Plant Physiol. (1980) 66, 696–703
0032-0889/80/66/0696/08/$00.50/0

Further Characterization of the *in Vitro* Binding of Phytochrome To a Membrane Fraction Enriched for Mitochondria[1]

Received for publication September 28, 1979 and in revised form May 20, 1980

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ABSTRACT

This study employs ^{125}I-labeled phytochrome (^{125}I-P) from oats to quantitate the binding of phytochrome to a membrane fraction from oats that is highly enriched for mitochondria, and it examines several parameters that influence this attachment. The binding of ^{125}I-Pfr to the mitochondrial fraction of unirradiated oat seedlings is significantly higher than that of ^{125}I-Pr. However, ^{125}I-Pfr and ^{125}I-Pr bind in equal quantities to mitochondrial preparations isolated from light-exposed seedlings. Maximum ^{125}I-Pfr binding to membranes from light-exposed plants occurs within 30 seconds and is optimized in a reaction buffer containing 5 millimolar $MgCl_2$ at pH 6.8. Scatchard plots of the binding data for Pfr indicate a single high-affinity site with an affinity constant of 1.79×10^{11} per molar. When optimal binding conditions are used, over 20% of the ^{125}I-P added is bound and a stoichiometry of about 100 molecules per mitochondrion is attained. When the specificity of binding is tested using competition experiments with a 15-fold excess of unlabeled phytochrome, ^{125}I-Pfr shows no specific binding to rat liver mitochondria.

An early response to the photoactivation of phytochrome in plant cells is a change in the ion permeability and electrical potential of membranes in the affected cells (16, 23, 30). One inference from these findings is that phytochrome physically interacts with plant cell membranes en route to inducing changes in the growth patterns of plants. Support for this hypothesis comes from a wide range of studies, as discussed in several recent reviews (15, 19, 26). As an extension of these studies, we developed a method to quantitate the amount of phytochrome that binds to purified membranes when it is reacted with these membranes *in vitro* (8). The method, using ^{125}I-P[2], allowed us to determine whether this binding met certain minimal criteria for the specific interaction of a ligand with a receptor site, such as those discussed by Cuatrecasas (4). Our initial results suggested that there were a saturable number of high affinity sites on purified oat mitochondria for ^{125}I-Pfr. However, the preliminary nature of these data raised certain questions about their interpretation, as pointed out in recent papers (22, 31). Also, confidence in the estimated number of binding sites per mitochrondrion was limited by the fact that these initial experiments made no attempt to optimize the binding in terms of pH, divalent cation concentration, or time of reaction. The experiments reported here examine these and other parameters of the binding and provide control data to further support the hypothesis that there are specific binding sites on oat mitochondria for oat phytochrome.

[1] This research was supported by National Science Foundation Grant PCM 78-08823 to S.J.R. and an Andrew W. Mellon Predoctoral Fellowship to T.E.C.

[2] Abbreviation: ^{125}I-P, ^{125}I-labeled phytochrome.

MATERIALS AND METHODS

Phytochrome Isolation and Iodination. The phytochrome used in these experiments was purified from 4-day-old etiolated oat seedlings by the method of Roux *et al.* (25). All phytochrome used was fully photoreversible, exhibited a peak *A* for Pr at 667 nm, and had a mol wt of 120,000 on SDS gels. For iodination, 80 to 95% pure samples were isotopically labeled by the method of Thorell and Johansson (27), modified as described by Georgevich *et al.* (8). Iodinated samples exhibited normal spectra (8).

Mitochondria Isolation. Mitochondria were isolated from 4-day-old etiolated seedlings of oats (*Avena sativa* L., cv. Garry) by the procedure of Douce *et al.* (6), except that 10 mM $NaHSO_3$ was substituted for cysteine in the extraction buffer. Approximately 1 kg of 4- to 5-cm long oat shoots was harvested after the plants had been chilled to about 4 C. Mitochondria isolated from unirradiated plants were termed "dark" mitochondria. "Light" mitochondria were isolated from etiolated oat shoots that were exposed to 15 min of white fluorescent light (irradiance of 0.1 mw cm^{-2}) while on ice after harvest and prior to homogenization. In both cases, all subsequent manipulations were carried out in green safelight at 3–5 C. The purified mitochondria were determined to be intact and functioning normally by electron microscopy and biochemical tests described by Douce *et al.* (6).

Mitochondria were isolated from rat liver by the method of Parsons *et al.* (17).

Morphometric Estimation of the Purity of the Isolated Mitochondria. The mitochondria were prepared for electron microscopy by the method described in Douce *et al.* (6). During the postfix in OsO_4 the mitochondrial pellet broke into five pieces, each of which was embedded separately in araldite after the usual intermediate processing steps. Several thin sections of each of these pieces were then cut, picked up on on copper grids, poststained in lead citrate as described by Venable and Coggeshell (28), then examined and photographed at random magnifications ranging from 3,000 to 10,000×. Photographic prints (20 × 25 cm) of sections from each piece of the original pellet were used for the morphometric analysis, following the general principles outlined by Baudhin (1) and Weibel (29). Each print contained between 250 and 1,000 mitochondria in the field.

Experimental Manipulation of the Mitochondria. Phytochrome binding experiments were conducted in 400 μl Beckman microfuge tubes. The tubes contained, on the bottom, 100 μl of 1.0 M sucrose, then 50 μl of silicon oil (a 3-mm oil layer), and the final top layer of 200 μl of the reaction medium. The general reaction medium,

medium R, consisted of 0.3 M mannitol, 10 mM Hepes, 0.1% BSA, and 10 mM KCl, at a pH adjusted for each experiment. The medium was supplemented with $MgCl_2$ or K_2HPO_4, depending on the type of experiment being conducted. The binding reaction was initiated by adding mitochondria to the centrifuge tube which already contained the components required for the binding experiment (^{125}I-P). After the requirement time period, the mitochondria were separated from the reaction medium by centrifugation through the silicon oil layer (11). Immediately after the centrifugation, the tubes were frozen at −70 C. While still frozen, the tubes were sliced with a razor through the center of the silicon oil layer. Gamma radiation in the top portion of the tube was counted to give the amount of free ^{125}I-P, and radiation in the bottom portion of the tube, which contained the mitochondrial pellet, was counted to determine bound ^{125}I-P. The mitochondrial pellets were then solubilized in 75 μl of 5% SDS and 10 mM EDTA. The exact method of solubilization was as follows: (*a*) thorough removal of the 1.0 M sucrose layer which was above the mitochondrial pellet; (*b*) solubilization of the remaining mitochondrial pellet in 25 μl of 5% SDS and 10 mM EDTA; and (*c*) washing of the tip of the centrifuge tube two more times with 25 μl of 5% SDS and 10 mM EDTA. This material was then combined to give a total volume of 75 μl, and counted again. Portions of the solubilized mitochondrial pellet were used for analysis on SDS gels and protein determinations.

