Phytochrome Modification and Light-enhanced, *In Vivo*-induced Phytochrome Pelletability¹, ²

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ABSTRACT

Phytochrome that was induced by red irradiation *in vivo* to pellet with subcellular material and that was released from the pellet by removal of divalent cations exhibited altered characteristics. Compared to phytochrome extracted in a soluble red-absorbing form from etiolated tissue, pelleted and released phytochrome, which was also assayed in the red-absorbing form even though pelleted in the far-red-absorbing form, showed 50% greater microcomplement fixation activity, eluted closer to the void volume of a Sephadex G-200 column, and electrophoresed more slowly on sodium dodecyl sulfate-polyacrylamide gels. Data presented here document that phytochrome pelleted in the far-red-absorbing form differs from soluble phytochrome extracted from nonirradiated tissue. These data, however, do not permit the conclusion that there is a causal relationship between pelletability and phytochrome modification.

Two fundamentally different red-light-induced associations of phytochrome with pelletable subcellular material have been described (8). One type is induced *in vitro* and represents, at least in part, an artificial, electrostatic association between phytochrome and 31S ribonuclear protein (12). The second type is induced only *in vivo*. The intracellular dark reaction leading to *in vivo*-induced pelletability is potentially an integral part of the mechanism of action of phytochrome (8, 13). Here we explore further this *in vivo*-induced pelletability phenomenon.

*In vivo*-induced pelletability has been interpreted as evidence for a biologically significant interaction between phytochrome and a membrane-bound "receptor," which represents an initial step in its mode of action (6, 15). Indirect physiological data, however, remain the strongest supportive evidence for this hypothesis (see ref. 8 for discussion). An investigation of possible changes in the properties of phytochrome, which might be correlated with its interaction with subcellular material, is one important approach to evaluating this hypothesis. Phytochrome photoinduced to pellet *in vivo* and subsequently released from the pellet by removal of divalent cations is reported to migrate closer to the void volume of a gel exclusion column that extracted in a soluble form from dark control plants (2, 19, 20). Our purpose here is to re-examine these observations by application of more sensitive techniques. We do not consider here the related, but considerably more complex, question of whether a causal relationship exists between phytochrome pelletability and the modification.

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MATERIALS AND METHODS

Phytochrome was isolated from 5-day-old oat (*Avena sativa* L., cv. Garry) shoots grown at 25 C and near saturating humidity in darkness on open, plastic cafeteria trays covered with cellulose packing material (Kimpak 6234, Kimberly Clark). Extractions, assay procedures, and experimental manipulations prior to immunoprecipitation of phytochrome were performed under green safelights (7). Extracts were maintained at 0 to 4 C. Final preparations were stored at −70 C.

**Actinic Irradiations.** Six unfiltered 40-w Gro-lux fluorescent lamps (Sylvania) were used as a "red" light source for tissue irradiation. Photostationary equilibrium was established in about 5 s and was indistinguishable from that obtained with a 660-nm Balzer B-40 interference filter. Far-red light was obtained by filtering the output of a tungsten lamp (Unitron LKR microscope illuminator) through a plastic cutoff filter 3.2 mm thick (Plexiglas, FRF-700).

**Phytochrome Measurement.** One unit of phytochrome, equal to about 1.7 mg (17), is that amount which, in 1.0 ml, gives $A_{660}^{1cm} = 1.0$ after saturating far-red irradiation (8, 9). Clear samples containing greater than about 0.05 unit/ml phytochrome were assayed as Pr at 667 nm with a Varian model 635 spectrophotometer. Opaque samples, with CaCO₃ used as a light-scattering agent (1), and clear samples containing less than about 0.05 unit/ml were assayed using a custom-built dual-wavelength spectrophotometer (4).

**Phytochrome Purification.** Control phytochrome was partially purified from nonirradiated tissue by chromatography of crude extracts through a brushite column (7) and by subsequent fractionation with 200 g/l (w/v) ammonium sulfate. The resulting pellet was dissolved in 0.1 M Na-phosphate (pH 7.8) and clarified at 40,000g for 15 min, yielding a phytochrome concentration of about 0.5 unit/ml.

