Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 5272–5276, September 1982
Botany

Native phytochrome: Inhibition of proteolysis yields a homogeneous monomer of 124 kilodaltons from *Avena*

(hot $NaDodSO_4$ extraction/protease inhibitors/solid-phase immunoprecipitation/one- and two-dimensional gel electrophoresis)

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Communicated by Winslow R. Briggs, May 19, 1982

ABSTRACT **Phytochrome purified from *Avena* as the red-absorbing form, Pr, by an established immunoaffinity column procedure is heterogeneous. Two major polypeptides and one minor polypeptide with apparent molecular masses of 118, 114, and 112 kilodaltons (kDal), respectively, are observed on $NaDodSO_4$/polyacrylamide gel electrophoresis. In contrast, only a single band of 124 kDal is obtained when phytochrome is rapidly immunoprecipitated after extraction either (*i*) as the far-red absorbing form, Pfr, in detergent-free buffer or (*ii*) in either spectral form in a 100°C $NaDodSO_4$-containing buffer. On two-dimensional gel electrophoresis the three column-purified species have pIs of 5.8, 6.0, and 6.0, whereas 124-kDal phytochrome is a single spot with a pI of 5.9. Incubation as Pr in extracts causes progressive conversion of the 124-kDal polypeptide to the 118- and 114-kDal species. This process is inhibited by phenylmethylsulfonyl fluoride, suggesting that Pr is susceptible and Pfr resistant to limited proteolysis during extraction. These data, and the fact that the cell-free translation product of phytochrome mRNA is also 124 kDal [Bolton, G. W. & Quail, P. H. (1982) *Planta*, in press], indicate that the native monomer from *Avena* is a single species of 124 kDal. Thus the heterogeneous preparations of slightly lower molecular weight ("large" or "120-kilodalton" phytochrome) previously extensively characterized appear to have consisted of a mixture of partially degraded molecules that have undergone limited proteolysis during purification as Pr, as is established practice. A reexamination of the molecular properties of phytochrome appears necessary.**

Phytochrome is a regulatory photoreceptor that has a central role in controlling plant development (1, 2). The molecule consists of a linear tetrapyrrole chromophore covalently linked to a polypeptide (3). Initial attempts to purify the pigment yielded a chromoprotein that migrated upon $NaDodSO_4$/polyacrylamide gel electrophoresis with an apparent molecular mass of 60 kilodaltons (kDal) (4). It was several years before Briggs and co-workers isolated a molecule with a monomer size of approximately 120 kDal and demonstrated that the 60-kDal species was derived proteolytically from the larger polypeptide during purification (5–7). Since that time it has been widely accepted that the approximately 120-kDal monomer ("large" phytochrome) represents the undegraded native chromoprotein, and much information on its molecular properties has been accumulated (2, 8).

Several observations have suggested that purified large phytochrome is heterogeneous. Multiple species have been detected by isoelectric focusing (9), by discontinuous $NaDodSO_4$/polyacrylamide gel electrophoresis (2, 10), by nondenaturing polyacrylamide gel electrophoresis (10), by NH_2-terminal amino acid analysis (7, 10), by flash photolysis studies of phototransformation kinetics (11), and by dark reversion studies (12). Contrasting potential explanations of these data include programmed intracellular production of multiple species and artifactual modification of a single species during purification. Boeshore and Pratt (13) have reported that phytochrome isolated as the far-red absorbing form, Pfr, has a lower mobility on $NaDodSO_4$/polyacrylamide gel electrophoresis than does phytochrome isolated as the red-absorbing form, Pr. The data were interpreted to indicate that Pfr is subject to some form of modification that results in an increased apparent molecular weight. The converse interpretation not precluded by the data is that it is Pr that is modified during isolation, resulting in greater mobility on $NaDodSO_4$ gel electrophoresis and that Pfr is less susceptible to this modification. One such modification might involve proteolysis. This point is important because all existing phytochrome purification protocols call for rigorous maintenance of the molecule as Pr (6–9, 14, 15).

In this report we explore the possibility that native phytochrome is a larger molecule than hitherto recognized and that previously observed heterogeneities are the result of limited proteolytic degradation during isolation.

MATERIALS AND METHODS

Plant Material. Seedlings of *Avena sativa* cv. Victory (gift from D. Wesenberg, U.S. Dept. of Agriculture, Aberden, ID) were grown in the dark at 25°C for 5 days and the apical 0.5 cm was harvested. Tissue for detergent-free buffer extraction was used fresh and that for extraction in $NaDodSO_4$-containing buffer was freeze-dried. All harvesting and subsequent manipulations were performed in dim green light. All extractions and immunoprecipitations were performed at 0–4°C unless otherwise noted.

