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Binding Properties *In Vitro* of Phytochrome to a Membrane Fraction

(stereospecific binding/cation effects/pH effects/phytochrome purification)

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ABSTRACT **Brief irradiation of a buffer extract of dark-grown zucchini squash seedlings with red light results in the binding of the far-red-absorbing form of phytochrome to a particulate fraction. A low concentration of magnesium (0.1 mM) permits partial far-red reversal of the binding. A higher concentration (10 mM) yields actually enhanced binding after the far-red treatment. Both magnesium and calcium have a strong effect on the vesicularization of the phytochrome-binding particles and on their aggregation into readily sedimentable complexes. At concentrations above 10 mM, binding of the far-red-absorbing form of phytochrome is inhibited. These effects were not observed with sodium or potassium. Increasing the H^+ concentration led to increased binding of the far-red-absorbing form. This form of phytochrome bound at pH 6.5 and 10 mM magnesium is released if either the pH is raised to 8.0 or the magnesium concentration is raised to 50 mM. These properties suggest a new method for phytochrome purification.**

In the past few years, strong evidence has been accumulating that an early consequence of the phototransformation of the red-light-absorbing and biologically inactive form of phytochrome (P_r) to the far-red-absorbing and biologically active form (P_{fr}) might be a change in the functional properties of membranes (1). Rubinstein *et al.* (2) first reported that a small fraction of the phytochrome extracted from dark-grown oat seedlings was pelletable. Marmé *et al.* (3) reported a correlation between phytochrome binding and the binding of naphthylphthalamic acid. Naphthylphthalamic acid binding has been proposed as a marker for the plasmalemma (4).

Irradiation of etiolated squash seedlings with 3 min of red light increased the amount of pelletable phytochrome by a factor of 10 (5, 6). With 10 mM magnesium present in the extraction medium, over 40% of the total extractable phytochrome was readily sedimented. The phytochrome-containing structures were characterized by sucrose density gradient centrifugation and electron microscopy (6). The size of the phytochrome-containing structures depended strongly upon the concentration of magnesium present in the extraction medium (6). A low concentration (0.1–1 mM) yielded particles too small to reveal structural detail by electron microscopy of negatively stained preparations (about 100 Å). Higher concentrations (3–10 mM) caused some vesicularization of particles (to structures 400–600 Å in diameter) and aggregation of the vesicles formed. Purified preparations of the vesicles contain phospholipids, steroids, glycolipids, and proteins, indicative of their membrane nature (J. M. Mackenzie, Jr. and J. Beck, personal communication). Evidence that the structures formed with higher magnesium concentration are vesicles comes from comparing their appearance with that of authentic negatively stained liposomes of a similar size (refs. 6 and 7; J. M. Mackenzie, Jr., personal communication).

The aim of the present paper is to demonstrate that P_{fr} can be bound *in vitro* to a particulate fraction obtained from dark-grown squash seedlings. The influence of divalent and monovalent cations and of pH on the binding of P_{fr} will also be considered.

MATERIALS AND METHODS

Seeds of zucchini squash (*Cucurbita pepo*, L., cv. Black Beauty) were germinated on moist absorbant paper and grown in darkness for 4 days at 25°. The seedlings were then chilled to 4°; hypocotyl hooks about 1 cm in length were harvested and placed immediately in Syracuse dishes on ice. The extraction buffer contained: 25 mM *N*-morpholino-3-propanesulfonic acid (MOPS), 3 mM disodium salt of ethylenediamine tetraacetic acid (EDTA), 14 mM 2-mercaptoethanol, 10 or 0.1 mM $MgCl_2$, and 250 mM sucrose, at pH 7.35. A ratio of 4 ml of extraction buffer to 1 g fresh weight of tissue yielded a final pH in the homogenate of 7.0. Except where noted below, the pH was always maintained thereafter at 7.0. Ten grams of tissue was chopped in the extraction buffer with a razor blade for 10 min and then ground gently in a chilled mortar for 4 min. The resulting brei was filtered through four layers of cheesecloth and one layer of Miracloth to remove larger cellular debris. Subsequent treatment is shown in Fig. 1. An additional 10-min centrifugation at 500 × *g* removed the remaining cell fragments. Centrifugation of the 500 × *g* supernatant (0.5 KS) at 17,000 × *g* for 30 min then removed the majority of the organelle fractions. Phytochrome in a supernatant or a pellet was estimated in a dual-beam difference spectrophotometer (Ratiospect R-2). The measuring wavelengths were 660 and 730 nm. To allow analysis of very small aliquots of samples, $CaCO_3$ powder was used to amplify sample absorbence (8). The maximum variation between replicate samples never exceeded 10%. The use of $CaCO_3$ also essentially eliminated any inherent light-scattering differences between supernatants and resuspended pellets. Each experiment contained at least two replicates for each

