Phytochrome Pelletability Induced by Irradiation in Vivo

TEST FOR IN VITRO BINDING OF ADDED [35S]PHYTOCHROME

LEE H. PRATT
Department of Biology, Vanderbilt University, Nashville, Tennessee 37235

ABSTRACT

Undegraded, highly purified [35S]phytochrome was immunoaffinity-purified either from dark control oat (cv. Garry) shoots or from etiolated oat shoots that were previously irradiated first with red and then with far-red light so that, if proper extraction conditions had been utilized, about 60% of the total phytochrome would have been pelletable. When [35S]phytochrome was added to extraction buffer immediately prior to homogenization of etiolated oat shoots, pelletability assays indicated that there was no preferential binding of [35S]phytochrome regardless of (a) whether it was purified from dark control or irradiated shoots, (b) whether it was added as phytochrome-red-absorbing form or phytochrome-far-red-absorbing form, or (c) whether it was added to dark control or red-irradiated shoots. Similarly, binding of [35S]phytochrome to resuspended pellets obtained from crude oat extracts was not specific for the source of [35S]phytochrome, for its form, or for the irradiation treatment given to intact shoots used to prepare the resuspended pellets. No evidence was obtained to support the hypothesis that phytochrome binds with specificity to particulate material in vitro under conditions used to assay for light-enhanced, in vivo-induced phytochrome pelletability.

About 95% of the phytochrome extracted from nonirradiated, dark-grown plants behaves as a soluble protein. In contrast, photoconversion of Pr to Pfr leads to pelletability of as much as 80% of the extractable phytochrome (8). This light-induced phytochrome pelletability is of at least two types (see ref. 10 for discussion). One type may be induced by irradiation of either intact tissue or crude extracts, whereas the other may be induced only by irradiation of intact tissue (11). This paper is concerned only with this second type of pelletability which is the result of an in vivo nonphotochemical reaction dependent upon Pfr and is expressed in vitro only in the presence of a divalent cation (11–13). Although in vivo-induced phytochrome pelletability itself may turn out to be biologically irrelevant, it is nevertheless important to understand because it results from a rapid (t1/2 at 25°C = 2 s; ref. 12) Pfr-mediated nonphotochemical reaction that might be an initial event in the mechanism of action of phytochrome.

It has been suggested that light-enhanced phytochrome pelletability represents binding in vivo between Pfr and a receptor or specific binding partner (7, 15). Nevertheless, one must consider at least three other possible explanations for light-enhanced phytochrome pelletability because it can be assayed only in vitro (12). First, pelletability may represent Pfr-mediated stabilization of a pre-existing association between Pr and pelletable subcellular constituents. Second, pelletability may arise because of an in vitro binding event involving phytochrome that is modified during the in vivo induction process. Third, the in vivo induction process may be a Pfr-mediated alteraton in some other component of the cell that leads to binding of phytochrome in vitro.

The experiments described here utilize exogenously added, highly purified [35S]phytochrome in an attempt to determine whether one or more of the above possible explanations for in vivo-induced pelletability might be excluded.

MATERIALS AND METHODS

Oats (Avena sativa L., cv. Garry) were grown for 4 days at 25°C in total darkness and near-saturating humidity (9). Whole shoots were harvested near the grain and used for all pelletability assays. All work was done under green light (9) until final pellets and supernatants had been separated.

Phytochrome Purification. Unlabeled undegraded oat phytochrome with a specific A ratio (A400/A280) with phytochrome as Pr) of 0.34 (estimated purity = 40%) was purified by brushite chromatography, ammonium sulfate fractionation, and DEAE-cellulose and Sephadex G-200 chromatography (4).