Irradiations. Red light (16 $\mu W \cdot m^{-2}$) was provided with a 660-nm interference filter and far-red light (900 $\mu W \cdot m^{-2}$) was provided with a cut-off filter (Corning type CS7-69), using a microscope illuminator as a light source.

Phytochrome Purification and Antibody Production. Phytochrome was purified by the immunoaffinity procedure of Hunt and Pratt (9) with initial assistance from L. H. Pratt. Monospecific anti-phytochrome immunoglobulins (IgG) were purified from rabbit antisera by immunoadsorption to immobilized phytochrome (9). Preimmune IgG was prepared by repeated precipitation with 50% saturated ammonium sulfate.

Detergent-Free Buffer Extraction and Immunoprecipitation. Tissue was homogenized in 35 mM *N*-morpholinopropanesulfonic acid/3 mM Na_4EDTA/250 mM sucrose/14 mM 2-mercaptoethanol/0.1% bovine serum albumin, pH 7.6 (23°C),

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Abbreviations: kDal, kilodalton(s); Pr, red-absorbing form of phytochrome; Pfr, far-red absorbing form of phytochrome; $PhMeSO_2F$, phenylmethylsulfonyl fluoride.

at 3 ml/g fresh weight with an Ultra Turrax blender (Tekmar, Cincinnati, OH). When phytochrome was to be extracted as Pfr a saturating dose of red light was given prior to homogenization. The homogenate was filtered and centrifuged for 10 min at 48,000 × g, and the supernatant was used for immunoprecipitation. The *Staphylococcus aureus* procedure was used for all immunoprecipitations (16). Six micrograms of purified antiphytochrome IgG or preimmune IgG and 20 μl of a 10% (vol/vol) suspension of the *S. aureus* cells were used for each 0.5 ml of extract.

$NaDodSO_4$-Buffer Extraction and Immunoprecipitation. Boiling $NaDodSO_4$-containing buffer (100 mM sodium phosphate, pH 7.8/140 mM 2-mercaptoethanol/1% $NaDodSO_4$) was added to freeze-dried tissue (1.5 ml/g fresh weight) and immediately placed in a boiling water bath. The tissue was homogenized at low speed with an Ultra Turrax blender for 5 min at 100°C, and the homogenate was cooled to 25°C and centrifuged for 10 min at 48,000 × g. The supernatant was diluted with 6–7 vol of 50 mM Tris·HCl, pH 7.5 (25°C)/150 mM NaCl/1% (vol/vol) Triton X-100 to sequester free $NaDodSO_4$, chilled, and clarified at 48,000 × g for 10 min. For immunoprecipitation, 8.4 μg of anti-phytochrome IgG or preimmune IgG and 20 μl of a 10% (vol/vol) suspension of *S. aureus* cells were used per ml of supernatant (16).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was conducted on 7% acrylamide gels according to Laemmli (17) and the gels were stained with Coomassie blue R. Molecular mass markers used were *Escherichia coli* RNA polymerase holoenzyme (β', 160 kDal; β, 150 kDal; σ, 90 kDal), *E. coli* β-galactosidase (116 kDal), rabbit phosphorylase *b* (94 kDal), bovine serum albumin (68 kDal), bovine catalase (58 kDal), porcine fumarase (50 kDal), rabbit aldolase (40 kDal), porcine malate dehydrogenase (36 kDal), porcine carbonic anhydrase (29 kDal), and soybean trypsin inhibitor (21 kDal). The molecular masses of the phytochrome bands were estimated by regression analysis of a plot of R_f versus molecular mass of protein standards coelectrophoresed with the phytochrome.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Two-dimensional gel electrophoresis was performed according to Horst *et al.* (18). Column immunoaffinity-purified phytochrome or immunoprecipitates from detergent-free extracts were boiled for 5 min in $NaDodSO_4$ gel electrophoresis sample buffer (17) and clarified in a Beckman Microfuge. $NaDodSO_4$ was eliminated by cold acetone precipitation of the protein from this supernatant. The protein was resolubilized in sample buffer containing 9.5 M urea, 5 mM K_2CO_3 (pH 10.3), 2% (vol/vol) Nonidet P-40, 0.5% (wt/vol) dithiothreitol, and 4% (vol/vol) pH 3–10 ampholytes (Bio-Rad). Isoelectric focusing toward the anode (150 V, 1 hr; 300 V, 15 hr; 700 V, 1 hr) was performed on 4.3% cylindrical acrylamide gels (0.3 × 10 cm) containing 2% (vol/vol) pH 6–8 ampholytes, 2% pH 5–7 ampholytes, and 1% pH 3–10 ampholytes. $NaDodSO_4$ gel electrophoresis in the second dimension was performed on 10% acrylamide gels.

