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Light-mediated changes in two proteins found associated with plasma membrane fractions from pea stem sections

(blue light/phytochrome/protein phosphorylation)

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ABSTRACT **Irradiation of etiolated pea (*Pisum sativum* L.) seedlings with white light affects two proteins, both of monomer molecular mass near 120 kDa. Both proteins have been detected in association with plasma membrane fractions. The first is identifiable in that it becomes heavily phosphorylated when the membranes are incubated with exogenous ATP. The second of these proteins is phytochrome, as determined by electrophoretic transfer (Western) blot analysis. Measurable phosphorylation and phytochrome (the latter detected by antigenicity) decline when the tissue is irradiated with white light prior to membrane isolation and *in vitro* phosphorylation. The phosphorylated protein is probably not phytochrome for three reasons. (*i*) It shows a slightly different distribution in sucrose gradients. (*ii*) Red light causes a gradual decline in the phytochrome that is associated with membrane fractions but has a negligible effect on the phosphorylatable protein; blue light, on the other hand, causes significantly slower loss of phytochrome than does red light but brings about a rapid decline in the phosphorylation signal. (*iii*) The molecular masses are not identical. The association of both proteins with membrane fractions is probably neither ionic nor, at least for the phosphorylatable protein, the consequence of entrapment of soluble proteins in vesicles formed during tissue extraction. Phytochrome is lost from the membrane fractions during irradiation, as judged by loss of antigenicity. Whether the phosphorylatable protein is lost, a specific kinase is lost, phosphatase activity increases, or phosphorylatable sites are blocked as a consequence of blue light treatment is not known.**

Plants depend heavily upon light signals to provide them with environmental information on light direction, intensity, duration, and spectral quality (1–5). Most responses showing action spectrum maxima in the blue and in the near-ultraviolet are thought to be mediated by a flavoprotein (6), whereas those in the red and far-red regions of the spectrum are mediated by the photoreversible pigment phytochrome (1–3).

The present study had its origin in the observation by one of us (S.G.) of a particular membrane-associated protein that became strongly phosphorylated *in vitro* on addition of [γ-^{32}P]ATP to microsomal membranes from pea seedlings. This protein could be detected on autoradiograms of sodium dodecyl sulfate (SDS) polyacrylamide gels of membrane proteins obtained from dark-grown seedlings. However, when the seedlings were exposed to white light for a few hours before preparation and phosphorylation of the membranes, the amount of ^{32}P incorporated into this band decreased dramatically. The molecular mass of the phosphorylatable protein was roughly 120 kDa. This molecular mass is near that of the pea phytochrome monomer (7), and preliminary electrophoretic transfer (Western) blots showed phytochrome antigenicity in this region of the gel. Since regulation of protein function by phosphorylation/dephosphorylation is widespread in living organisms (8), including plants (9, 10), the light-induced changes in phosphorylation we observed could represent steps in a transduction chain activated either by a blue light photoreceptor or by phytochrome.

In view of this possibility, we undertook studies addressing four questions. (*i*) Is the association with membrane fractions that is observed for phytochrome and the phosphorylatable 120-kDa protein ionic, hydrophobic, or simply the result of entrapment of soluble protein in vesicles formed during membrane isolation (see ref. 11)? (*ii*) If the phytochrome and phosphorylatable protein are particulate for reasons other then entrapment, with what membrane fraction(s) are they associated? (*iii*) Is the readily phosphorylated, nonsoluble protein detected in microsomal preparations from dark-green seedlings itself phytochrome (known to be a phosphorylated protein; ref. 12)? (*iv*) What is the nature of the light effect(s)?

MATERIALS AND METHODS

Chemicals. Acrylamide, methylenebisacrylamide, and SDS (all two times crystallized) were obtained from Serva. Tris and glycine (Ultra-Pure grade) were from Bethesda Research Laboratories; urea (electrophoresis grade) was from Schwarz/Mann. Molecular mass standards were from Sigma. All other chemicals were standard enzyme grade. The [γ-^{32}P]ATP was obtained from Amersham.