Abbreviations: 0.5 KS, 500 × *g* supernatant; 17 KS, 17,000 × *g* supernatant; 17 KP, 17,000 × *g* pellet; 50 KS, 50,000 × *g* supernatant; 50 KP, 50,000 × *g* pellet; P_r, red-absorbing form of phytochrome; P_{fr}, far-red-absorbing form of phytochrome.

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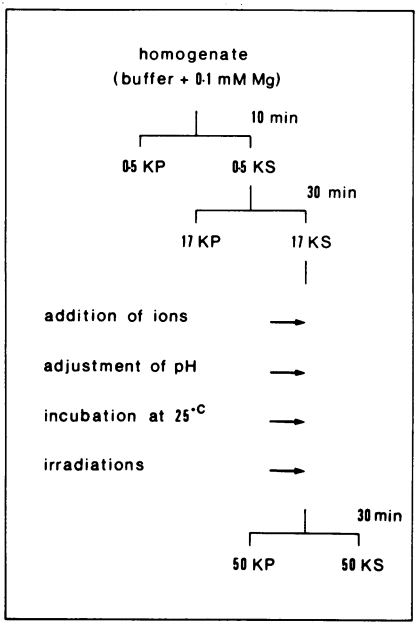

FIG. 1. Extraction procedure for phytochrome and phytochrome-binding particles from squash seedlings, used to study the influence of various factors on phytochrome binding. Order of treatments not necessarily that shown. Incubations at 25° were for 15 min. *0.5 KP* and *0.5 KS*, 500 × *g* pellet and supernatant, respectively; *17 KP* and *17 KS*, 17,000 × *g* pellet and supernatant, respectively; *50 KP* and *50 KS*, 50,000 × *g* pellet and supernatant, respectively.

measurement, and all experiments were repeated at least once. The manipulations were all carried out at 4° under dim green light except as specified below. The percent of P_{fr} bound was calculated as the amount in the pellet divided by the total of that in the pellet plus that in the supernatant, times 100.

RESULTS

In Vivo and In Vitro Induction and Reversal of Binding. If intact squash seedlings are irradiated with 3 min of red light (saturating for P_r to P_{fr} phototransformation) just before extraction, about 40% of the phytochrome is found in the pelletable fraction 17,000 × *g* pellet (17 KP), provided that the magnesium concentration in the extraction medium is 10 mM (Table 1). Further centrifugation of the 17,000 × *g* supernatant (17 KS) for 30 min at 50,000 × *g* brings down only an additional 4%. With 0.1 mM magnesium, only 20% is pelletable, and in the absence of any light treatment, only 4% is pelletable (Table 1). Light treatment and magnesium concentration affected only the distribution of phytochrome between pellet and supernatant. The total was not altered.

TABLE 1. *Recovery of phytochrome in pelletable form after red irradiation in vivo, with different magnesium concentrations in extraction buffer*

Light treatment	$[Mg^{++}]$, mM	$\%P_{fr}$ in 17 KP	$\%P_{fr}$ in 50 KP
3-min red	10.0	40	4
3-min red	0.1	20	—
None	10.0	4	—

