfr, or Pcyc by endogenously derived protease(s) or when purified phytochrome was degraded as Pr by added trypsin.

Phytochrome Degraded as:	Δ(ΔA) (% of unincubated control)	K_{av}	Estimated Mol Wt	Photoreversibility	Recognized by:	
					Antilarge serum	Antismall serum
Pr	61	0.0 (27)[1]	≥150,000	little or none	?[2]	yes
		0.056 (32)	130,000	yes	yes	yes
		0.153 (39)	92,000	little or none	yes	no
		0.225 (47)	67,000	yes	yes	yes
Pfr	49	0.0 (23)	≥150,000	little or none	?	yes
		0.068 (26)	125,000	yes	yes	yes
		0.255 (40)	58,500	yes	yes	yes
Pcyc	37	0.0 (25)	≥150,000	little or none	?	yes
		0.049 (29)	133,000	little or none	yes	no
		0.246 (45)	61,000	yes	yes	yes
		1.0 (102)	<11,700	little or none	?	yes
Pr (trypsin)	...	0.0 (26)	≥150,000	little or none	?	yes
		0.012 (27)	140,000	little or none	yes	no
		0.257 (47)	57,500	yes	yes	yes

[1] Numbers in parentheses indicate fraction number.

[2] The "?" indicates that recognition by the antilarge phytochrome serum is uncertain.