Plant Material. Pea seeds (*Pisum sativum* L.) were imbibed and then grown in moist vermiculite for 7 days in complete darkness at 24°C (±1°C) (except for preliminary experiments, see *Results*). For all experiments except those involving gradient fractionation, 8-mm stem segments were taken from the third growing internode, beginning 2 mm below the hook. The seedlings were moved into a cold room and harvested under dim green light at 4°C. The terminal ≈10 mm of dark-grown seedlings was used for the cell fractionation studies.

Isolation of Membrane Fractions. Harvested tissues (30–100 sections) were placed into a chilled mortar on ice and ground with a pestle in 5 ml of chilled 30 mM Tris/Mes, pH 8.0/0.4 M sucrose/10 mM KCl/1 mM EDTA/0.1 mM $MgCl_2$/1 mM dithiothreitol. The resulting homogenate was filtered through 20-μm mesh nylon cloth and centrifuged for 10 min at 6000 × *g* (SS-34 rotor, Sorvall RC-5 refrigerated centrifuge). The pellet was discarded. The 6000 × *g* supernatant was then centrifuged for 30 min at 100,000 × *g* (Beckman type 65 rotor, Sorvall OTD-2 ultracentrifuge) to yield a soluble fraction and a microsomal membrane pellet.

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Abbreviation: ER, endoplasmic reticulum.

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The pellet was resuspended in 100–200 μl of 10 mM Tris/Mes, pH 7.2/1 mM dithiothreitol/0.25 M sucrose (resuspension buffer) to give a protein concentration of 10–20 mg/ml. The fractions were either used immediately or stored at −80°C. All manipulations were carried out in a cold room under dim green light.

To determine subcellular localization, membrane fractionation was performed by rate-zonal followed by isopycnic centrifugation, as described by Ray (13) except as follows. Homogenization was with 0.25 M sucrose containing 20 mM Mes, pH 6.5/1 mM EDTA/0.1 mM $MgCl_2$/1 mM dithiothreitol. Rate-zonal centrifugation was for 20 min at 10,000 rpm into linear 13–25% (wt/wt) sucrose gradients containing the other components just mentioned in an SW 28 rotor (Beckman Instruments; 13,250 × *g* at r_{av}). Isopycnic centrifugation was into linear 20–50% sucrose gradients made up in 80 mM Tris (pH 8.0) containing 10 mM KCl/0.1 mM $MgCl_2$/1 mM EDTA for 90 min at 28,000 rpm in the same rotor (104,000 × *g* at r_{av}). The top 5 mm of each rate-zonal gradient plus the lowest 5 mm of homogenate overlay were used for isopycnic fractionation primarily of endoplasmic reticulum (ER) and plasma membrane vesicles; the zone from 15 to 30 mm below the top of the rate-zonal gradients was used for isopycnic fractionation primarily of Golgi dictyosomal membranes plus some mitochondria. Marker enzyme assays were as described (13), except for Ca^{2+}/spermine/digitonin-stimulated glucan synthase activity (here called glucan synthase III, but possibly just an activated form of glucan synthase II), which was as in ref. 14 but with 500 μM UDPG.

***In Vitro* Phosphorylation.** The phosphorylation procedure is a modification of that by Shulman (15). For microsomal membrane proteins, amounts of protein as noted below were added to the standard phosphorylation assay mixture [30 mM Tris/Mes, pH 7.0/5 mM $MgCl_2$/0.2 mM EGTA/0.12 mM Ca (total ≈ 0.2 μM Ca free)] (minus ATP) and prewarmed to 30°C for 30 s. The reaction was started by addition of 2 μl of 5 mM ATP containing 1.33 Ci of [γ-^{32}P]ATP per mmol (1 Ci = 37 GBq) to give a final concentration (in 100 μl) of 100 μM ATP. After 30 s at 30°C, the reaction was terminated by addition of 100 μl of 2× SDS/PAGE sample buffer (0.125 M Tris·HCl, pH 6.8/10% SDS/0.2 M dithiothreitol/10% glycerol/0.004% bromophenol blue). The samples were heated to 100°C for 60 s, cooled on ice, and analyzed by SDS/PAGE. Proteins were estimated by the method of Lowry, as modified by Peterson (16).