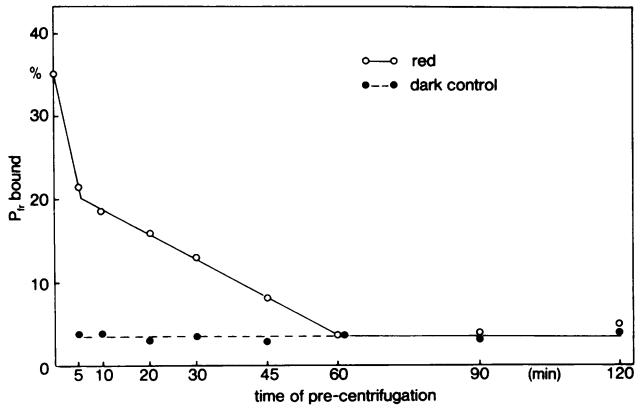

FIG. 2. Effect of precentrifugation of the 0.5 KS before red irradiation on subsequently pelletable phytochrome after red treatment and a second centrifugation of 30 min at 50,000 × *g*.

In order to determine whether phytochrome binding could be induced *in vitro*, hooks from dark-grown seedlings were extracted in the presence of 10 mM magnesium. The 0.5 KS was then warmed to 25° and irradiated with saturating red light, before it was rechilled and centrifuged at 17,000 × *g* for 30 min. As Table 2 shows, 36% of the phytochrome appeared in the pellet (17 KP). A second centrifugation at 50,000 × *g* for 30 min brought down an additional 10%. Dark controls contained negligible photochrome in the pellets. Clearly, binding could be induced *in vitro*. As before, there was no loss of total phytochrome. The 46% pelletable after red irradiation *in vitro* of the 0.5 KS compares favorably with the 44% pelletable after red treatment *in vivo* (Table 1).

If red-light treatment *in vitro* was delayed to the 17 KS, the subsequent 50 KP contained 34% of the total phytochrome (Table 2). As before, the sample was warmed, irradiated, and rechilled before centrifugation. Evidently the 17,000 × *g* centrifugation had removed about 25% of the structures to which P_{fr} could bind. If red-light treatment was further delayed to the 50 KS, a second 50,000 × *g* centrifugation pelleted only 16% of the phytochrome (Table 2). Thus, the first 50,000 × *g* centrifugation had removed almost half of the phytochrome-binding structures remaining in the 17 KS.

From the results shown in Tables 1 and 2, an important property of the phytochrome-binding fraction is seen. If P_{fr} becomes bound *in vivo*, it will sediment by centrifugation at 17,000 × *g* for 30 min (40% of the phytochrome in the 17 KP). However, if phytochrome is not present as P_{fr} through this centrifugation, the binding structures remain in the 17 KS; if the 17 KS is treated with red light, there is still enough binding material to yield 34% of the phytochrome pelletable during a 50,000 × *g* centrifugation (50 KP). The sedimenta-

TABLE 2. *Recovery of phytochrome in pelletable form after red irradiation of the supernatants after each of the three centrifugation steps*

Supernatant irradiated	$[Mg^{++}]$, mM	$\%P_{fr}$ in 17 KP	$\%P_{fr}$ in 50 KP
0.5 KS	10	36	10
17 KS	10	—	34
50 KS	10	—	16*

* After a second 50,000 × *g*, 30-min centrifugation.

TABLE 3. *Effects of the relative sequence of magnesium addition and red and far-red treatments on amount of phytochrome pelletable from the 17 KS*

[Mg^{++}], mM in extraction buffer	Sequence of treatments	%Phytochrome* in 50 KP
10.0	Red	34
10.0	Red, far-red	48
0.1	Mg^{++}, red	54
0.1	Mg^{++}, red, far-red	59
0.1	Mg^{++}, far-red	7
0.1	Red, Mg^{++}	48
0.1	Red, far-red, Mg^{++}	15
0.1	Far-red, Mg^{++}	2

* If final light treatment was red, phytochrome in 50 KP was P_{fr}. If it was far red, phytochrome in 50 KP was P_r. First two lines, Mg^{++} at 10 mM from start of extraction.

tion properties of the binding fraction are evidently altered by complexing with phytochrome.

The influence of precentrifugation of the 0.5 KS at 50,000 × *g* for periods of from 5 to 120 min before red irradiation on subsequently pelletable phytochrome was investigated in more detail. The results are shown in Fig. 2. In each case, the supernatant obtained after precentrifugation was irradiated as before and then centrifuged again at 50,000 × *g* for 30 min to assay for bound P_{fr}. One hour of such precentrifugation is sufficient to remove almost all of the binding structures, reducing the pelletable phytochrome to the level of the dark control.