RESULTS

Column-immunopurified phytochrome, which normally appears as a single band on continuous phosphate buffer gels (9), is readily resolved into a doublet (or triplet) with Laemmli's (17) discontinuous gel system (Fig. 1, lanes A and F, and refs. 2 and 10). The molecule is purified routinely as Pr in this column procedure, as is true of all other conventional protocols (6, 7, 14, 15). In contrast, when phytochrome is extracted as Pfr into a normal detergent-free buffer (Fig. 1, lane B) or in either spectral form (Pr here) into a hot $NaDodSO_4$-containing buffer (Fig. 1, lane D) and is then isolated by rapid immunoprecipitation, it behaves as only a single band on $NaDodSO_4$/polyacrylamide gel electrophoresis and migrates more slowly than the column-immunopurified species (Fig. 1, lanes A and F). These mobility differences are retained when the column-purified molecule is run in the same gel lane with either the buffer-extracted (Fig. 1, lane C) or $NaDodSO_4$-extracted (Fig. 1, lane E) polypeptides, indicating the absence of electrophoresis artifacts. The mobilities of the buffer-extracted and $NaDodSO_4$-extracted polypeptides are the same within the resolution of the system (Fig. 1, lanes B–E) and correspond to a molecular mass of 124 kDal. The mobilities of the major bands in the column-purified preparation correspond to 118 and 114 kDal. A fourth minor band at 112 kDal is present in all three preparations.

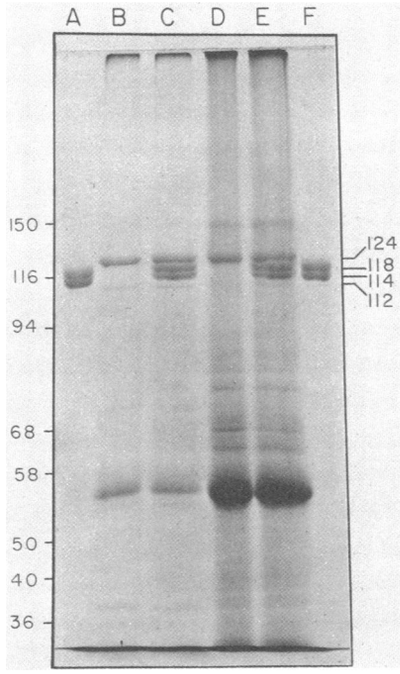

FIG. 1. Comparison of electrophoretic mobility of various phytochrome preparations in a $NaDodSO_4$/7% polyacrylamide gel. Lanes A and F, 1 μg of phytochrome purified by column immunoaffinity chromatography; lane B, phytochrome extracted as Pfr from fresh tissue in detergent-free buffer and rapidly immunoprecipitated; lane C, mixture of material in lanes A and B; lane D, phytochrome extracted as Pr from freeze-dried tissue in boiling $NaDodSO_4$ buffer and immunoprecipitated after sequestering of free $NaDodSO_4$ by Triton X-100; lane E, mixture of material in lanes A and D. Molecular masses (kDal) of protein standards (see Fig. 2, lane J) are shown on the left, here and in Figs. 2–5. Estimated molecular masses of the four phytochrome bands are to the right.

Control experiments in Fig. 2 show that the 124-kDal polypeptide extracted either from freeze-dried tissue in hot $NaDodSO_4$-containing buffer or from fresh tissue as Pfr in detergent-free buffer is immunospecifically adsorbed by anti-phytochrome-coated *S. aureus* cells (Fig. 2, lanes B and D) and not by preimmune IgG-coated cells (Fig. 2, lanes C and H). These data confirm that the bands exhibiting the mobility changes discussed here are in fact phytochrome by the criterion of immunospecificity. The minor 112 kDal polypeptide is absent from the preimmune serum controls (Fig. 2), but it has not been determined whether this band [observed also by Hunt and Pratt (10)] is a phytochrome polypeptide or results from contaminant antibodies in the immune sera. Other Coomassie blue-positive bands visible in both the immune samples and the preimmune serum controls (apart from the major IgG band) are derived predominantly from the *S. aureus* cells. The greater levels of IgG and *S. aureus* proteins relative to the phytochrome band for $NaDodSO_4$-extracted (Fig. 1, lanes D and E; Fig. 2,