Electrophoresis. Concentration gradient SDS/PAGE (5–20% acrylamide) was done as described (17, 18). All stock solutions used for preparing the gels were filtered through a 0.45-μm (pore size) filter prior to use. The acrylamide/bisacrylamide stock solution was treated with a mixed ion-exchange resin (Serva Serdolite-MB) prior to filtration. For 1-mm-thick gels, 30 μg of protein in 30 μl of SDS/PAGE sample buffer was used per lane (6.5 mm wide) except where noted below. Electrophoresis was normally for 12–14 hr at 4 mA per gel. To detect phytochrome and phosphorylated proteins, the gels were equilibrated for 30 min in 1% SDS in blotting transfer buffer (20 mM Tris/200 mM glycine/20% methanol, pH 8.3) and electroblotted onto 0.2-μm (pore size) nitrocellulose (19) in a Hoefer Scientific Instruments TE52 electroblot apparatus.

Newly obtained polyclonal rabbit antiserum against pea phytochrome was prepared as described (20). Antigen-specific antibodies, which were affinity-purified as described elsewhere (21), were used for detection of phytochrome on blots. Antibody was then detected with a VectaStain ABC kit (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. The substrate was diaminobenzidine with $NiCl_2$. High-resolution autoradiograms were obtained by exposing the nitrocellulose to Kodak X-AR film without intensifying screens at room temperature for 1–14 days. Approximate levels of phosphorylated protein and phytochrome were estimated by scanning autoradiograms or Western blots with a Hoefer GS-300 scanning densitometer (transmittance mode for autoradiogram; reflectance mode for Western blots). Amounts of proteins were such that there was reasonable linearity between concentration and densitometer reading. Typical autoradiograms and Western blots are shown in Fig. 5.

Experiments Testing Nature of Membrane Association. In one set of experiments, a total of 1.2 mg of membrane-associated protein (from dark-grown seedlings) in resuspension buffer was phosphorylated as described above, but the reaction was quenched with EDTA and EGTA (final concentrations, 15 and 4 mM, respectively) without boiling, leading to a final volume of 780 μl. Aliquots of 100 μl each were diluted to 0.5 ml with distilled water, resuspension buffer, 1.25 M KCl (to yield a final concentration of 1 M), SDS to yield protein:SDS ratios (wt/wt) of 10:1, 5:1, or 2.5:1, or Triton X-100 to give protein:Triton X-100 ratios (wt/wt) of 1:1, 1:2, 1:4, or 1:8, respectively. Osmotic shock was induced by diluting a 100-μl aliquot to 5 ml with distilled water, by diluting a 40-μl aliquot into 3 ml, or diluting a 200-μl aliquot into 7 ml, giving dilutions from 35- to 75-fold. The samples were incubated at room temperature for 5 min and then pelleted by centrifugation at 100,000 × *g* for 20 min. Supernatants were removed, and the pellets were gently rinsed with ice-cold distilled water and resuspended in 100 μl of resuspension buffer. After removal of aliquots for protein assay, SDS sample buffer (18) was added, the samples were boiled for 1 min, 50-μl aliquots were loaded for electrophoresis, and the gels were run and electroblotted.