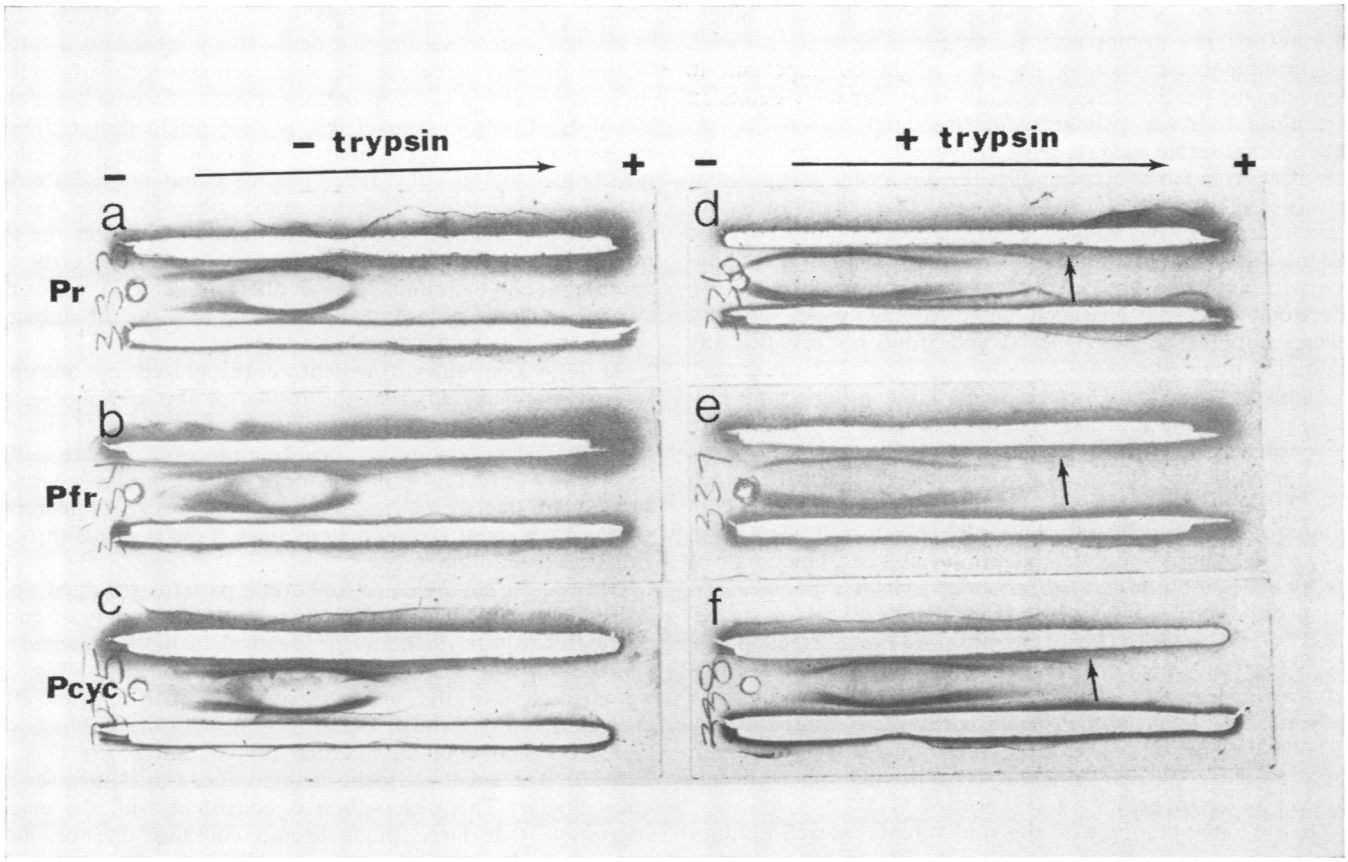

FIG. 5. Immunoelectrophoretic plates of purified large oat phytochrome (1 unit/ml; specific absorbance ratio = 0.23) degraded as Pr (d), Pfr (e), or Pcyc (f) by incubation at 4 C for 20 hr in the presence of 10 μg/ml of trypsin. Corresponding controls (a–c) were incubated in an identical fashion but without added trypsin. Undiluted antilarge phytochrome serum placed in upper trough; undiluted antismall phytochrome serum placed in lower trough.

to those obtained when crude phytochrome preparations were degraded by endogenously derived protease(s) (Fig. 1). Controls incubated in the absence of added trypsin exhibited negligible degradation (Fig. 5, a–c). In all cases there were in addition to the fragment recognized by both sera, two fragments that were recognized only by antilarge serum.

The sample degraded as Pr by trypsin was fractionated by the same calibrated Bio-Gel P-200 column used above (Fig. 6). Three distinct fragments were detected. The largest was associated with the void volume and was recognized with certainty only by antismall serum. A slightly smaller fragment eluted approximately 2 ml (one fraction) beyond the void volume and reacted only with antilarge serum. There was a small peak of photoreversibility in this region, but because of the proximity of these first two fragments, it could not be assigned to either (or both) with certainty. The majority of the photoreversibility, however, was found associated with fraction 47. This fragment had an apparent mol wt of 57,500 and was recognized by both antisera.

These results are compared with those obtained from the degradation of Pr by endogenously derived protease(s) in Table I.

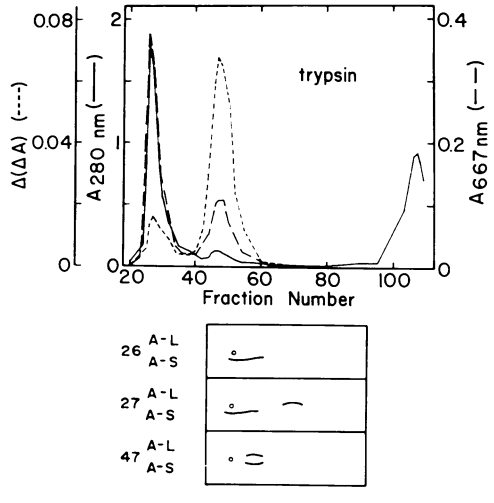

FIG. 6. As in Figure 2, except that a 2-ml sample of purified large oat phytochrome (1 unit/ml; specific absorbance ratio = 0.23) incubated as Pr with 10 μg/ml of trypsin is chromatographed.

DISCUSSION

It is now well established that native ("large") phytochrome is unusually susceptible to proteolysis. One of the products is a large chromopeptide ("small" phytochrome) with spectral properties very similar to those reported for phytochrome *in vivo* (see ref. 1 for discussion). In addition to the widely used spectral assay for phytochrome, three laboratories have recently reported the use of antismall phytochrome serum as an alternate assay system (10, 14, 16). However, neither these antisera nor the dual wavelength, photoreversibility assay (2) could be expected to detect all peptides derived from large

phytochrome by proteolysis, especially those not containing a photoreversible chromophore. Because it is possible that such nonchromophore-containing peptides might be important in phytochrome-mediated responses, an assay for their detection is important. It was primarily for this latter reason that we obtained antiserum against large Garry oat phytochrome, and it is clear from earlier reports that the antiserum is capable of detecting such peptides (7, 8). In particular, immunoelectrophoretic evidence has already led to the conclusion that at least two such peptides, in addition to that detectable by precipitation with antismall phytochrome serum, are produced (7, 8). Electrophoresis of degraded phytochrome using polarity reverse to that used here (Figs. 1 and 5) has not revealed any additional fragments.