For competition experiments, mitochondria were incubated with ^{125}I-P and unlabeled phytochrome. Unless otherwise noted, a 15-fold excess of unlabeled phytochrome was used. The volumes of the reaction medium were adjusted so that the concentration of ^{125}I-P remained constant, and the experiments were then conducted as described in the previous paragraph.

Radioactivity Measurements. Radioactivity of samples containing ^{125}I-P was measured with a Beckman 4000 gamma counter. The efficiency of counting was approximately 60%. Mol wt characterization of bound radioactive species in the pellet was determined by exposing dried SDS polyacrylamide slab gels on Kodak SB5 x-ray film for 2 to 7 days. Mol wt was calculated using R_F values and comparing these values to standards.

SDS Gel Analysis and Protein Determinations. Two-mm-thick polyacrylamide slab gels with 7.5% resolving and 3% stacking gels were cast and run as described by Georgevich *et al.* (8).

Protein was measured by the method of Lowry *et al.* (13), modified as described by Georgevich *et al.* (8).

Analysis of Immunoprecipitates of ^{125}I-Phytochrome. The goat anti-phytochrome IgG used in this experiment was obtained from Cappell Laboratories as a gift from Dr. F. Mumford of the Central Research Department, E.I. du Pont Co. The specificity and titer of the antibody were determined using double diffusion immunoprecipitation on Ouchterlony plates and by radioimmune assays using ^{125}I-P.

To prepare the immunoprecipitates analyzed in Figure 1, ^{125}I-P was incubated with antiphytochrome IgG in 400 μl polyethylene centrifuge tubes at 4 C for 24 h on a shaker table. The precipitates were pelleted in a Beckman Microfuge for 10 min at 4 C, then the Microfuge tubes were frozen and the pellet was removed by slicing it off just below the pellet-supernatant interface. The immunoprecipitate was boiled in sample buffer and analyzed on SDS polyacrylamide gels as described by Georgevich *et al.* (8).

Operational Definitions: Specific and Nonspecific Binding; Purified Mitochondria. For the purposes of discussion in this paper, we will define specific binding of phytochrome as that portion of the total ^{125}I-P bound that can be competitively inhibited from binding by a 15-fold excess of unlabeled phytochrome reacted with the membranes simultaneously with ^{125}I-P. Accordingly, nonspecific binding is defined as the portion of the total ^{125}I-P bound that cannot be competitively inhibited from binding by the 15-fold excess of unlabeled phytochrome. The assumption is made

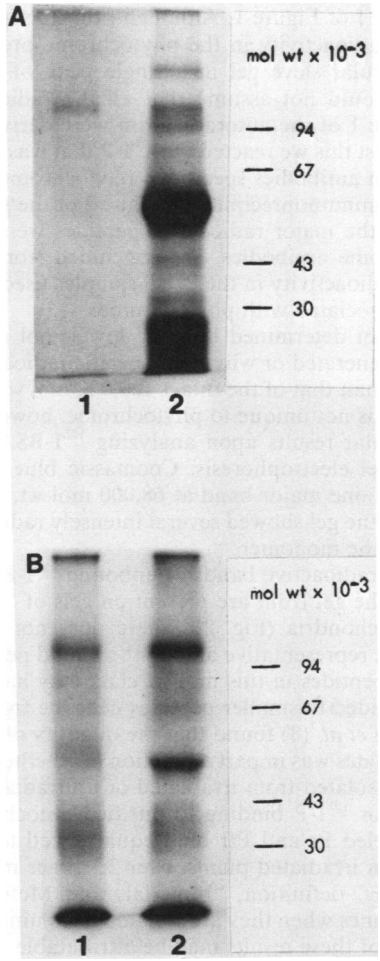


FIG. 1. A: Coomassie blue-stained slab gel showing the pattern of protein bands produced after SDS gel electrophoresis of: lane 1, 10 μg ^{125}I-P; lane 2, proteins associated with the immunoprecipitate of 20 μg ^{125}I-P from the same phytochrome preparation used for lane 1. B: Autoradiogram of the gel shown in A.

(and supported by the Scatchard plot of Fig. 9b), that ^{125}I-Pfr binds with a high affinity to its attachment site, and so for Figure 6b, specific binding is defined as that portion of the total bound which is not readily washed off the membranes, and nonspecific binding is that portion which is readily washed off.

Although we argue under "Discussion" that mitochondria are certainly the most likely site of the high affinity binding of phytochrome, there is no direct evidence given in the paper that this is so. Thus, the term "purified mitochondria" as used here refers to a preparation of membranes that is greatly enriched for mitochondria; and "binding to purified mitochondria" strictly means binding to sites located in the preparation of purified mitochondria.

RESULTS AND DISCUSSION

The point of departure for the experiments reported here was the earlier report of Georgevich *et al.* (8) on the binding of ^{125}I-P to cell particulates and to purified mitochondria. Data in that paper indicated that after electrophoresis on SDS polyacrylamide gels, the 120,000 mol wt band of ^{125}I-P represented more than 90% of the protein content of the gel, as judged by Coomassie blue stain, but only about 30% of the total radioactivity as judged by autoradiography. The phytochrome used for the experiments reported here gave similar results after analysis on SDS gels, as

shown in lane 1 of Figure 1. Although prior to separation of SDS gels, all the radioactivity in the phytochrome preparation eluted from a molecular sieve gel in a single peak of photoreversible protein, we could not assume that all the radioactive peptides shown in lane 1 of the autoradiogram were derived from phytochrome. To test this we reacted the ^{125}I-P that was electrophoresed on lane 1 with antibodies specific to phytochrome, then analyzed the resultant immunoprecipitate on lane 2 of the gel. As shown in Figure 1, all the major radioactive peptides were recognized by the phytochrome antibodies. We concluded from this that over 90% of the radioactivity in the ^{125}I-P samples used for the binding assays was associated with phytochrome.