Light Sources and Spectrophotometry. Broad spectral band blue light was obtained from blue fluorescent lamps (GE 20 W, F20T12-B) wrapped in one layer of Cinemoid (no. 857). Broad spectral band red light was obtained from red fluorescent lamps (Sylvania F40/R and F20T12/R) filtered through 3 mm of colored plastic filter (Shinkolite, Mitsubishi Rayon). Fluence rates at the level of the epicotyl hooks were ≈2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for red light and ≈15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for blue light. Phytochrome was measured with a Ratiospect R-2 difference spectrophotometer (Agricultural Specialties). Measuring wavelengths were 730 and 800 nm.

RESULTS

Membrane Protein Phosphorylation. Preliminary experiments with microsomal membranes isolated from plants grown in darkness (save for brief exposures to dim red light for watering) exhibited a heavily labeled protein near 120 kDa when phosphorylated either *in vivo* with $^{32}P_i$ or *in vitro* as described above (results not shown). Several labeled proteins were found, ranging in molecular mass from 270 to 10 kDa. There was considerable heterogeneity of phosphorylated proteins in the 120-kDa region, but the component near 120 kDa was by far the most heavily labeled (see dark control, Fig. 5 *Upper*). A strong phosphorylated band in this region of the gel was apparent only in membrane and not in supernatant fractions. If the pea seedlings were exposed to bright white light in a growth chamber for 4 hr prior to membrane isolation, the ^{32}P labeling of this component almost disappeared (data not shown).

Similar membrane preparations were tested immunologically on Western blots for the presence of phytochrome. Membranes from the dark-grown seedlings showed strong phytochrome antigenicity, also near 120 kDa, whereas those from light-treated seedlings showed only a faint band (data not shown). As we could not detect this phytochrome spectrophotometrically with our instrumentation, it probably represents at most a few percent of the total phytochrome.

Table 1. Effect of solubilization treatments on total protein and pelletable phytochrome and phosphorylation of the 120-kDa polypeptide

	1 M KCl (4)	Protein:SDS 10:1 (2)	Protein:SDS 10:2 (2)	Protein:SDS 10:4 (2)	Protein:Triton X-100 1:1 (2)	Protein:Triton X-100 1:2 (1)	Protein:Triton X-100 1:4 (2)	Protein:Triton X-100 1:8 (1)	Buffer (3)	Osmotic shock (3)
Protein	114	64	47	34	81	93	79	38	91	75
Phytochrome	96	46	19	16	102	101	104	119	110	113
Phosphorylation	98	73	49	32	83	115	95	80	98	63

Phytochrome and phosphorylation measurements represent peak heights from densitometric scans, and all numbers are normalized as percentage of H_2O control. Absolute protein content of resuspended H_2O control pellet was 77.4 μg, representing 50% of the initial 154-μg protein suspension before treatment and repelleting. KCl and detergents were in H_2O or resuspension buffer. Osmotic shock experiments were performed by diluting membrane preparations in 35–75 volumes of distilled H_2O and repelleting. Numbers in parentheses represent the number of replicate experiments performed.

Nature of Membrane Association. Experiments were next carried out to determine whether the observed membrane associations were the consequence of ionic binding, hydrophobic interaction, or entrapment. Membranes were phosphorylated and then treated with KCl, SDS, or Triton X-100 prior to electrophoresis, blotting, immunostaining, and autoradiography, as described under *Materials and Methods*. Resuspension either in water, in resuspension buffer, or in 1 M KCl gave comparable protein and phytochrome yields, with water causing a modest loss of the detected phosphorylation (Table 1). Hence, neither the 120-kDa phosphorylated protein nor the phytochrome appeared to be ionically bound to the membranes. The persistence of both signals in the presence of the EDTA and EGTA used to quench the phosphorylation reaction also argues against ionic binding. Increasing concentrations of SDS remove only a little more phosphorylation and phytochrome than total protein, and Triton X-100 concentrations sufficient to remove over half of the total protein had little or no effect on either phosphorylation or phytochrome, suggesting that both components are firmly bound to the membrane.