To obtain highly purified, undegraded, 35S-labeled phytochrome, oats were first germinated for 2 days, as described above, so that their roots were about 1 cm long and their shoots were just beginning to emerge. Fifty seedlings were transferred to a polystyrene, 10-cm diameter Petri dish to which 3 ml H2O containing carrier-free [35S]SO42− (ICN 64040) was added. After 2 days further growth as described above, whole shoots were harvested and phytochrome was purified by an immunoaffinity procedure (3). Control [35S]phytochrome was purified from 50 shoots (2.2 g) obtained from 50 nonirradiated plants that were grown 15 mCi 35S. Pelletable [35S]phytochrome was purified from 100 shoots (3.7 g) obtained from plants grown in the presence of 30 mCi 35S. Immediately before homogenizing the harvested shoots, they were irradiated for 5 min with red light, incubated in darkness at 3°C for 5 min, and then irradiated for 5 min with far-red light. [35S]Phytochrome was extracted by three 10-s bursts in a small Waring Blender bowl into 10 ml extraction buffer [9 ml H2O + 1 ml 0.5 M Tris (pH 8.5 at 25°C) + 0.15 ml 2-mercaptoethanol] to which 50 μl (control phytochrome) or 25 μl (pelletable phytochrome) of 3 M Mops was added to maintain the pH of the extracts near 7.8. Extracts were filtered through Miracloth (Calbiochem), made 15 mm in CaCl2, incubated on ice for 10 min, and clarified by centrifugation at 12,000 rpm for 10 min in a Sorvall SS-34 rotor. Supernatants were made 5 mm in PO42− and 5 mm in EDTA and chromatographed through 8-ml bed volume brushite columns (9). Fractions eluted from the columns were monitored by liquid scintillation counting and it was assumed that phyto-

---

1 This work was supported by National Science Foundation Grant PCM77-23584.
2 Present address: Botany Department, University of Georgia, Athens, Ga. 30602.

3 Abbreviation: Mops, N-morpholino-3-propanesulfonic acid.
chrome was in the peak fractions of radioactivity eluted with 60 mM K-phosphate (pH 7.8), 14 mM 2-mercaptoethanol. These fractions were pooled and phytochrome was precipitated from them by 33% saturation ammonium sulfate. After centrifugation, pellets were dissolved in 100 mM Na-phosphate (pH 7.8). The redissolved pellets were clarified by centrifugation.

Phytochrome was further purified by the immunooaffinity procedure described in detail elsewhere (3). The peak of radioactivity eluted by 3 mM MgCl₂ from the immunooaffinity column was assumed to be phytochrome and was made 1 to 2% in sheep serum to provide nonradioactive carrier protein. Purified [³⁵S]phytochrome was precipitated by 50% saturation ammonium sulfate and dissolved in 550 μl (control phytochrome) or 300 μl (pelletable phytochrome) 100 mM Na-phosphate (pH 7.8). Radioimmunoassay of phytochrome content (4) in these final preparations indicated that about 65 μg control phytochrome at a specific radioactivity of 2 cpm/ng and about 8 μg pelletable phytochrome at a specific radioactivity of 6 cpm/ng were obtained. Purified [³⁵S]phytochrome samples were stored in 1-ml glass microcentrifuge tubes at -70°C and were clarified by centrifugation for 10 min at 13,000 rpm in a Sorvall HB-4 rotor before each use.

"Pelletable" phytochrome is referred to as such because, if it was extracted under the proper conditions, approximately 65% of the extracted phytochrome would have been expected to pellet (11; see ref. 10 for discussion).

Actinic Irradiation. Intact plant material was irradiated with red light as described elsewhere (16). Extracts, purified phytochrome, and shoots for extraction of pelletable [³⁵S]phytochrome were irradiated by filtering the output of a Unitron LKR microscope illuminator through either a Balzers B-40 663-nm interference filter or 3.2 mm of far-red-transmitting plastic (Plexiglas FRF-700). All irradiations were saturating.

Pelletability Assays. Shoots were extracted into 25 mM Mops-Tris, 20 mM MgCl₂, 14 mM 2-mercaptoethanol (pH 7.5), at a ratio of 4 ml buffer to 1 g tissue (11). Although this concentration of MgCl₂ induced nonspecific aggregation of subcellular debris, phytochrome pelletability of the type studied here is not a trivial co-precipitation of phytochrome with this nonspecifically aggregated subcellular debris (see ref. 10 for discussion). This phytochrome pelletability is dependent upon a Pfr-mediated process that obligatorily takes place in vivo (11, 12). Unfortunately, if the MgCl₂ levels used here had been low enough to prevent nonspecific aggregation of subcellular debris, it would not have been possible to assay for in vivo-induced phytochrome pelletability (13). Two different experimental protocols were used.