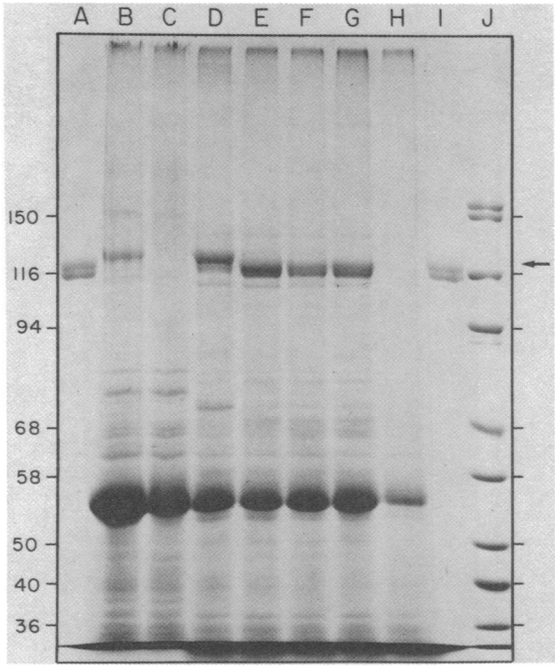

FIG. 2. Anti-phytochrome and preimmune control immunoprecipitates from *Avena* extracts prepared using $NaDodSO_4$-containing and detergent-free buffers. Samples were subjected to gel electrophoresis on $NaDodSO_4$/7% polyacrylamide. Lanes A and I, phytochrome (1 μg) purified by column immunoaffinity chromatography. Lanes B and C, immunoprecipitates from $NaDodSO_4$/buffer extracts. Freeze-dried tissue was extracted in boiling $NaDodSO_4$-containing buffer, free $NaDodSO_4$ was sequestered with Triton X-100, and immunoprecipitates were prepared by using anti-phytochrome-coated (lane B) or preimmune IgG-coated (lane C) *S. aureus* cells. Lanes D–H, immunoprecipitates from detergent-free buffer extracts. Phytochrome was extracted from fresh tissue as Pfr in detergent-free buffer and aliquots were warmed to 25°C for incubation according to the protocol below prior to immunoprecipitation with either anti-phytochrome-coated (lanes D–G) or preimmune IgG-coated (lane H) *S. aureus* cells. Incubations: D, Pfr, 60 min; E, Pr, 60 min; F, Pr, 60 min, then Pfr, 60 min; G, Pr, 120 min; H, Pfr, 60 min. For incubations of phytochrome as Pr (lanes E–G) aliquots were given saturating irradiations (3 min) of far-red light at the start and of red light at the end of the incubation period. Samples were cooled to ice temperature again before immunoprecipitation. Lane J, protein markers used for molecular mass calibration. Arrow indicates 124-kDal polypeptide.

lane B) than for buffer-extracted (Fig. 1, lanes B and C; Fig. 2, lane D) preparations reflects a lowered affinity of the $NaDodSO_4$-denatured molecule for anti-phytochrome (unpublished data).

The phytochrome obtained by extraction and immunoprecipitation as Pfr in detergent-free buffer appears as a single spot upon two-dimensional gel analysis (Fig. 3*A*). This result strongly indicates that the phytochrome isolated in this manner is a homogeneous preparation as regards both molecular mass (124 kDal) and isoelectric point (pI = 5.9). The phytochrome isolated by column immunoaffinity purification exhibits heterogeneity of isoelectric point as well as of apparent molecular weight (Fig. 3*B*). The 118- and 114-kDal species have pIs of 5.8 and 6.0, respectively. The separation of the 124-, 118-, and 114-kDal polypeptides run simultaneously on the same gel verifies that each has a distinct pI (Fig. 3*C*). The minor 112-kDal polypeptide is barely visible in each of the magnified regions of the gels (Fig. 3) and appears to have a pI similar to that of the 114-kDal species (6.0).

Phytochrome isolated as Pr by rapid immunoprecipitation from detergent-free buffer extracts consists predominantly of the 118- and 124-kDal polypeptides (data not shown). This observation is consistent with the report of Boeshore and Pratt (13), who found, using continuous rather than discontinuous $NaDodSO_4$/polyacrylamide gel electrophoresis, that the molecule isolated as Pfr has a lower average mobility than does that isolated as Pr under identical conditions. Boeshore and Pratt proposed that these differences in mobility result because Pfr undergoes some form of modification to which Pr is not subjected. However, two lines of indirect evidence suggested to us that the converse is more likely. First, the mobility of the polypeptide extracted into hot $NaDodSO_4$-containing buffer (designed to minimize posthomogenization modifications) is independent of the spectral form at extraction and matches the mobility of the molecule extracted as Pfr in detergent-free buffer (Fig. 1, lanes B and D) rather than that of Pr. Second, the mobility of phytochrome synthesized *in vitro* is also identical to that of the $NaDodSO_4$-extracted polypeptide (19). Thus all procedures yield a 124-kDal polypeptide except extraction as Pr in detergent-free buffer.