Since cytoplasmic phytochrome is present in great excess over that associated with membranes, the question of possible entrapment was investigated. Membranes were osmotically shocked by dilution with distilled water as described in *Materials and Methods* (Table 1). This treatment, which might be expected to burst vesicles, had no major effect on the phosphorylation and phytochrome bands (Table 1).

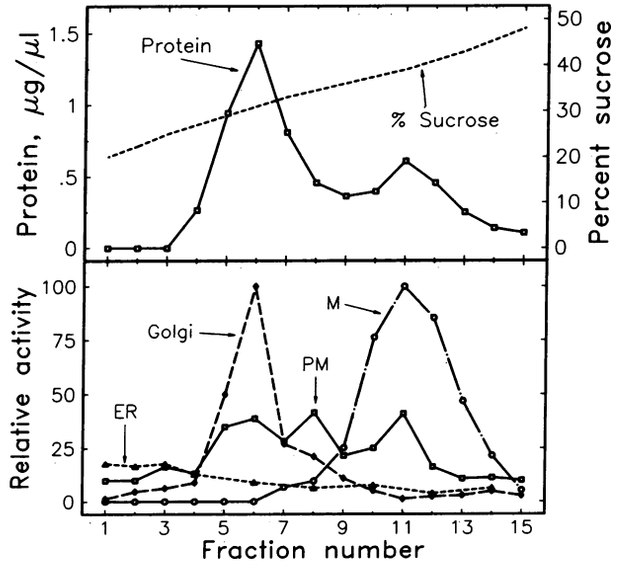

FIG. 1. Distribution of total protein and of marker enzymes for ER, Golgi, plasma membrane (PM), and mitochondria (M) after isopycnic gradient centrifugation of the fast-sedimenting zone from rate-zonal gradients. One-hundred percent activity for ER, NADH: cytochrome *c* reductase, 0.0395 ΔOD/min at 550 nm; for mitochondria, cytochrome *c* oxidase, 0.0675 ΔOD/min at 550 nm; for PM, 755 cpm of [^{14}C]glucan synthesized per 7 min for glucan synthase III; for Golgi, 2997 cpm of [^{14}C]glucan synthesized per 10 min for glucan synthase I.

Membrane Localization. Membrane vesicles in pea homogenate were first subfractionated by rate-zonal centrifugation to separate microsomal membranes (primarily ER and plasma membrane) from faster-sedimenting organelles (primarily Golgi cisternae and mitochondria). The principal membrane components of these subfractions were then separated by isopycnic sucrose density gradient fractionation. The microsomal subfraction gave the profiles shown in Fig. 1 and the Golgi/mitochondrial subfraction gene those shown in Fig. 3. (For these figures and for Figs. 2 and 4, the maximal measured activity for any component is arbitrarily set at 100% and the activity in the other gradient indicated proportionally.)

Aliquots of these gradient fractions were also phosphorylated prior to electrophoresis and Western blotting. Figs. 2 and 4 compare the distribution of the 120-kDa phosphorylated protein and of phytochrome with that of glucan synthase III, a Ca^{2+}/spermine/digitonin-activated glucan synthase that seems to be localized to plant plasma membranes (22). Phosphorylated protein and phytochrome activities are distributed over the same fractions from the respective gradients, a distribution shared with that of the plasma membrane marker. However, the relative activities do not show precise matching, except for an almost perfect correspondence between the distribution of phytochrome and of 120-kDa phosphorylation in the plasma membrane- and ER-enriched gradient (Fig. 4). Comparison of the marker profiles with those for phytochrome and phosphorylatable 120-kDa protein suggests that these two components are not associated in any major way with ER, mitochondria, or Golgi membranes; their distribution within and between the two gradients matches relatively closely that of a plasma membrane marker, glucan synthase III. The broad distribution within a single gradient is consistent with a heterogeneity of density

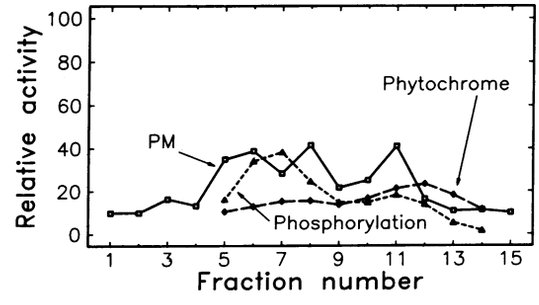

FIG. 2. Distribution of plasma membrane (PM) marker, phosphorylation detectable near 120 kDa on SDS gel, and phytochrome detectable by Western blotting among fractions from the gradient illustrated in Fig. 1.