For some experiments, either about 300 ng control [³⁵S]phytochrome or about 80 ng pelletable [³⁵S]phytochrome was added to the extract buffer immediately prior to homogenization by three 10-s bursts at full speed in a VirTis homogenizer. Crude extracts were immediately filtered through Miracloth. Exactly 5 min after homogenization, measured 3-ml aliquots were centrifuged in 15-ml Cortex tubes for 5 min at 13,000 rpm in a Sorvall SS-34 rotor. Pellets were dissolved by heating to 100°C in 3 ml 25 mM Mops-Tris, 5 mM EDTA, 2% (w/v) SDS (pH 7.5). Radioactivity in pellets and supernatants then was determined by counting for 10 min in 15 ml scintillation cocktail (4) with a Searle Mark III liquid scintillation counter on a variable quench program.

For the other experiments, shoots were homogenized by three 10-s bursts in a small Waring Blender bowl. Crude extracts were filtered through Miracloth and centrifuged as above, except without special concern for the time between homogenization and centrifugation. Supernatants were assayed spectrophotometrically for phytochrome content. Pellets were resuspended in 3 ml extraction buffer/g tissue extracted with a loose-fitting glass-glass tissue homogenizer. An aliquot of the resuspended pellet was saved for spectrophotometric phytochrome assay, whereas 2-ml aliquots were added to 15-ml Corex centrifuge tubes. Labeled phytochrome, as in the other experiments, was added to appropriate Corex tubes. Samples were incubated on ice for the times indicated and then centrifuged for 5 min at 13,000 rpm in a Sorvall SS-34 rotor. Radioactive pellets were dissolved as above but in 2 ml buffer with SDS. Pellets and supernatants were then assayed for radioactivity as above. Phytochrome pelletability at the end of a 4-h incubation was determined spectrophotometrically in aliquots to which no [³⁵S]phytochrome was added.

SDS-Polyacrylamide Gel Electrophoresis. Samples were electrophoresed by a modification of the procedure of Weber and Osborn (17) as described earlier (4). Protein bands were stained with Coomassie brilliant blue R, A profiles were measured, and gels were sliced and counted for radioactivity as before (4). Photoreversibility Assays. Phytochrome photoreversibility was measured at 666 versus 727 nm with a custom-built, dual-wavelength spectrophotometer (6) that had an EMI 9658R photomultiplier and was adapted for automated measurement. Samples of 0.4 ml were mixed with 0.5 g CaCO₃ as a light-scattering agent (2) in a cuvette with vertical optical path and a cross-sectional area of 1 cm². Although Boisard et al. (1) have documented that use of CaCO₃ can lead to measurement artifacts when one is using cuvettes with a horizontal light path, the conditions used here did not lead to detectable artifacts (M.-M. Cordonnier and L. H. Pratt, unpublished observations).

RESULTS

The purity and undegraded state of the [³⁵S]phytochrome preparations are indicated by the predominant 120,000-dalton bands observed after SDS-polyacrylamide gel electrophoresis (Fig. 1, a and b).

When aliquots of these [³⁵S]phytochrome preparations are added to extraction buffer immediately before tissue homogenization, it is evident that a significant level (about 30%) of the exogenous phytochrome is pelletled (Table 1). It is also evident that the level of pelletability of exogenous [³⁵S]phytochrome is independent of whether the tissue was irradiated prior to extraction, whether the added [³⁵S]phytochrome was control or pelletable, or whether the [³⁵S]phytochrome was added as Pfr or Pfr. Parallel controls without [³⁵S]phytochrome documented that the levels of pelletability observed in homogenates of dark control and red-irradiated shoots were the same (about 7 and 65%, respectively) as reported earlier (11).

When aliquots of [³⁵S]phytochrome are added to resuspended pellets obtained from either red-irradiated or red/red-irradiated shoots and allowed to incubate for 4 h on ice, approximately 20% of the exogenous phytochrome was observed to pellet (Table II). Again, the level of pelletability observed was independent of tissue pretreatment or of the kind (control or pelletable) or form (Pfr or Pfr) of [³⁵S]phytochrome added. Controls indicated that 76% of the endogenous phytochrome pelletled during the first centrifugation, whereas 93 to 95% of the phytochrome in the resuspended pellets, to which [³⁵S]phytochrome was added, pelletled during the second centrifugation at the end of the 4-h incubation.

The time course for the association of exogenous phytochrome with pelletable material was measured by centrifuging resuspended pellets at different times after the addition of [³⁵S]phytochrome (Fig. 2). The level of pelletability of [³⁵S]phytochrome increases during the first 100 min of incubation and stabilizes thereafter. As much [³⁵S]phytochrome associates with resuspended pellets obtained from extracts of dark control shoots (Fig. 2), in which the level of pelletability of endogenous phytochrome is very low (data not shown, see ref. 11), as in the case of resuspended pellets of extracts obtained from irradiated shoots, in which the level of pelletability of endogenous phytochrome is high.