In vivo studies (5) have shown that the binding of P_{fr} to membranes could be partially reversed by subsequent far-red irradiation *in vivo*, just before extraction. After red plus far-red treatment, about 12% of the phytochrome (as P_r) was pelletable (10 mM magnesium in the extraction medium) compared to 40% after red treatment only. The question therefore arose as to whether one could reverse P_{fr} binding *in vitro* with far-red light. The results are shown in Table 3. When 10 mM magnesium was present from the start of extraction, far-red treatment of the 17 KS after red treatment actually increased the percentage of bound phytochrome from 34 to 48%. If phytochrome and the binding fraction were extracted in the presence of 1/100 the magnesium concentration (0.1 mM), and magnesium was increased to 10 mM in the 17 KS just before red or red plus far-red treatment, far red also yielded a slight increase in binding (from 54 to 59%). Far red alone yielded 7% bound. However, when the red plus far-red treatment *preceded* the increase in magnesium, only 15% of the phytochrome was bound. Thus with low magnesium concentrations, the binding of P_{fr} was partially reversible by far-red light. Far-red light alone, before magnesium addition, gave only 2% binding.

When P_{fr} is bound *in vitro* as described above, the complex is remarkably stable. There was no measurable destruction of phytochrome, nor was there any release to the soluble fraction during a 4-hr incubation in the dark at 25°.

The Effects of Monovalent and Divalent Cations on Pelletable P_{fr}. The influence of K^+, Na^+, Mg^{++}, and Ca^{++}, all applied as chlorides, on the pelletability of P_{fr} was investigated following the procedure described in Fig. 1. All experiments were done starting with the same 17 KS. The ions were added,

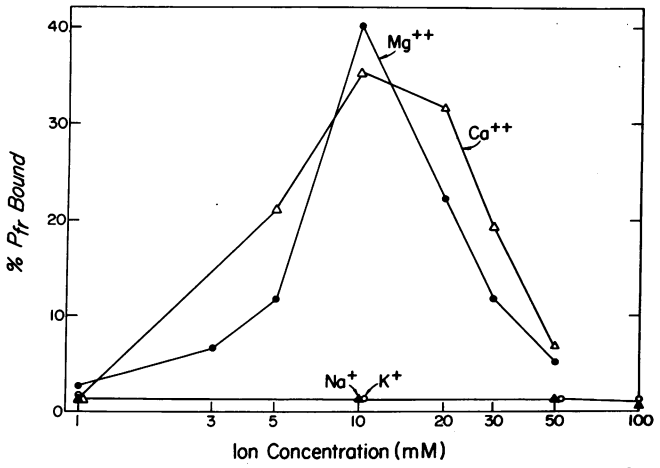

FIG. 3. Influence of monovalent and divalent cations on the pelletability of P_{fr} from the 17 KS by centrifugation at 50,000 × *g*. Bound P_{fr} was measured in 50 KP.

the pH was readjusted to 7.0, the sample was warmed to 25°, and red-light treatment given, before the second centrifugation. It was shown elsewhere (6) that magnesium had a specific effect on phytochrome pelletability; it altered the sedimentation properties of the P_{fr}-binding membrane fraction. Increasing its concentration from 0.1 to 3 mM caused formation of vesicles; increase from 3 to 10 mM caused the membrane vesicles to form large aggregates. This effect is also shown by results obtained after *in vitro* induction of binding at various magnesium concentrations (Fig. 3). From 10 to 50 mM, the amount of bound phytochrome decreased remarkably, although the pellets remained the same size. Calcium showed a similar behavior through the whole concentration range. Thus, the binding of P_{fr} seems to be inhibited by concentrations of calcium or magnesium higher than 10 mM. Sodium and potassium did not cause formation and aggregation of P_{fr}-binding vesicles (Fig. 3). Pellets were scarcely visible even with concentrations of 100 mM. The effect of these ions on binding itself needs more investigation.