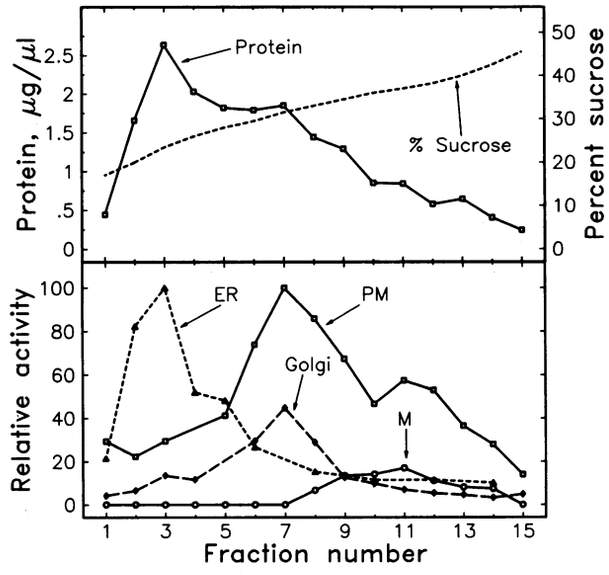

FIG. 3. Distribution of membrane marker enzymes after isopycnic centrifugation as for Fig. 1, but from the slower-sedimenting zone from the rate-zonal gradients. One-hundred percent activity values and abbreviations as for Fig. 1.

reported for plasma membrane fractions from other plants (23).

Effects of Red and Blue Light. In the sucrose gradient experiments just described, although phytochrome and phosphorylation activity coincide in the sample in which they are most enriched (Figs. 2 and 4), phytochrome predominates at higher densities and phosphorylation activity predominates at lower densities in the other rate-zonal sample (Figs. 1 and 3). These results indicate the phosphorylated protein may not be phytochrome. Indeed, small air bubbles during electroblotting in one experiment left sharply defined clear circles near 120 kDa. These flaws permitted precise alignment of the immunostained nitrocellulose with the autoradiogram made from it, and the phytochrome was about 0.5 mm above the phosphorylated band on the blot. Finally, in maize coleoptile membranes, where an analogous light-sensitive phosphorylatable protein is found, the molecular mass of the membrane-associated phytochrome is near 124 kDa, and that of the phosphorylated protein only 112 kDa (results not shown).

To explore other possible differences, sections were either harvested from dark-grown seedlings or from seedlings exposed, respectively, to 2 or 4 hr of continuous blue light or to 3 or 6 hr of continuous red light prior to homogenization, membrane isolation, and phosphorylation. The resulting Western blots and autoradiograms (Fig. 5) show that the two

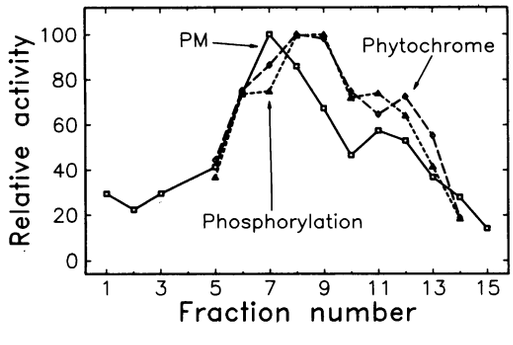

FIG. 4. Distribution of plasma membrane (PM) marker, phosphorylation detectable near 120 kDa on SDS gel, and phytochrome detectable by Western blotting among fractions from the gradient illustrated in Fig. 3.