The specificity for the association of exogenous phytochrome with pelletable material was tested further by adding to resus-
pended pellets, which were obtained from extracts of dark control shoots, an excess quantity of nonradioactive, purified phytochrome (as Pr) immediately prior to the addition of [35S]phytochrome (control Pr) and the onset of a 4-h incubation. Without addition of the nonradioactive competitor, 17.1 ± 0.4% (mean ± se) of the [35S]phytochrome was pelletable. Addition of a 10-fold excess of nonradioactive competitor yielded 17.7 ± 0.4% pelletability of [35S]phytochrome, whereas a 100-fold excess (about equal to the endogenous level) gave 16.5 ± 0.7%.

The status of the [35S]phytochrome preparations was determined at the completion of the experiments by SDS-polyacrylamide gel electrophoresis (Fig. 1, c and d). Since each [35S]phytochrome preparation was kept in a single tube with aliquots taken for each experiment, the radioactivity profiles, which indicate little degradation of the protein moiety when compared to the initial profiles (Fig. 1, a and b), represent the maximum extent of degradation.

Another control was used to determine whether significant proteolysis of [35S]phytochrome occurred during the 4-h incubation used in the exchange experiments. Approximately, 3,000 cpn of a mixture of control and pelletable [35S]phytochrome was added to a resuspended pellet as in the other exchange experiments. After 4-h incubation on ice, the sample was centrifuged in the usual way. [35S]Phytochrome in the supernatant from this second centrifugation was immunoprecipitated by anti-oat-phytochrome serum. The immunoprecipitate, after collection by centrifugation, was washed by resuspension in 0.13 M NaCl, 1% (v/v) Triton X-100, and 1% (w/v) sodium desoxycholate followed by centrifugation over a 200-µl cushion of 5% sucrose in a 1-ml conical tip centrifuge tube. The washed immunoprecipitate was resuspended in 0.145 M NaCl and again collected by centrifugation. It then was dissolved and electrophoresed by the standard procedure. Only little proteolysis was evident (Fig. 3).

### Table 1. Pelletability of [35S]Phytochrome Added to Extraction Medium Used for Homogenization of Oat Shoots

<table>
<thead>
<tr>
<th>Oat Shoots</th>
<th>[35S]Phytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Form</td>
</tr>
<tr>
<td>Dark control</td>
<td>Control Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
<tr>
<td></td>
<td>Pelletable Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
<tr>
<td>Red-irradiated</td>
<td>Control Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
<tr>
<td></td>
<td>Pelletable Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
</tbody>
</table>

### Table 2. Pelletability of [35S]Phytochrome Added to Resuspended Pellets and Incubated 4 h at 0°C

<table>
<thead>
<tr>
<th>Oat Shoots</th>
<th>[35S]Phytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Form</td>
</tr>
<tr>
<td>Red-irradiated</td>
<td>Control Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
<tr>
<td></td>
<td>Pelletable Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
<tr>
<td>Red-, far-red-irradiated</td>
<td>Control Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
<tr>
<td></td>
<td>Pelletable Pr</td>
</tr>
<tr>
<td></td>
<td>Pfr</td>
</tr>
</tbody>
</table>
FIG. 2. Time course for the binding of phytochrome to aliquots of resuspended pellets obtained from crude extracts of either dark control (O) or red-irradiated (●) oat shoots. The percentage of added [35S]phytochrome found in the pellet was determined by centrifugation at the indicated time after addition of pelletable [35S]phytochrome and by dividing the radioactivity recovered in the pellet by that recovered in the pellet and supernatant combined. Mean ± se is shown for the 4-h points. The data are from two independent experiments.

FIG. 3. A and radioactivity profiles of an SDS-polyacrylamide gel after electrophoresis of an immunoprecipitate obtained as described in the text. The highest peak in the A profile is the heavy chain of immunoglobulin G; the peak to the right is the light chain of immunoglobulin G; the peak to the left is the undegraded phytochrome monomer. Origin is to the left. A standard curve obtained with replicate gels as described in the legend to Fig. 1 is superimposed. The gel beyond the bromophenol blue band was discarded.