Pelleted Pfr was partially purified from oats that had been irradiated with red light for 3 min at 25 C. Shoots were homogenized with a Waring Blender in 25 mm Mops³-Tris, 14 mM 2-mercaptoethanol, 20 mM MgCl₂ (pH 7.5; obtained by mixing 25 mm Mops with 25 mm Tris, each containing 14 mM 2-mercaptoethanol and 20 mM MgCl₂ at a ratio of 2 ml buffer/1 g fresh-weight oat shoots. Crude extracts were filtered through cheesecloth and centrifuged 15 min at 20,000g. Pellets were resuspended in a volume of extraction buffer equal to that used for tissue homogenization. Following a second 15-min 20,000g centrifugation, pellets were resuspended in about one-half the extraction volume of 25 mm Mops-Tris (pH 7.5), 14 mM 2-mercaptoethanol, 5 mM EDTA and incubated in darkness for 15 min. MgCl₂ then was added back to a concentration of 20 mM from a 1 M stock solution. In this way, upon centrifugation again at 20,000g for 15 min, phytochrome was released to the supernatant (11) and nonspecific aggregation of subcellular debris was reintroduced to yield a less

³ Abbreviation: Mops: N-morpholinopropanesulfonic acid.
contaminated phytochrome preparation. Typically, 67% of the pelleted phytochrome pool was released to the supernatant following resuspension in the EDTA-containing buffer. This once-pelleted, but now soluble, phytochrome was concentrated and further purified by precipitation with 200 g/l (w/v) ammonium sulfate. The pellet was dissolved in 0.1 M Na-phosphate (pH 7.8) and clarified (40,000 g, 15 min), yielding a phytochrome concentration of about 0.15 unit/ml. Before storage and further use, pelleted Pfr samples were given a saturating dose of far-red light.

Phytochrome labeled with $^3$H or $^{35}$S was obtained by adding the appropriate radioisotope ([3H]leucine, 5–10 mCi/ml, 62 Ci/mmol, Schwarz/Mann, or [$^{35}$S]H$_2$SO$_4$, carrier-free, ICN Pharmaceuticals) to intact oat seedlings prior to harvest. [$^{35}$S]H$_2$SO$_4$ (1.0 mCi, using 1- to 2-μl drops) was applied to coleoptile tips of 3-day-old plants. [$^{35}$S]H$_2$SO$_4$ (1.0 mCi) was added to 3.0 ml H$_2$O in a plastic Petri dish containing 50 2-day-old oat seedlings. Thus, phytochrome was labeled by substitution during synthesis rather than by chemical modification after synthesis. In both cases, shoots were harvested when 4 days old, and respective phytochrome preparations were made as described above.

**Gel Filtration.** Phytochrome preparations were chromatographed through a 2.4 × 98-cm Sephadex G-200 column with 0.1 M Na-phosphate (pH 7.8). A void volume marker, blue dextran, and a total volume marker, tryptophan, were added to each sample before application to the column. Phytochrome mobility was expressed as $K_v$ (7).

**Microcomplement Fixation.** Microcomplement fixation assays were performed as before (7) by the method of Levine and Van Unnikas (5).

**Polyacrylamide Gel Electrophoresis.** SDS-polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (18) using either slab (1.5 × 130 × 110 mm) or cylindrical (5 × 110 mm) gels. Gel composition was 5% (w/v) acrylamide, 0.14% (w/v) $N$,$N'$-methylenebisacrylamide or 0.64% (w/v) diallyltartardiamide (used in gels to be sliced for liquid scintillation counting), and 0.1% (w/v) SDS in 0.1 M Na-phosphate (pH 7.1 to 7.2). Electrophoresis was performed at 800 mamp/cylindrical gel and at 95 mamp/slab gel for about 5 h at room temperature. Samples were prepared by heating to 100°C in 0.01 M Na-phosphate (pH 7.1 to 7.2), 0.73 M 2-mercaptoethanol, 0.1% (w/v) SDS, 0.2% (v/v) glycerol, and 0.0015% (w/v) bromphenol blue for 5 min. Electrophoresis chamber buffer was 0.1 M Na-phosphate (pH 7.1 to 7.2) and 0.1% (w/v) SDS.