We have not determined how the lower mol wt phytochrome bands were generated or why their specific radioactivity is apparently higher than that of the intact 120,000 mol wt monomer. The phenomenon is not unique to phytochrome, however, as we have observed similar results upon analyzing ^{125}I-BSA on SDS polyacrylamide gel electrophoresis: Coomassie blue stain of the gel revealed only one major band at 68,000 mol wt, whereas autoradiography of the gel showed several intensely radioactive peptides smaller than the monomer.

The major radioactive bands of unbound ^{125}I-P at 120,000 mol wt and near the gel front are present on gels of ^{125}I-Pfr bound to purified mitochondria (Fig. 2). There does not appear to be a 60,000 mol wt representative among the bound peptides, although some of the peptides in this mol wt class may have been proteolytically degraded to smaller peptides near the front.

Georgevich *et al.* (8) found that the quantity of ^{125}I-P bound to crude particulates was in part a function of whether the particulate fraction was isolated from irradiated or unirradiated plants. This is also true for ^{125}I-P binding to purified mitochondria (Fig. 3). Whereas labeled Pr and Pfr bind equally well to purified mitochondria from irradiated plants, over 2.5 times more Pfr than Pr specifically (*cf.* definition, "Materials and Methods") binds to these membranes when they are isolated from unirradiated plants. At least part of these results may be attributable to differences in

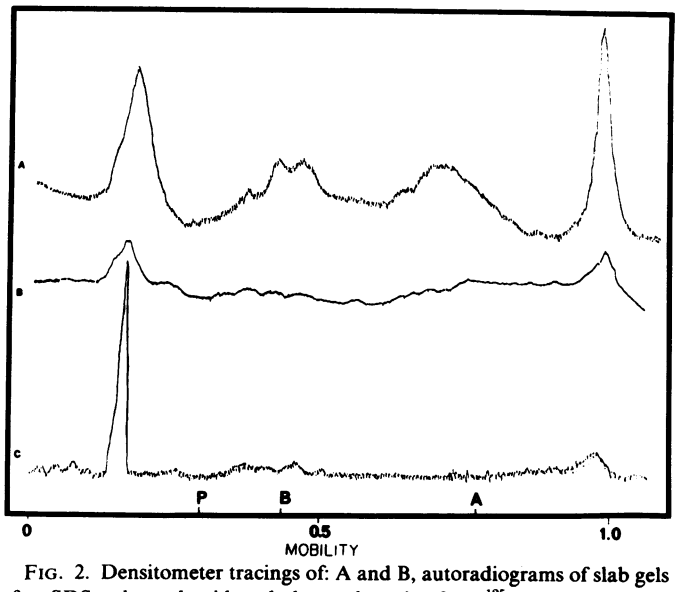


FIG. 2. Densitometer tracings of: A and B, autoradiograms of slab gels after SDS polyacrylamide gel electrophoresis of (a) ^{125}I-P (free) and (B) labeled protein from the same sample of ^{125}I-P as used in A, only after it was bound to sites in a preparation of purified mitochondria and then pelleted through a silicon oil layer as described under "Materials and Methods"; C, the Coomassie blue staining pattern of proteins on the same gel as analyzed in A. Molecular weight markers are: P, phosphorylase, 94,000 mol wt; B, BSA, 67,000 mol wt; A, carbonic anhydrase, 30,000 mol wt.

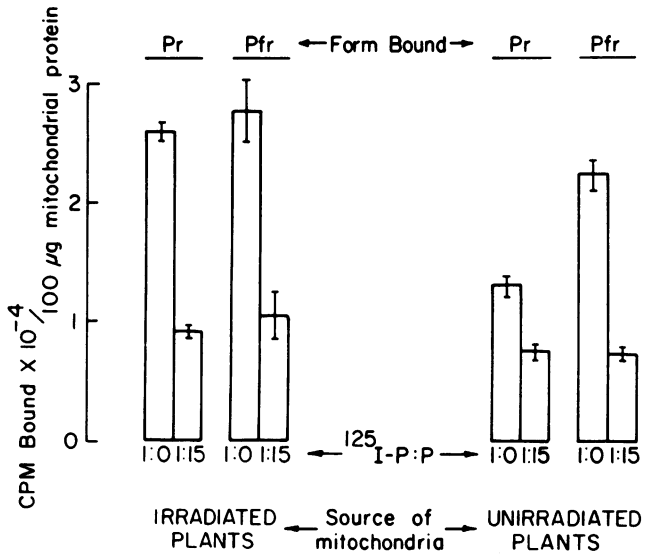


FIG. 3. Comparison of phytochrome binding to mitochondria from irradiated and unirradiated plants. The reaction medium for these experiments included 2.5 mM $MgCl_2$ and 5.0 mM K_2HPO_4 in medium R at pH 7.4. The specific activity of the ^{125}I-P used was 65 cpm/ng. The harvest of dark-grown shoots, 1.5 kg, was divided in half, one-half placed in a beaker on ice and irradiated with 15 min of white fluorescent light, and the other half kept dark in a cold room at 2 C. After the light exposure, tissue homogenization and all subsequent steps were carried out under a green safelight. The mitochondria from the irradiated and unirradiated tissue were isolated in parallel. For the binding experiments, the mitochondria were added to the reaction mixture and incubated for 6 min at 22 C. Vertical bars denote 1 SD.

what experimental conditions optimize specific binding of phytochrome to light *versus* dark mitochondria, especially if the *in vivo* irradiation induced some alteration in the composition or arrangement of the membranes in the mitochondrial fraction. Such an alteration is not unlikely (10). We have no direct information on what this alteration might be, although R induced binding of phytochrome to mitochondria *in vivo*, as reported for pea (7), is a potential candidate. *In vivo* the binding of Pfr necessarily would be to irradiated membranes. This fact plus our observation that exogenous phytochrome alters mitochondrial NADH-dehydrogenase activity only in mitochondria from irradiated plants (3), led us to choose these mitochondria over those from unirradiated sources for the detailed characterization of the binding described below.