To test this alternative interpretation directly, phytochrome was extracted as Pfr in detergent-free buffer, photoconverted back to Pr in the crude extract, and incubated for increasing periods before being reconverted once more to Pfr and immunoprecipitated. Increasing periods of incubation as Pr result in the progressive disappearance of the 124-kDal polypeptide with the concomitant appearance of new, more rapidly migrating, bands (Fig. 4, lanes A–G) initially at 118 kDal and then increasingly at 114 kDal. Phytochrome retained as Pfr for the full incubation period (Fig. 4, lane I) shows only relatively minor conversion of the 124- to the 118-kDal molecule. This result provides strong evidence that Pr is susceptible and Pfr relatively resistant to an *in vitro* modification that leads to increased electrophoretic mobility. The rate at which this modification occurs is temperature dependent. The present experiments were conducted at 25°C to accelerate the process but qualitatively identical results are obtained at the lower temperatures (0–4°C) and longer times (≥12 hr) required for purification (unpublished data). It is uncertain whether the minor levels of the 118- and 114-kDal bands present in samples isolated and incubated as Pfr (Fig. 2, lane D; Fig. 4, lane I; Fig. 5, lanes A and B) arise (*i*) from the phytochrome that remains as Pr at the photoequilibrium established by the red irradiation [15–25% (2, 20)] or (*ii*) from slower modification of Pfr itself. All samples were in the Pfr form at the time of preparation for electrophoresis, eliminating the trivial possibility that the observed mobility differences are caused by differences in the spectral form of the molecule at that point in the procedure. Further controls in Fig. 2, lanes D–G, establish that the induced mobility change is unidirectional. The mobility increase caused by incubation as Pr for 60 min (Fig. 2, lane E) is not reversed by reconversion to Pfr and further incubation for another 60 min under the same conditions (Fig. 2, lane F).

The serine protease inhibitor phenylmethylsulfonyl fluoride ($PhMeSO_2F$) strongly inhibits the conversion of the 124-kDal polypeptide to the more rapidly migrating species during 60-min incubation as Pr (Fig. 4, lanes G and H). Fig. 5 shows the effectiveness of increasing concentrations of $PhMeSO_2F$ in inhibiting this conversion. Another protease inhibitor, iodoacetimide (1 mM), was ineffective, whereas 20 mM EDTA reduced the degradation rate by about 50%.

DISCUSSION

The present data indicate that the native phytochrome monomer is larger than previously recognized and may be a single polypeptide species rather than the heterogeneous combination of variants reported in the past. It is apparent that column-im-