A more detailed characterization of the products of proteolysis by gel filtration and immunochemical assay indicates the existence of at least four distinguishable peptides (Figs. 2–4, and 6; Table I). Small phytochrome is detected in each case. However, only one of the two fragments identified in immunoelectrophoresis only by antilarge serum is seen (Figs. 2, 4, and 6). In addition, degradation of phytochrome as Pr or Pfr by endogenously derived protease(s) yields a photoreversible peptide, larger than small phytochrome, which is recognized by both antisera (Fig. 2, fraction 32; Fig. 3, fraction 26). The apparent size of this fragment (about 125,000–130,000 daltons) suggests that it may be the peptide selectively purified by Roux, who reported that his phytochrome is approximately 120,000 daltons (17). The precise relationship of this larger, photoreversible fragment to either large or small phytochrome is as yet undetermined.

The fragment eluting with the void volume in each instance may represent a small amount of residual large phytochrome, possibly physically altered by denaturation. Because the antilarge serum has a much lower titer than antismall serum (6), and because this antigen was only weakly detected even with the antismall serum, it is reasonable that such a denatured form of large phytochrome might not be detected by the antilarge serum. The absence of the typical rapid migration rate (14) may also result from the possible denaturation of the molecule. Bio-Gel P-200 was chosen for these assays because we were primarily interested in the smaller proteolytically derived fragments. Thus, we are unable to resolve this minor residual activity associated with the void volume.

One other fragment was detected in the case of phytochrome incubated under cycling conditions (Fig. 4). This fragment is quite small, appearing at $K_{av} = 1.0$, and may account for the very small mol wt estimates (as low as 10,000) reported by Walker and Bailey (18, 19).

Finally, it should be expressly noted that the estimated sizes, properties, and relative amounts of the different products may be a function of both the concentration of proteases and extent of incubation as well as the conditions under which degradation is carried out. However, the three endogenously degraded samples examined here are strictly comparable because they are identically handled (except for light exposure) aliquots of the same preparation. The differences observed (Figs. 2–4; Table I) are probably a reflection of the previously reported differential reactivities of Pr and Pfr (3).

The identification of a large fragment derived from large phytochrome but not detected by antismall serum and possessing little or no photoreversibility (Fig. 2, fraction 39; Fig. 4, fraction 29) suggests that small phytochrome in fact may represent, at best, only about one-half of the native molecule. This large, nonphotoreversible peptide, which has an estimated size of from 90,000 to 135,000 daltons depending upon how large phytochrome is degraded (Table I), is not recognized at all by antismall serum, and hence likely represents a totally distinct primary structure. Because each subunit of the native molecule is about 125,000 daltons (6, 15), it would seem that this distinct, nonphotoreversible peptide might thus represent at least one-half of the native molecule. The most likely possibilities are that this latter peptide could represent either one of two nonidentical phytochrome subunits or one-half of each of two identical subunits. The mol wt estimates for this nonphotoreversible peptide may be in considerable error because large phytochrome gives an aberrant value by gel exclusion chromatography (1, 8). Except for the use of antilarge serum, this fragment would be undetected.

At least one other fragment, unrecognized by antismall serum, is anticipated from the results of the immunoelectrophoretic analyses before chromatography (Figs. 1 and 5). However, identification of this fraction will require either higher titer antilarge serum or more refined immunochemical assays. This undetected fragment of unknown size is apparently greater than about 10,000 daltons since it did not pass through a dialysis membrane (6).

Because the immunoelectrophoretic patterns obtained upon degradation of large phytochrome with a wide variety of endopeptidases (8) are qualitatively identical to those obtained by degradation with endogenously derived proteases (Fig. 1), we characterized the products obtained with trypsin after chromatography through P-200 (Fig. 6) with the products obtained by incubation of a crude preparation of Pr (Fig. 2; Table I). The small phytochromes produced in both cases are quite similar. This observation is consistent with the earlier suggestion that large phytochrome contains one or more regions which are uniquely susceptible to the action of endopeptidases (8). The other fragments are significantly different. Again, it is possible that the products of trypsin degradation are dependent upon both protease concentration and incubation time, although it has already been found that the immunoelectrophoretic pattern obtained is relatively independent of these two parameters (6, 7). A relatively high concentration of trypsin was used here to ensure maximal hydrolysis (at 4 C in 60 mM potassium phosphate, pH 7.8) of the native molecule (7, 8).

Clearly, antilarge serum will serve as a useful assay system for the fragments of phytochrome proteolysis. It is also clear that small phytochrome is probably produced by a variety of endopeptidases and is exceptionally resistant to further hydrolysis. Thus, as already suggested by Gardner *et al.* (9), it is not surprising that the first report of purified, homogeneous phytochrome in fact described the properties of this proteolytic product. It is just as clear that at least one other large peptide, distinct from small phytochrome in all respects examined, is also produced. The possible relationship of this and other phytochrome-derived peptides to morphogenic activity are as yet unknown. The availability of antilarge phytochrome serum provides, at present, the only means for their assay.

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