The Effect of pH on P_{fr} Binding In Vitro. The effect of alteration of pH in the presence of various concentrations of magnesium in the 17 KS was investigated following the protocol shown in Fig. 1. The results are shown in Fig. 4. For all five magnesium concentrations, decreasing the pH increased the percentage of bound phytochrome in the 50 KP. The optimum concentration of magnesium at pH 6.5 yielded more than 80% of the phytochrome bound. In the absence of binding structures, the phytochrome itself did not precipitate under these conditions. P_r binding (no light treatment) was measured at two different magnesium concentrations. In both cases (1 and 10 mM), P_r binding was more than an order of magnitude lower than comparable P_{fr} binding.

Magnesium Reversal of the pH Effect, and pH Reversal of the Magnesium Effect. A large volume of 17 KS was initially adjusted to pH 6.5 and 10 mM magnesium to yield maximum binding (Fig. 4). Binding was induced as usual by red-light treatment. Six aliquots were taken. One remained as the control; three others were readjusted to pH 7.0, 7.5, and 8.0; and two others were kept at pH 6.5, but the magnesium concentration was increased to 30 and 50 mM. After subsequent centrifugation, the 50 KP and 50 KS were assayed for phyto-

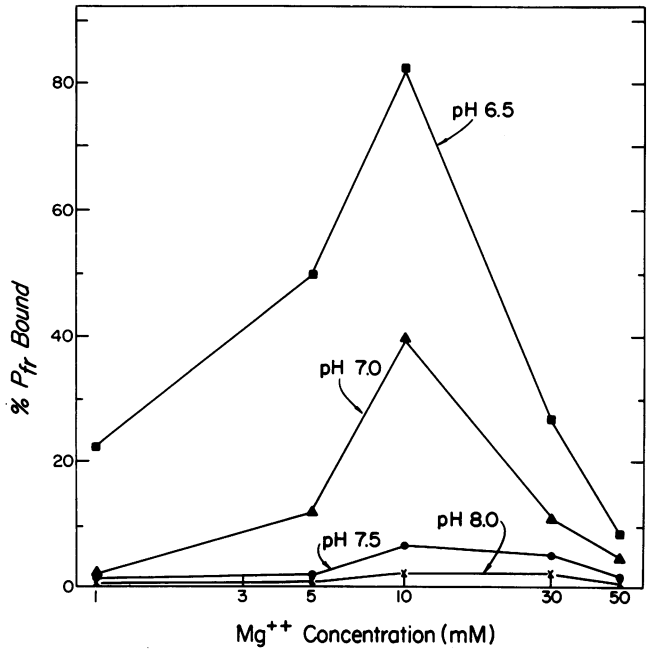

FIG. 4. Influence of pH at several different magnesium concentrations on the pelletability of P_{fr} from the 17 KS. Ion and pH adjustments were made before incubation and irradiation. Bound P_{fr} was measured in 50 KP.

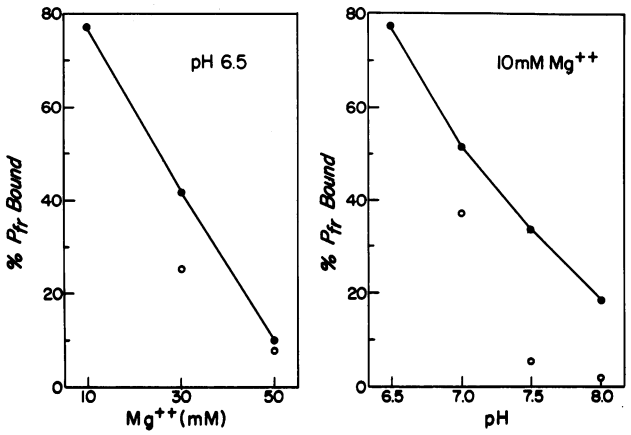

FIG. 5. Influence of increasing magnesium concentration or pH in releasing phytochrome bound as P_{fr} at pH 6.5, magnesium 10 mM. (*Left*) pH constant, magnesium concentration increased; (*right*) magnesium concentration constant, pH increased. *Open circles* represent the percent binding obtained when pH and magnesium concentration were adjusted before irradiation (from Fig. 3).

chrome. The percent pelletable was clearly reduced either by increasing the pH or by increasing the magnesium concentration (Fig. 5). The open circles show the amount bound when the given conditions of pH and magnesium concentration were achieved *before* red light treatment, and are taken from Fig. 4. It is conceivable that a longer incubation period might be needed to release the maximal bound phytochrome after an increase either in pH or magnesium concentration.