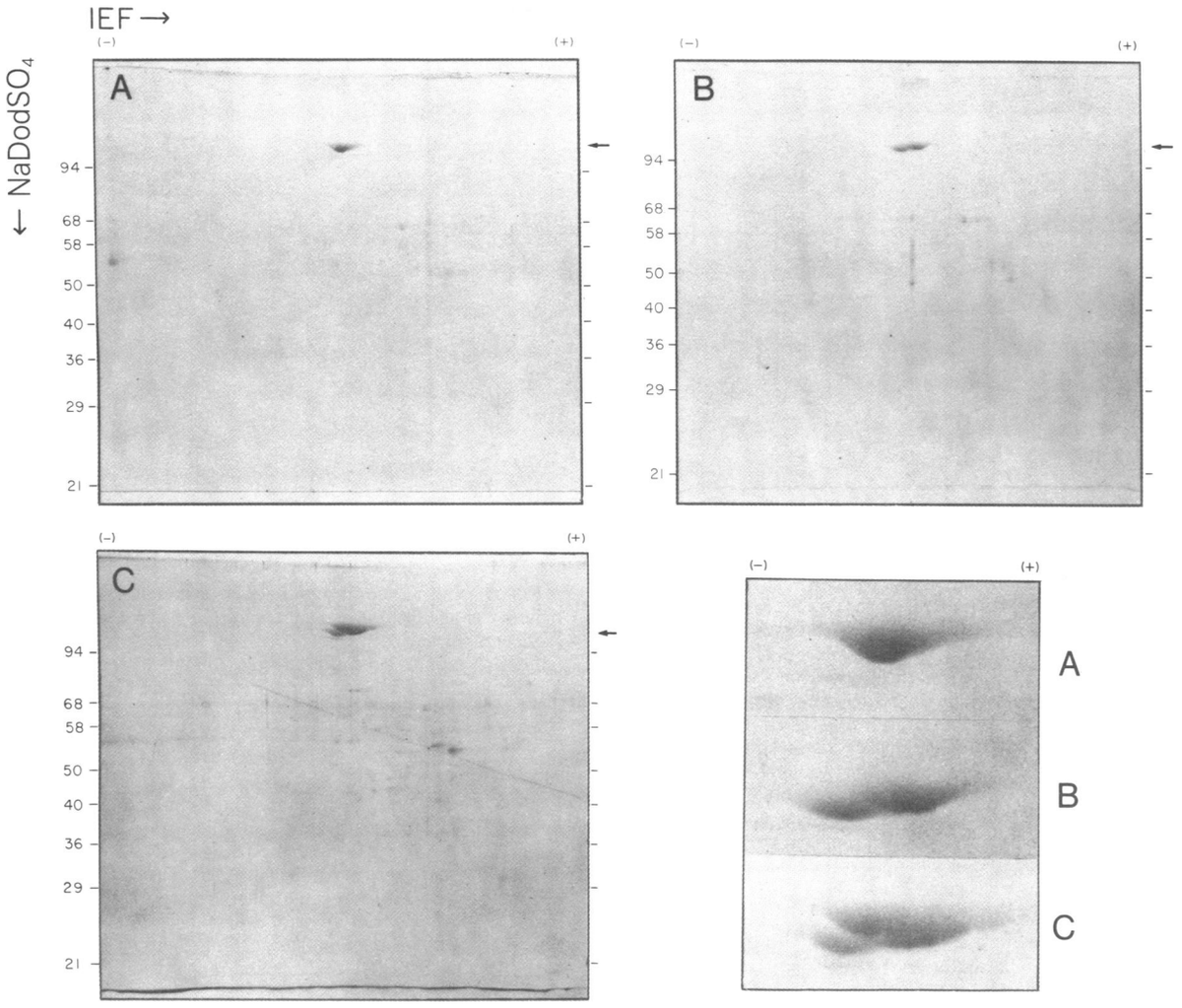

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis of phytochrome extracted as Pfr from fresh tissue in detergent-free buffer and rapidly immunoprecipitated (*A*); 4 μg of phytochrome purified by column immunoaffinity chromatography (*B*); and mixture of materials in *A* and *B* (*C*). Arrows to the right indicate position of phytochrome spots. (*Lower Right*) Enlargements of phytochrome spots found in *A*, *B*, and *C*. Samples were subjected to isoelectric focusing (IEF) in the first dimension and electrophoresis on discontinuous $NaDodSO_4$/10% acrylamide gels in the second dimension. The pIs of the 124-, 118-, 114-, and 112-kDal phytochrome species are 5.9, 5.8, 6.0, and 6.0, respectively.

munopurified phytochrome from *Avena* has been modified from its native state, artifactually during purification, to a form(s) with lower apparent molecular mass on $NaDodSO_4$/polyacrylamide gel electrophoresis. The evidence indicates that Pr is much more susceptible to this modification than is Pfr. Thus the lower electrophoretic mobility of phytochrome isolated as Pfr than of that isolated as Pr, reported previously by Boeshore and Pratt (13), results not from selective modification of Pfr as suggested by these authors but rather the converse. Indirect evidence from the use of $PhMeSO_2F$ suggests that this modification probably involves limited proteolytic degradation caused by one or more serine proteases. The process is relatively rapid and can proceed to completion even at ice temperature during the time required for most existing purification procedures. Because it is routine with these procedures to maintain phytochrome exclusively in the more susceptible Pr form throughout purification, the probability that degradation will occur is maximized. Indeed, we have found that the progressive decrease in apparent molecular mass of phytochrome occurs with the molecule in the Pr form with all the currently available purification protocols that we have tested with *Avena* (9, 14, 15).

Evidence that the 124-kDal polypeptide observed here is in fact the undegraded native monomer is provided by the apparent homogeneity and identical mobilities on $NaDodSO_4$/polyacrylamide gel electrophoresis of the cell-free translation product (19) and the polypeptides isolated either as Pfr in detergent-free buffer or by hot $NaDodSO_4$ extraction in either spectral form. Together these data indicate that the phytochrome polypeptide is subject neither to intracellular proteolytic processing nor to posthomogenization proteolysis under the conditions used. The detection of only a single molecular species in all three cases indicates that there is only one type of phytochrome.