Figure 4 documents the Mg^{2+} requirement for specific binding of ^{125}I-Pfr to purified mitochondria at pH 7.4. At zero mM $MgCl_2$ there is no specific binding of Pfr but significant nonspecific binding. Both specific and nonspecific binding rise with increasing $MgCl_2$, but the specific rises more rapidly, so that the maximum amount of specific binding is achieved at 5 mM. In data provided elsewhere (2), 10 mM, 15 mM, and 20 mM $MgCl_2$ do not increase the total ^{125}I-P bound but they do significantly increase the level of nonspecific binding. Thus, 5 mM $MgCl_2$ supports the highest level of specific binding over the range of concentrations tested.

Figure 5 tests the pH dependence of the binding, and the data there show optimal specific binding at about pH 6.8. The level of nonspecific binding seems to be relatively unaffected by pH. The isoelectric point of undegraded phytochrome is between about pH 5.9 and 6.2 (19); and it is fully photoreversible and soluble at pH 6.8, so artifacts arising from pH-induced insolubility are not likely to be involved in the *in vitro* association of phytochrome with mitochondria.

Table I measures the level of specific binding as a function of

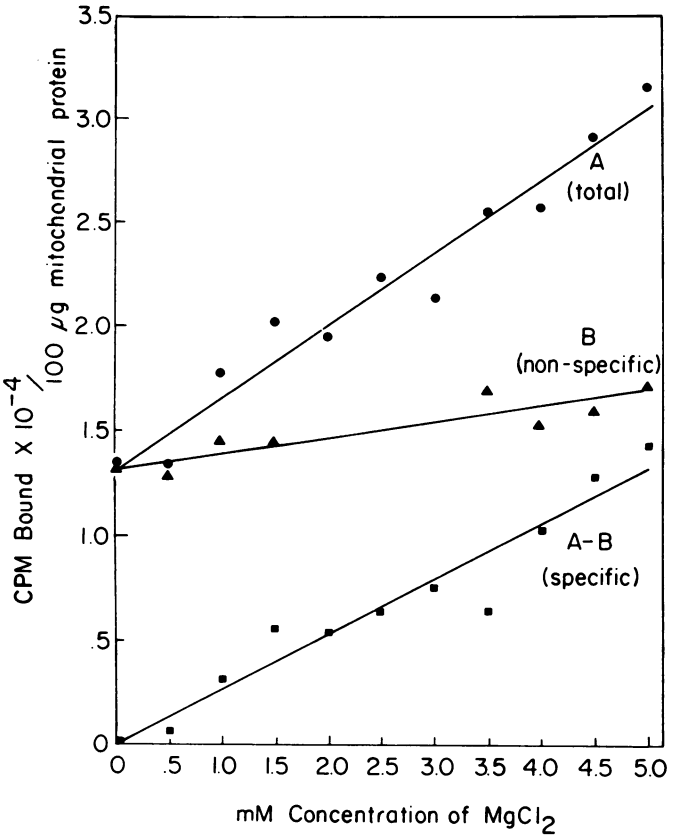


FIG. 4. The effect of Mg^{2+} on the binding of labeled phytochrome to purified mitochondria. The reaction medium for these experiments was medium R at pH 7.4 with or without $MgCl_2$ as indicated in the abscissa of the Figure. The reaction temperature was 25 C, the specific radioactivity of the ^{125}I-Pfr used was 450 cpm/ng, and the amount of mitochondria in each reaction was 114 ± 23 µg mitochondrial protein.

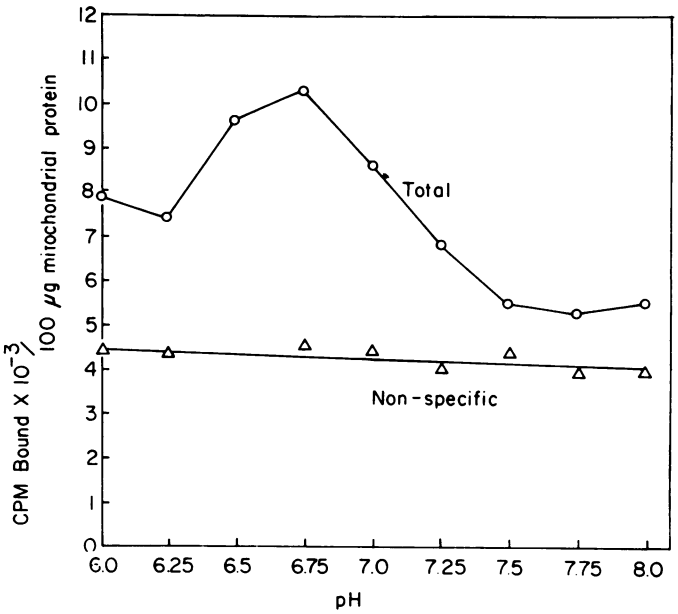


FIG. 5. The effect of pH on the binding of labeled phytochrome to purified mitochondria. The reaction medium for these experiments was medium R plus 5 mM $MgCl_2$ at pH values indicated in the abscissa. The reaction temperature was 25 C, the specific radioactivity of the ^{125}I-Pfr used was 170 cpm/ng, and the amount of mitochondria in each reaction was 95 ± 10 µg mitochondrial protein.

Table I. *Effect of Reaction Time on the Quantity of ^{125}I-Pfr Bound*

The reaction medium for these experiments was medium R plus 2.5 mM $MgCl_2$ and 5 mM K_2HPO_4 at pH 7.4 and 25 C. The ^{125}I-Pfr used had a specific activity of 482 cpm/ng, and for each experiment 95 ± 17 µg mitochondrial membranes (protein) were used.

Reaction Time	Specific cpm of ^{125}I-Pfr Bound/100 µg Mitochondrial Protein
min	
0.5	6,800
6	6,250
10	6,250
14	6,750
18	6,800

time starting at about 30 s and testing points every 4 or 5 min thereafter through 18 min. The results indicate that this binding is exceedingly rapid, apparently saturating at 30 s or less. The data in Table 1 and in Figures 3 to 5 were obtained to discover optimal conditions of [Mg^{2+}], pH, and reaction time, but were themselves not obtained under these conditions. All of the data described below were obtained from experiments carried out for 6 min, in a reaction medium that contained 5 mM $MgCl_2$ at pH 6.8. These reaction conditions are only optimal for those three parameters, and so the levels of specific binding obtained could presumably be raised further by other adjustments of the reaction medium. The rapidity of binding, the pH optimum, and the $MgCl_2$ optimum are remarkably similar to the values for these parameters found by workers studying the copelletability of phytochrome with cell particulates following *in vivo* irradiations (20, 22).