Immunoprecipitates for electrophoretic analysis were prepared by adding antiphloem serum to solutions of phytochrome at equivalence and incubating overnight at 0°C. After collection by centrifugation, immunoprecipitates were resuspended in 0.13 M NaCl containing 1.0% Triton X-100, and 1.0% sodium deoxycholate. The resuspended pellet was layered over a 200-μl, 5% sucrose pad in a conical tip centrifuge tube of 1, 2, or 3 ml capacity and again centrifuged. This second pellet was resuspended in saline and detergent and centrifuged as above. After washing once or twice more with saline or with saline and detergent, the final immunoprecipitate was dissolved by heating in SDS sample buffer as described above.

Following electrophoresis, peptide bands were stained with Coomassie brilliant blue R (18). Absorbance scans of cylindrical gels were made with a Shimadzu MPS-50L recording spectrophotometer equipped with a custom-built gel transport.

Polyacrylamide gels were sliced and counted as elsewhere (3). To minimize counting error, slices in the region of the phytochrome monomer band were counted for 100 min.

**Immunoelectrophoresis.** Ouchterlony double diffusion and immunoelectrophoresis were performed as before (7).

**RESULTS**

During Sephadex G-200 gel exclusion chromatography, pelleted Pfr elutes more rapidly than dark control phytochrome (Fig. 1), confirming earlier reports (2, 19, 20). One explanation of the faster migration rate of pelleted Pfr through a Sephadex G-200 column is a size increase resulting from a possible continued association of phytochrome with a putative binding partner. To test this possibility, an immunoprecipitate was prepared from fractions at the phytochrome peak ($K_v$ = 0.066) and examined by SDS-gel electrophoresis (Fig. 2a). No additional bands, which might be attributed to a phytochrome binding partner, were observed with immunoprecipitates of pelleted Pfr when compared with those of controls (Fig. 2b).

Comparison of control phytochrome preparations to pelleted Pfr by Ouchterlony double diffusion and immunoelectrophoresis (7) indicated no differences (data not shown). The microcomplement fixation assay, a sensitive indicator of antigenic activity, did detect a difference between control and pelleted Pfr (Fig. 3).

A comparison of mobility on SDS-polyacrylamide gels shows that pelleted Pfr migrates more slowly than control Pfr (Figs. 4 and 5). The small, but definite, mobility change of pelleted Pfr translates to an assumed monomer weight difference of approximately 5,000. Pelleted Pfr and control Pfr electrophoresed as 123,000- and 118,000-dalton monomers, respectively. Co-electrophoresis of pelleted Pfr and control Pfr yields the expected increase in band width (Figs. 4, c and d, and 5e). Selective modification of pelleted Pfr due to immunoprecipitation is not seen, as electrophoresis of brushite-purified preparations shows the same difference (Fig. 5, a and b).

The decreased mobility of pelleted Pfr on SDS-polyacrylamide gels is highly reproducible. Mobilities for the phytochrome monomer in partially purified preparations are the means of four independent electrophoretic experiments with a standard error of 0.002 (Fig. 5). Mobilities for the phytochrome monomer from the immunoprecipitates are the means of 13 independent electrophoretic experiments with standard errors of 0.004 for Pfr and 0.003 for pelleted Pfr. Confidence limits for the significance of the difference obtained by both paired and unpaired Student's $t$ tests were greater than 99%. When pelleted Pfr and control Pfr mobilities
were compared to the mobility of the 50,000-dalton heavy chain of immunoglobulin G in the same gels, rather than to bromphenol blue, the observed difference was the same.