The principal consequence of these findings is that it is highly probable that much of the data accumulated on the molecular properties of purified phytochrome was obtained with a partially degraded molecule (see refs. 2 and 8 for reviews) and therefore requires reexamination. The reductions in apparent molecular mass of 6 and 10 kDal, respectively, for the 118- and 114-kDal species represent substantial polypeptide segments. Thus some properties such as amino acid composition (2, 8, 10) will obviously require revision, whereas others such as the variety of proposed differences between Pr and Pfr (21–23), the purported capacity for specific *in vitro* binding to such structures as mitochondria (23), liposomes, and protoplasts (24), and

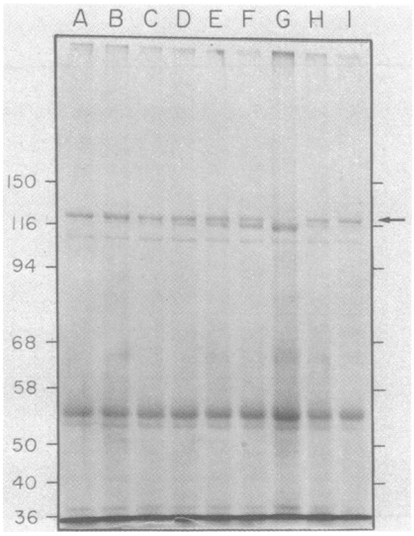

FIG. 4. Time course of increase in electrophoretic mobility of phytochrome during incubation as Pr in crude extracts at 25°C. Samples were subjected to electrophoresis on $NaDodSO_4$/7% polyacrylamide. Lane A, phytochrome extracted as Pfr from fresh tissue in detergent-free buffer and rapidly immunoprecipitated. Lanes B–H, same extract as in lane A was warmed to 25°C and the phytochrome was converted to Pr by saturating (3-min) far-red irradiation. After various intervals the phytochrome was converted back to Pfr again by a saturating (3-min) red irradiation, chilled to ice temperature, and rapidly immunoprecipitated as above. Intervals (min) between far-red and red irradiations were B, 0; C, 2; D, 5; E, 10; F, 20; G, 60; and H, 60 with 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) added prior to warming and far-red irradiation. Lane I, Phytochrome extracted as Pfr, incubated at 25°C for 60 min, and immunoprecipitated. Arrow indicates approximate position of phytochrome bands.

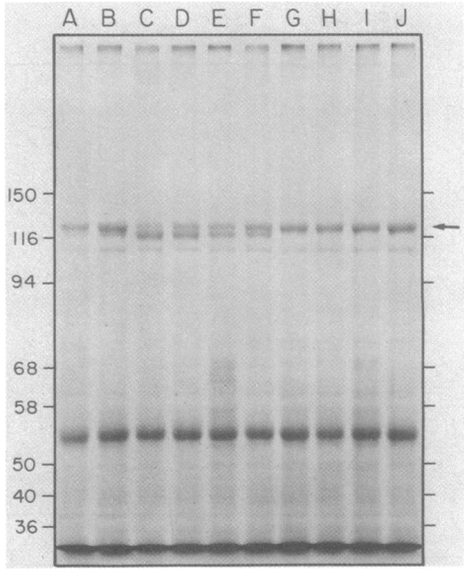

FIG. 5. Inhibition by $PhMeSO_2F$ of increase in electrophoretic mobility of phytochrome incubated as Pr in crude extracts at 25°C. Phytochrome was extracted as Pfr from fresh tissue in detergent-free buffer and immunoprecipitated from aliquots of the extract after 60-min incubation as follows: lane A, Pfr, 25°C, no $PhMeSO_2F$; lane B, Pr, ice temperature, no $PhMeSO_2F$; lane C, Pr, 25°C, no $PhMeSO_2F$; lane D, Pr, 25°C, 50 μM $PhMeSO_2F$; lane E, Pr, 25°C, 100 μM $PhMeSO_2F$; lane F, Pr, 25°C, 200 μM $PhMeSO_2F$; lane G, Pr, 25°C, 500 μM $PhMeSO_2F$; lane H, Pr, 25°C, 1 mM $PhMeSO_2F$; lane I, Pr, 25°C, 2 mM $PhMeSO_2F$; and lane J, Pfr, 25°C, 2 mM $PhMeSO_2F$. For incubations of phytochrome as Pr, (lanes C–I) aliquots were given saturating irradiations (3 min) of far-red light at the start and of red light at the end of the 60-min period. Aliquots incubated at 25°C were cooled to ice temperature again before immunoprecipitation. $PhMeSO_2F$ (in isopropyl alcohol) was added prior to warming to 25°C or far-red irradiation where appropriate. Equal aliquots of isopropyl alcohol were added to the minus $PhMeSO_2F$ controls. Immunoprecipitates were subjected to electrophoresis on $NaDodSO_4$/7% polyacrylamide.