Before attempting to quantitate and obtain binding constants for the specific binding of ^{125}I-P to purified mitochondria under the newly established reaction conditions, we ran certain controls to test the validity of our competition experiments. Figure 6a shows that a 10- to 15-fold excess of unlabeled phytochrome provides a level of competitive inhibition that is adequate and not significantly increased by higher excesses of unlabeled phytochrome. In related experiments described elsewhere (2), neither ovalbumin, BSA, or hemoglobin competitively inhibited phytochrome binding at all, even when added in a 30× molar excess over ^{125}I-P.

The data in Figure 6b were obtained using the same preparations of ^{125}I-P and purified mitochondria as used for Figure 6a. They show that about 50% of the total ^{125}I-P bound can be readily removed in a single wash, indicating that it has a low affinity for its binding site(s). The remaining ^{125}I-P bound appears to have a high affinity for its site(s), since subsequent washes remove very little more of it. The levels of specifically bound phytochrome estimated by the two quite different methods in Figure 6 (17,000 *versus* 14,000 cpm) agree within the standard deviations obtained.

Figure 7 examined what amount of nonspecific binding could be ascribed to some volume of the reaction mixture that was trapped in spaces between the sedimenting membranes and carried into the pellet. The data indicate that for reaction mixtures containing more than about 10 µg mitochondrial protein, the volume trapped increases linearly with increasing concentrations of mitochondria, averaging about 1.3 µl per 100 µg of mitochondrial protein. Thus, under the conditions of the plant mitochondria experiments described in this paper, "trapped volume" cpm would never amount to enough in any experiment to contribute significantly to the binding and competition data.

In our first report of ^{125}I-P binding to purified mitochondria, the pH, [$MgCl_2$], and reaction time were not optimized; consequently, 1% or less of the total ^{125}I-P reacted with the mitochondria bound to them (8). Figure 8 indicates that when the improved reaction conditions are used and enough mitochondrial sites are

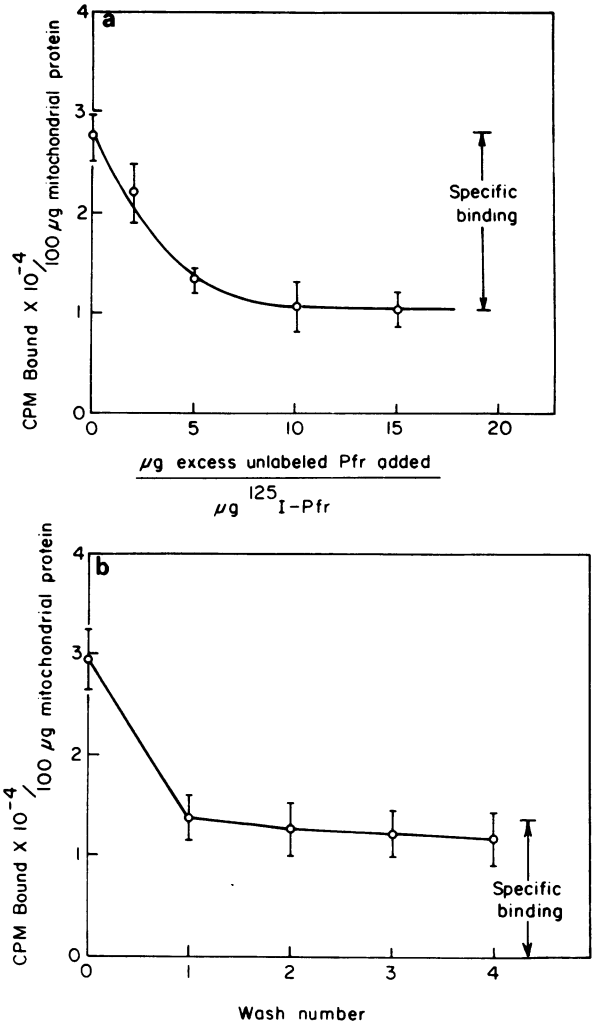


FIG. 6. Quantity of nonspecific binding as tested by (a) competition and (b) by wash experiments. a: The reaction medium for these experiments was medium R plus 5 mM $MgCl_2$ at pH 6.8. The reaction temperature was 24 C, the specific radioactivity of the ^{125}I-P used was 200 cpm/ng, and the amount of mitochondria in each reaction was 90 ± 8 μg mitochondrial protein. One μg of ^{125}I-Pfr was used for all the experiments, and the amount of unlabeled phytochrome added was varied from 0 μg to 15 μg. Vertical bars denote 1 SD. b: The reaction medium, reaction temperature, and specific radioactivity of the ^{125}I-Pfr used for these experiments were the same as for Figure 6a. One μg ^{125}I-Pfr was added to approximately 200 μg mitochondrial protein and the binding reaction was carried out as outlined under "Materials and Methods." After the mitochondria had been centrifuged through the silicon oil layer, separating bound from free ^{125}I-Pfr, the centrifuge tube was sliced at the oil layer. Radioactivity in the bottom portion of the tube containing the mitochondrial pellet was counted to determine the amount of bound ^{125}I-Pfr without any wash. After counting, the remaining silicon oil and 1.0 M sucrose were removed from above the mitochondrial pellet. The pellet was resuspended in 200 μl of medium R plus 5.0 mM $MgCl_2$, and the solution was then centrifuged for 15 min. Next, the supernatant from this centrifugation was removed and measured for radioactivity, as was the mitochondrial pellet that remained. This gave the amount of ^{125}I-Pfr that remained bound after the first wash. These procedures were repeated for each additional wash. Vertical bars denote 1 SD.