DISCUSSION

Undegraded phytochrome of both high purity and relatively high specific activity is required to determine whether exogenous phytochrome will bind in a specific fashion to pelletable material in crude plant extracts. It is apparent that the [35S]phytochrome prepared for these experiments meets both criteria (Fig. 1). In addition, it is important that proteolysis, which is a potentially serious problem (see ref. 10 for review), does not occur to a significant extent during the course of the experiments. Appropriate controls (Figs. 1, c and d, and 3) indicate that proteolysis is not a problem here.

In searching for possible in vitro binding, it is crucial that the exogenous phytochrome preparations used not be significantly modified during preparation. Although it can never be shown conclusively that purified phytochrome preparations, such as those used here, contain phytochrome indistinguishable from that present in a crude extract, there is reason to believe that these [35S]-phytochrome preparations have not been significantly modified in vitro. First, as shown elsewhere (3, 5), immunoaffinity-purified phytochrome appears indistinguishable from conventionally purifi ed undegraded phytochrome. Second, the immunoaffinity purification utilizes relatively mild conditions and requires less than 10 h for completion beginning with intact shoots. Thus, time during which in vitro modifications might occur has been minimized. Third, phytochrome was labeled by substitution during synthesis, rather than by modification in vitro after synthesis. Fourth, no loss of [35S]phytochrome was evident during the frequent centrifugations prior to each experiment nor was any substantial degradation evident upon completion of the experiments (Figs. 1, c and d, and 3), both of which might have been expected if [35S]phytochrome had been denatured. It is evident that a greater percentage of [35S]phytochrome pellets than in the case of endogenous phytochrome, which indicates that the purified phytochrome might be different in some unknown way. If this is the case, then appropriate caution must be used in the interpretation of all in vitro binding experiments.

Direct assays designed to detect in vitro binding of phytochrome as a function of a prior light treatment given to intact shoots (Table I) indicated that none was observed. Assuming that the [35S]-phytochrome preparations were not substantially modified in vitro during purification, it is possible to conclude that phytochrome pelletability induced by irradiation in vivo does not occur by binding in vitro in response (a) to a change in some component of the cell other than phytochrome or (b) to a change in phytochrome itself (as might be anticipated to have occurred with pelletable [35S]phytochrome) unless the change in phytochrome leading to in vitro binding were transient, being lost by the [35S]phytochrome soon after tissue homogenization. Inasmuch as the amount of [35S]phytochrome added was less than about 0.1% of the endogenous phytochrome level, it is evident that the lack of in vitro binding observed here does not result from a hypothetical limitation in the number of "binding sites."

A second approach to determine whether in vivo-induced binding can occur in vitro is to determine whether exogenous phytochrome will exchange with phytochrome that is already bound. The experiments presented here (Table II; Fig. 2) indicate that this is not the case. Although a relatively high percentage of the exogenous phytochrome does bind in vitro, the binding exhibits characteristics of nonspecificity. First, even a large excess of nonradioactive exogenously added phytochrome did not displace [35S]phytochrome. Second, the binding level obtained was independent of the pretreatment of the tissue used and of the form of [35S]phytochrome added. Third, even when the exogenous phytochrome was purified from that which was potentially pelletable (that is, would have pelleted given the appropriate extraction conditions, see ref. 11), it does not exchange in a specific fashion with endogenously bound Pr or Pr(II; Fig. 2).

The data reported both here and in the accompanying paper by Quail and Briggs (14) appear to support the hypothesis that phytochrome that is induced to pellet by irradiation in vivo is already bound prior to tissue homogenization. As Quail and Briggs (14) also emphasize, these experiments do not completely eliminate the possibility that in vivo-induced phytochrome pelletability arises from an in vitro binding event. The possibility that the [35S]phytochrome had been modified after extraction remains. Even if the [35S]phytochrome had not been modified, it is still possible that in vitro binding occurs in response to a transient change in phytochrome that permits binding only within the first few min after extraction (12). If this transient change then disappeared, binding could no longer occur in vitro even though all other conditions remained suitable. Such a transient change would not be expected to persist in the [35S]phytochrome preparations. Finally, even if binding were to occur in vivo, neither these nor any other data thus far reported permit the conclusion that binding is to a membrane. For example, self-aggregation of phytochrome (18) and co-precipitation with other subcellular debris remains a credible possibility.
LITERATURE CITED