Co-electrophoresis of pelleted Pfr and control phytochrome immunoprecipitates isotopically labeled in vivo confirms the mobility difference. Regardless of which preparation was labeled with $^{35}$S and which with $^3$H, the expected isotope displacement was observed (Figs. 6 and 7), thereby eliminating the possibility of an isotope effect. The radioisotope profiles in Figure 7 show the radioactivity observed in the 10 slices centered about the phyo-

discussion

Results presented here are in agreement with earlier reports (2, 19, 20) which indicated that pelleted Pfr exhibits changed characterics. Compared with control Pr, pelleted Pfr elutes closer to the void volume of a Sephadex G-200 column (Fig. 1). This difference in $\Delta v$, is exaggerated by variability in fraction sizes and
Fig. 5. A scans of cylindrical gels after electrophoresis of partially purified pelleted Pfr (a) and control Pr (b) preparations and after electrophoresis of immunoprecipitates of pelleted Pfr (c), control Pr (d), and an equal mixture of pelleted Pfr and control Pr (e). Approximately 10 μg phytochrome was electrophoresed in each case. Labeled bands are the 118,000-dalton phytochrome monomers; mobilities relative to bromophenol blue are indicated. The major bands to the right are lower mol wt impurities (a, b) or heavy and light chains of immunoglobulin (c-e).

is consistent with a possible increase of 90,000 in mol wt if the altered elution rate is attributed solely to a size increase. In light of the subunit size for phytochrome (14, 16) and results of SDS-polyacrylamide gel electrophoresis (Figs. 4–8), however, the possibility of a size increase as an explanation for the Kav difference appears unlikely. In contrast to the observations of Grombein and Rudiger (2), our data do not indicate that a significant portion of the eluted pelleted Pfr is “very large” in size.

No additional bands were detected by Coomassie brilliant blue staining of gels in which pelleted Pfr immunoprecipitates were electrophoresed following elution (Fig. 2). This observation indicates that a potential size increase ascribed to a binding partner was not detected. The small band to the left of the phytochrome monomer in Figure 2a is not unique to pelleted Pfr immunoprecipitates, as we have observed it in polyacrylamide gels of control Pr immunoprecipitates as well. A limitation in identifying additional bands by Coomassie brilliant blue staining of polyacrylamide gels in which immunoprecipitates have been electrophoresed is the possibility that an additional band might co-migrate with an immunoglobulin G band and, therefore, might not be detected. We suggest that a conformational change leading to an increase in the Stokes radius for phytochrome is a likely explanation for the increased elution rate from the Sephadex G-200 column.

The increased microcomplement fixation activity of pelleted Pfr (Fig. 3) is indicative of a change in one or a few of the antigenic determinants of phytochrome, which leads to the conclusion that any possible conformational changes are restricted to one or a few discrete sites on the molecule (5). The identical equivalence points show that all classes of immunoglobulins recognize both phytochrome preparations (5). Pratt et al. (10) have shown that denaturation of phytochrome by exposure to 5 M urea leads to increased microcomplement fixation activity. A comparable conformational change might be related to the modification leading to the observed faster elution rate from the gel exclusion column.

Because the modification appears prior to extraction or rapidly in vitro (Fig. 8), it does not result from prolonged exposure of Pfr to endogenous materials released during extraction to which phytochrome has no exposure in vivo. Before electrophoresis, samples were exposed to rigorous denaturing conditions. Therefore, the results further indicate that the decreased mobility of pelleted Pfr results from a covalent modification.

It is evident that phytochrome bound to and released from pelletable material in the Pfr form exhibits altered properties
FIG. 8. Photograph of SDS-polyacrylamide slab gel after electrophoresis of immunoprecipitates of control phytochrome (C) and rapidly extracted pelleted Pfr (P). Origin is at top.

compared to control phytochrome. These data, however, do not permit any conclusion concerning the question of whether there is a causal relationship between the modification and pelletability. Since the form of phytochrome is different during preparation of pelleted Pfr, as compared to control phytochrome, it is possible that they differ because of differential sensitivity to conditions of extraction and partial purification. We are not, however, describing differences arising from the use of different forms of phytochrome during assay since pelleted Pfr was photoconverted back to Pr before assay. The nature of the modification of phytochrome that leads to these differences is unknown.

LITERATURE CITED