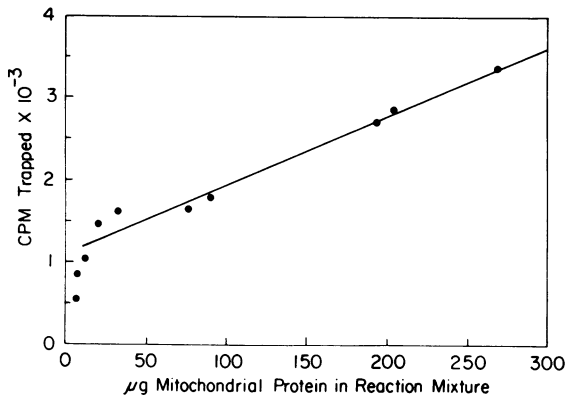


FIG. 7. Volume of reaction medium trapped in spaces between sedimenting membranes *versus* quantity of membranes sedimented. The reaction medium for these experiments was medium R plus 5 mM $MgCl_2$ at pH 6.8. $Na^{125}I$ was substituted for ^{125}I-P and maintained at 2.7×10^5 cpm per 200 μl of reaction medium. The amount of mitochondrial membrane (protein) present was varied from 0 μg to 270 μg as indicated on the abscissa. The reaction temperature was 25 C.

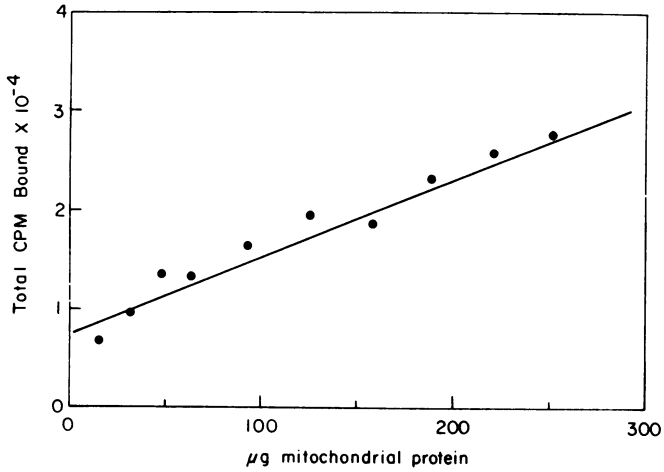


FIG. 8. Amount of ^{125}I-Pfr bound to purified mitochondria as the amount of mitochondria is increased while keeping the level of ^{125}I-Pfr constant at 1 μg (1.4×10^5 cpm). The reaction medium for these experiments was medium R plus 5 mM $MgCl_2$ at pH 6.8, and the reaction temperature was 25 C.

provided, over 20% of the ^{125}I-Pfr added will bind to the mitochondria. Since even the improved reaction parameters are not optimal in an absolute sense, this level of binding is significant and begins to approach levels reported in the pelletability studies (20).

To obtain a binding constant for the association of ^{125}I-P with mitochondria, we derived Scatchard plots (Fig. 9b and 10b) from the saturation binding curves of Figures 9a (Pfr) and 10a (Pr). The fact that Figure 9b gives a linear plot indicates that there is a single class of sites with the same relative affinity for Pfr; and, from the slope of the line, that affinity can be calculated to be very high: $Ka = 1.7 \times 10^{11}$ M^{-1} (5). The saturation binding curve of Pr, in contrast, is significantly different (Fig. 10a), and produces a nonlinear Scatchard plot, or, at best, a nearly horizontal line, indicating low-affinity binding (Fig. 10b). The data in Figures 9 and 10 were obtained under conditions that maximize specific binding, and they show that about 2.5 × more Pfr than Pr binds specifically to the "light" mitochondria. The data in Figure 3 showed about equal specific binding of Pfr and Pr to "light" mitochondria, but they were obtained under conditions that gave much lower levels of specific binding, so they were biased differently. Figures 9 and 10 allow a recalculation of the stoichiometry of the binding, revised upward from the initial estimate (8). Using

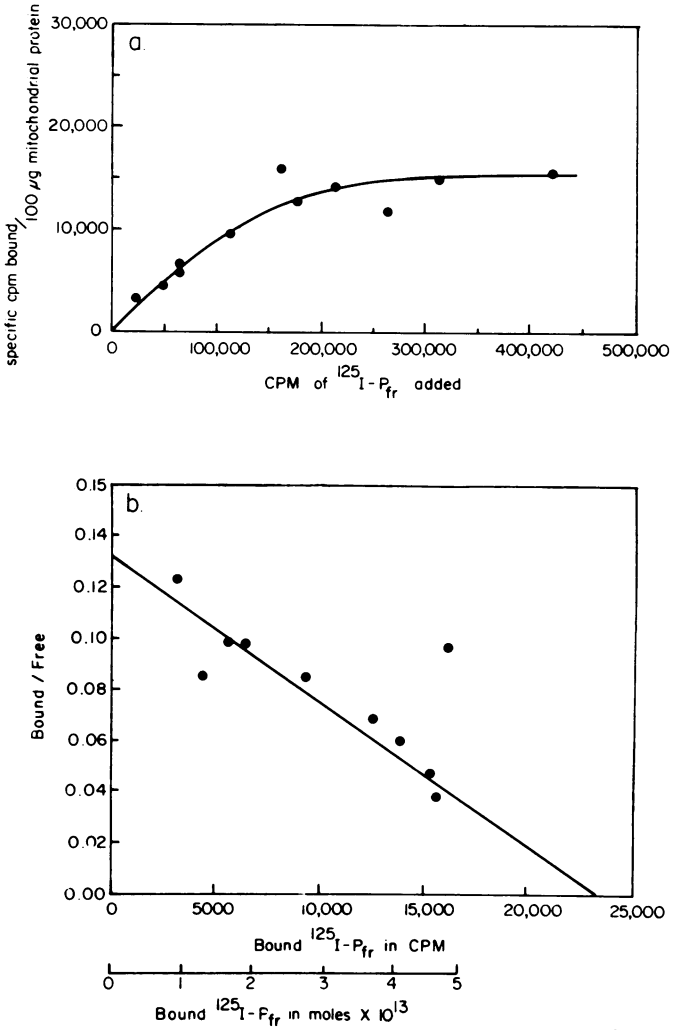


FIG. 9. Saturation binding curve (a), and Scatchard plot (b) for ^{125}I-Pfr binding to purified mitochondria. The reaction medium for these experiments was medium R plus 5 mM $MgCl_2$ at pH 6.8, and the reaction temperature was 23 C. The mitochondrial concentration was held constant at 63 ± 9 μg mitochondrial protein for each experiment. The specific radioactivity of the ^{125}I-P used was 142 cpm/ng.