DISCUSSION

After brief irradiation of etiolated zucchini squash seedlings, about 40% of the total extractable phytochrome can be pelleted. It was shown elsewhere (6) that this fraction of P_{fr} was bound to a membrane fraction that could be isolated and purified. The recognition of P_{fr} by the receptor sites at the membrane surface must be highly stereospecific (5). The sites distinguish between P_r and P_{fr} despite the apparently small conformational difference between the two forms of phytochrome (9). The present work demonstrates that even in an *in vitro* system, the membrane fraction is still able to recognize P_{fr} stereospecifically.

The *in vitro* binding of P_{fr} to this membrane fraction and its sedimentation properties are strongly affected by magnesium and calcium. Potassium and sodium have no obvious effect on the pelletability of the phytochrome-binding structures. As was shown in studies on the induction of binding *in vivo* (6), magnesium seems to have at least three effects: vesicularization, aggregation of the vesicles, and at higher concentrations, inhibition of P_{fr} binding. The inhibition might be explained by a change in the affinity between the ligand and the receptor on the vesicles caused by intra- or interchain crosslinking through protein carboxyl or lipid phosphate groups (10) or simply by ionic strength. At constant magnesium concentration P_{fr} binding depends strongly on the pH (Fig. 4). At lower pH values the binding increases. This effect could be explained by charge neutralization, and thus compensation of repulsive forces (10). There is no obvious effect of pH on the pelletability of the binding structures. Although one cannot presently say for certain that the phytochrome is bound to the vesicles seen in the electron microscope, the parallelism between vesicle formation and phytochrome sedimentability is striking.

For the reconstitution of membranes from *Mycoplasma laidlawii* (11) and for the recombination of erythrocyte membranes and ATPase (12), increasing the magnesium concentration had the same effect as increasing the H^+ concentration. In the present case, the effect is reversed: increasing magnesium concentration has the same effect as decreasing the H^+ concentration (Fig. 5).

It was shown elsewhere (5) that far-red irradiation *in vivo* subsequent to red irradiation would partially reverse the red effect, reducing bound phytochrome from 40 to 12%. Partial far-red reversibility of P_{fr} binding can also be demonstrated *in vitro* (Table 3) at low magnesium concentration (0.1 mM). With higher magnesium concentration, however (10 mM), far red subsequent to red actually increases the amount of phytochrome bound (Table 3). This phenomenon requires further investigation.

The various effects described in this paper suggest a simple procedure for purification of phytochrome, directly based on Fig. 1. Dark-grown plant material is homogenized in buffer at low magnesium concentration (0.1 mM). The pH of the 17 KS is adjusted to 6.5 and the magnesium concentration to 10 mM before saturating red plus far-red irradiation, to yield maximum binding. The 50 KP is then washed in buffer with a low magnesium concentration (0.1 mM) at pH 7.5 to disaggregate the vesicles and release the phytochrome. Magnesium is then added to 50 mM to reaggregate the vesicles under conditions in which phytochrome does not bind. Subsequent centrifugation removes the membrane fraction while the phytochrome remains in the supernatant. The advantages of this procedure would be potentially high yield and rapid preparation of highly purified phytochrome. This procedure has been used successfully both at Rouen and at

Harvard. In the former case, yields between 40 and 50% were obtained, and the phytochrome had a ratio A_{280}/A_{665} between 13 and 17. Precipitation with 33% saturated ammonium sulfate, followed by redissolving in 0.1 M sodium phosphate buffer and Sephadex G-200 gel filtration, has given preparations with a ratio near 2.5 (present authors with J. M. Mackenzie, Jr. and David W. Hopkins, personal communication). For comparison, purified rye phytochrome has a ratio near 1.3 (13).

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