the spectral and photochemical properties of the pigment (21) will require careful reevaluation. Our present data show directly that the reported heterogeneity in molecular mass and pI of *Avena* phytochrome (8–10) is generated artifactually during purification and suggest that other heterogeneities, including multiple NH_2-terminal amino acids (7, 10) and multiple kinetic populations detected in flash photolysis studies of phytochrome phototransformation (11) and in dark reversion experiments (12), may also result from the mixture of differentially degraded species present. We have recent evidence that proteolytic degradation of the 124-kDal molecule to the 118/114-kDal doublet is responsible for the shift to shorter wavelength of the Pfr absorption maximum long observed with existing purification schemes (25). This observation also explains the results of Epel (26), who attributed similar spectral data to "activation" of Pfr.

We thank L. H. Pratt for assistance in initiating the immunopurification procedures; W. R. Briggs, in whose laboratory much of the anti-phytochrome production was carried out; R. Burgess for the gift of RNA polymerase; M. Werner-Washburn for assistance with the two-dimensional polyacrylamide gel electrophoresis; and K. Keegstra, K. Cline, and H. Hershey for critically reading the manuscript. This work was supported by National Science Foundation Grants PCM8003921, PCM7723584, and PCM7514161.

1. Smith, H. (1975) *Phytochrome and Photomorphogenesis* (McGraw-Hill, London).
2. Pratt, L. H. (1979) *Photochem. Photobiol. Rev.* **4**, 59–124.
3. Fry, K. T. & Mumford, F. E. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1466–1473.
4. Mumford, F. E. & Jenner, E. L. (1966) *Biochemistry* **5**, 3657–3662.
5. Gardner, G., Pike, C. S., Rice, H. V. & Briggs, W. R. (1971) *Plant Physiol.* **48**, 686–693.
6. Rice, H. V., Briggs, W. R. & Jackson-White, C. J. (1973) *Plant Physiol.* **51**, 917–926.
7. Rice, H. V. & Briggs, W. R. (1973) *Plant Physiol.* **51**, 927–938.
8. Pratt, L. H. (1982) *Annu. Rev. Plant Physiol.* **33**, 557–582.
9. Hunt, R. E. & Pratt, L. H. (1979) *Plant Physiol.* **64**, 332–336.
10. Hunt, R. E. & Pratt, L. H. (1980) *Biochemistry* **19**, 390–394.
11. Cordonnier, M. M., Mathis, P. & Pratt, L. H. (1981) *Photochem. Photobiol.* **34**, 733–740.
12. Pike, C. S. & Briggs, W. R. (1972) *Plant Physiol.* **49**, 514–520.
13. Boeshore, M. L. & Pratt, L. H. (1981) *Plant Physiol.* **68**, 789–797.
14. Bolton, G. W. (1979) Dissertation (Univ. of Minnesota, St. Paul, MN).
15. Smith, W. O., Jr., & Daniels, S. M. (1981) *Plant Physiol.* **68**, 443–446.
16. Kessler, S. W. (1975) *J. Immunol.* **115**, 1617–1624.
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
18. Horst, M. N., Basha, S. M. M., Baumbach, G. A., Mansfield, E. H. & Roberts, R. M. (1980) *Anal. Biochem.* **102**, 399–408.
19. Bolton, G. W. & Quail, P. H. (1982) *Planta*, in press.
20. Yamamoto, K. T. & Smith, W. O., Jr. (1981) *Plant Cell Physiol.* **22**, 1159–1164.
21. Song, P. S., Chae, Q. & Gardner, J. D. (1979) *Biochim. Biophys. Acta* **576**, 479–495.
22. Hunt, R. E. & Pratt, L. H. (1981) *Biochemistry* **20**, 941–945.
23. Cedel, T. E. & Roux, S. J. (1980) *Plant Physiol.* **66**, 696–703.
24. Kim, I. S. & Song, P. S. (1981) *Biochemistry* **20**, 5482–5489.
25. Vierstra, R. D. & Quail, P. H. (1982) *Plant Physiol.* **69**, S85 (abstr.).
26. Epel, B. (1980) in *Photoreceptors and Plant Development*, ed. DeGreef, J. (Antwerpen Univ. Press, Antwerp, Belgium), pp. 467–479.