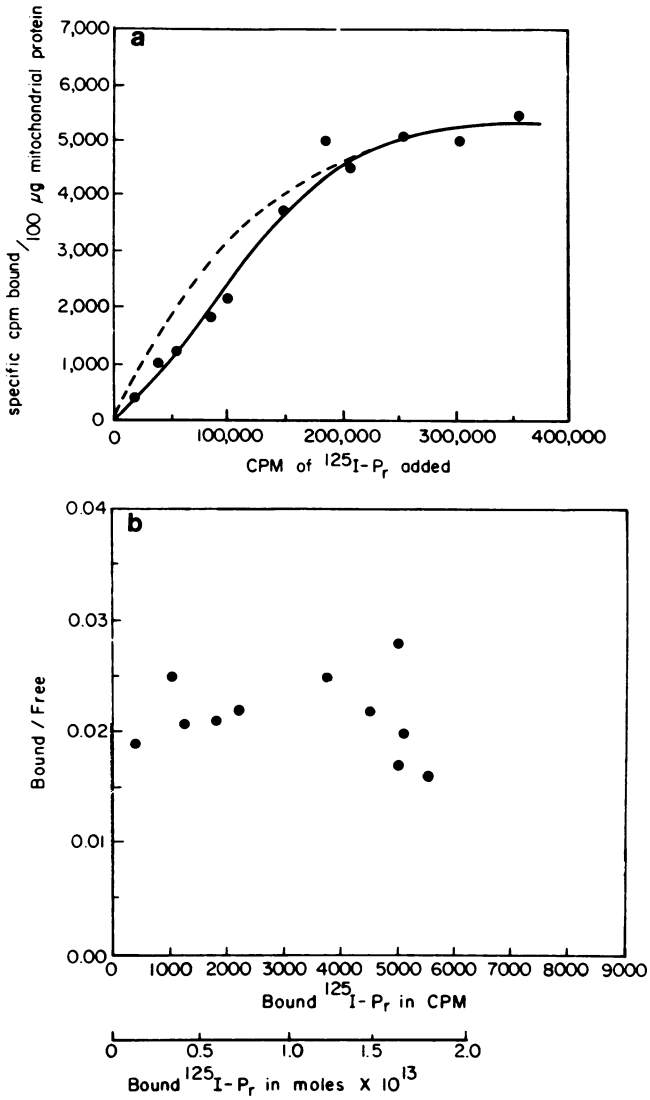


FIG. 10. Saturation binding curve (a), and Scatchard plot (b) for ^{125}I-Pr binding to purified mitochondria. The reaction medium for these experiments was medium R plus 5 mM $MgCl_2$ at pH 6.8, and the reaction temperature was 23 C. The mitochondrial concentration was held constant at 56 ± 7 μg of mitochondrial protein for each experiment. The specific radioactivity of the ^{125}I-P used was 133 cpm/ng.

5×10^{-14} g as an approximation of the average protein weight per mitochondrion (12, 14), and 240,000 as the mol wt of phytochrome, there are about 100 molecules of Pfr bound per mitochondrion under the experimental conditions used in Figure 9. This is a very small number in comparison to the total number of phytochrome molecules that could physically fit on the surface of a mitochondrion, but still a significant number, considering it would provide at least 30,000 phytochrome binding sites per cell on mitochondria alone (9).

All the data above point to the conclusion that there are specific binding sites in the preparation of oat mitochondria for oat phytochrome. Another approach to probing the specificity of the sites is to ask whether they would be found in preparations of purified mitochondria from totally unrelated organisms. Figure 11 investigates this question, using purified rat liver mitochondria as the test organelles. The results show that the total binding of ^{125}I-P to rat liver mitochondria is much lower than it is to oat mitochondria and that none of the phytochrome bound can be competitively inhibited by a 15× excess of unlabeled phytochrome; *i.e.*, none of the total ^{125}I-P bound is specifically bound. Thus, there are no specific binding sites on rat liver mitochondria for phytochrome. A more rigorous test of how species-specific the binding sites are would be to test oat phytochrome binding to mitochondria from dicots, such as pea, that have phytochrome that is immunologically distinguishable from oat phytochrome (18, 24).

Given the evidence above for the high affinity binding of phytochrome to specific sites in the mitochondrial membrane preparation, we need to ask, finally, to what membrane or particulate in the preparation is phytochrome binding. Table II addresses itself to the question by estimating the purity of the mitochondria preparation by quantitative morphometry of electron micrographs of the preparation. Biochemical tests, reported elsewhere (2), showed that there was no NADPH-dehydrogenase activity in the preparation, and so little or no contamination by ER membranes (21). Biochemical tests also showed that the preparation had a high level of mitochondrial activity, indicated by ADP/O ratios > 2.5, and respiratory control ratios > 4.0; and they indicated that 85 to 90% of the mitochondria present had intact outer membranes, suggesting a low degree of breakage in

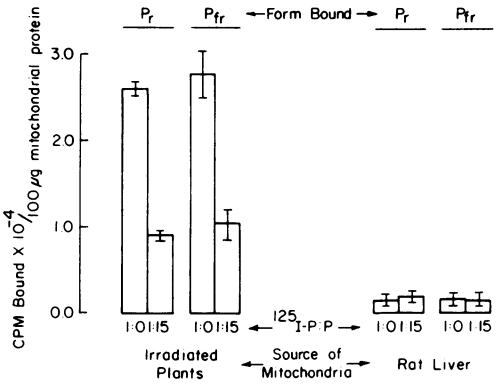


FIG. 11. Comparison of the binding of ^{125}I-P to oat *versus* rat liver mitochondria. The reaction medium for these experiments was Medium R plus 5 mM $MgCl_2$ at pH 6.8 and 24 C. The ^{125}I-P used had a specific radioactivity of 120 cpm/ng and for each experiment 150 ± 19 µg of mitochondria (protein wt) were used.

Table II. *Estimation of the Purity of the Mitochondrial Preparation by Quantitative Morphometry*

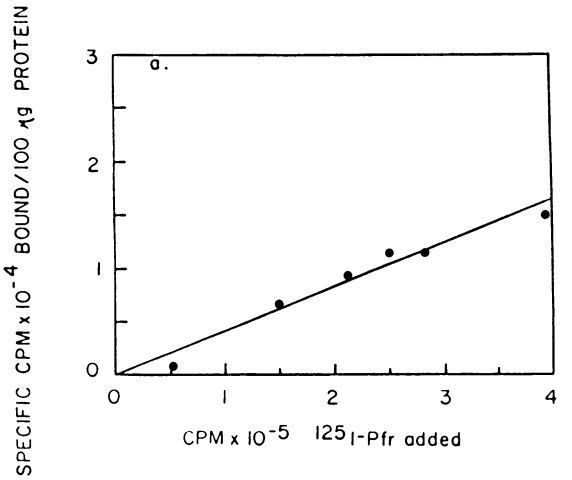
Purified mitochondria were pelleted by centrifugation then fixed for electron microscopy. The fixed pellet broke into five fragments, each of which was embedded, sectioned, and examined separately.

Fragment No.	% Mitochondria[a]	% Other Organelles[b]	% Undefined[c]
1	70	10	20
2	80	8	12
3	76	10	14
4	82	5	13
5	77	7	16

[a] Double-membrane vesicles enclosing cristae-like membranes.
[b] Vesicles without cristae.
[c] Membrane fragment.

the preparation. The electron micrographs cannot give an accurate measure of intactness because some mitochondria are broken during the centrifugation used to concentrate them in preparation for fixation. This centrifugation breaks between 10 and 15% of the mitochondria in the preparation as estimated by an intactness assay. This breakage, in fact, must be considered one of the contributing factors to the fairly large percentage of "undefined" membranes in Table II. But breakage problems aside, Table II indicates that about 77% of the area filled by membranes in the photomicrographs examined can be assigned unambiguously to mitochondria. Only about 8% of the area is filled by membrane-enclosed structures that are either clearly not mitochondrial or are at least not unambiguously mitochondrial in origin. These structures include microbodies (about 3%), etioplasts (about 1%), and small, single-membrane vesicles that probably originate from plasma membrane, ER membrane, and vesiculated pieces of broken outer membranes from mitochondria (about 4%). Since some portion of the 15% "undefined" membranes is mitochondrial in origin, the preparation overall is highly enriched for mitochondria. And if the data were expressed as mitochondria *versus* any other one specific kind of membrane, the purity would surely exceed 90%.

On the basis of relative membrane area available, one could reasonably expect that the mitochondria in the membrane preparation were the major binding site for ^{125}I-P. Figure 12 provides support for this conclusion. For the binding experiments described there, a preparation of membranes was used, that, relative to the purified mitochondria preparations, was less enriched for mitochondiral membranes but more enriched for most other cellular



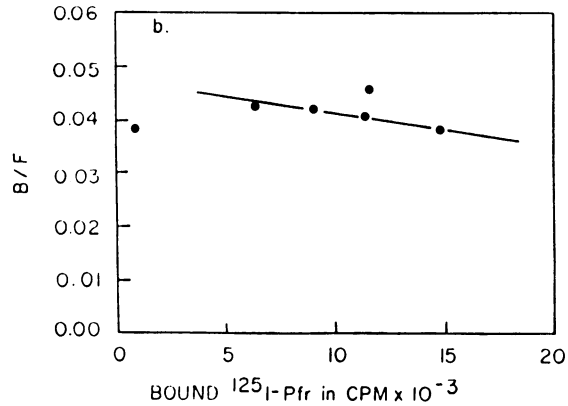


FIG. 12. Saturation curve (a) and Scatchard plot (b) of the binding of ^{125}I-Pfr to a crude membrane fraction isolated from light-exposed oat shoots. The membrane material used in these experiments was obtained from homogenized oat tissue prepared as described under "Materials and Methods" for the initial steps in the isolation of plant mitochondria. The filtered homogenate was centrifuged at 1,000*g* for 10 min and the pellet discarded. The 1,000*g* supernatant was centrifuged for 20 min at 20,000*g*. The pellet from this centrifugation was resuspended in 0.3 M mannitol, 10 mM Hepes and 0.1% BSA at pH 7.4. This material was then used to conduct binding experiments as described under "Materials and Methods," using medium R plus 5 mM $MgCl_2$ at pH 6.8 and 24 C. The specific radioactivity of the phytochrome used was 86 cpm/ng, and approximately 86 µg of particulates (protein weight) from the 20,000*g* pellet was used in each experiment.

membranes. Only about 12% of the ^{125}I-P added to this preparation was specifically bound as judged by competition experiments. The binding showed no evidence of saturability even after 5 µg of ^{125}I-P was added; thus, it was clearly a low-affinity binding. Except for the heterogeneity of the membranes used, the assay conditions were the same as those used for Figure 9. The results in Figure 12 agree with the predictions of Pratt and Marmé (20) that there would be little meaningful binding of phytochrome to crude preparations of membranes *in vitro*, but they contrast sharply with the results in Figure 9 and suggest that when one enriches for mitochondria in the preparation, the probability of high affinity, saturable binding of phytochrome to membranes is dramatically increased.

Given the relatively low number of high affinity sites for phytochrome in the mitochondrial preparation, any amount of

contaminant membrane would compromise the confident identification of the binding site, if this identification were based solely on the purity of the preparation. A more important basis for identifying mitochondria as a probable binding site for ^{125}I-P in the data reported here is the fact that exogenously added phytochrome photoreversibly alters a mitochondrial function, NADH-dehydrogenase, under the same experimental conditions as used for these binding studies (3). These physiological data in combination with the data in Table II argue strongly for the working hypothesis of this report: That the specific binding of ^{125}I-P to sites in the mitochondrial membrane preparation was to sites on the mitochondria in the preparation. As a further test of this hypothesis, we have obtained ferritin-conjugated goat antiphytochrome antibodies and have used these antibodies to identify the sites of phytochrome binding in the mitochondrial membrane preparation. In work to be reported elsewhere (Slocum and Roux, in preparation), we found that the vast majority of the label was found associated with the mitochondria of the membrane preparation.

Acknowledgments—We thank Drs. J. Mauseth and R. Ramirez-Mitchell for advice on the quantitative morphometry of the isolated mitochondria, and C. Hale II and K. McEntire for performing the radioimmune assays and the gel analyses of the immunoprecipitates.

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