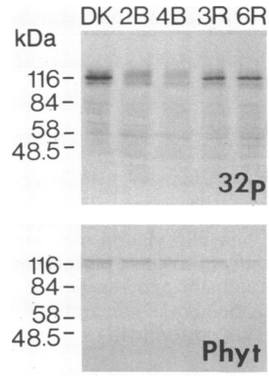

FIG. 5. Autoradiogram (*Upper*) and Western blot (*Lower*) showing effect of red and blue light on membrane-associated phosphorylation and phytochrome detectable near 120 kDa on SDS gel. DK, dark-grown; 2B or 4B, 2 or 4 hr of continuous blue light; 3R or 6R, 3 or 6 hr of continuous red light.

entities do not exhibit identical behavior. Blue light for 2 hr eliminates the 120-kDa phosphorylation completely, while having little effect on the level of phytochrome detectable on the blot. Indeed, in the experiment illustrated the blue light fluences were almost three orders of magnitude higher than those needed to eliminate the phosphorylation. In subsequent experiments we have repeatedly found that 10 s of blue light of the same fluence is sufficient. By contrast, 6 hr of red light dramatically reduces the detectable phytochrome but has little effect on the amount of phosphorylation present at 120 kDa. Spectrophotometric measurements of phytochrome in the tissue of the variously treated seedlings showed the expected result (see ref. 24): 6-hr red light treatment reduced tissue phytochrome to 18% of the dark control, whereas 4 hr of blue light only reduced it to 50%.

DISCUSSION

With respect to the 120-kDa phosphorylated protein, its association with the membrane fraction is almost certainly hydrophobic. Neither osmotic shock nor mild detergent treatment releases it from a particulate fraction (Table 1). Furthermore, since is was not detected in the soluble fraction (data not shown), it is unlikely that it is merely residual cytoplasmic protein still entrapped after the above treatments. Since 1 M KCl fails to release any activity to the soluble fraction, it is also unlikely that the association is ionic.

With respect to phytochrome, ionic association is also unlikely since 1 M KCl has no effect on its pelletability. However, the case for a hydrophobic association is more difficult to make for phytochrome. In this case the pelletable fraction represents a very small percentage of a largely cytoplasmic protein. Pupillo and Del Grosso (11) have shown that hypotonic conditions and Triton X-100 concentrations similar to those used here release malic enzyme entrapped by plasma membrane vesicles extracted from *Cucurbita pepo* hypocotyls. As phytochrome was not significantly released by these treatments, entrapment seems unlikely. Nevertheless, since we cannot exclude the possibility that phytochrome could form tighter nonspecific associations with membranes than malic enzyme, and because of artifactual problems that have plagued earlier studies of particulate phytochrome (see ref. 25), our conclusion that the phytochrome–membrane association reported here is not entrapment is tentative.

Regardless of the nature of the relationship, the 120-kDa phosphorylated protein and phytochrome are most likely associated with the plasma membrane fractions. Their dis-

tribution on gradient fractionation agrees reasonably well (though not perfectly) with that of a plasma membrane marker, glucan synthase III (22). Furthermore, the broad distribution seen on isopycnic sucrose gradient centrifugation contrasts sharply with the considerably more restricted distribution of mitochondrial, Golgi, and ER marker enzymes. Although a tonoplast marker was not included in the present study, it is unlikely that the associations seen were with tonoplast membranes. These membranes normally band near or above the ER in sucrose gradients (26) and do not show the broad distribution reported here.

It seems unlikely that the pelletable phytochrome itself is the phosphorylated protein. (*i*) They differ by about 1% in molecular mass on SDS/PAGE. Indeed, in membrane preparations from maize the difference appears nearer 12 kDa. (*ii*) The particulate phytochrome is only a minute fraction of total cellular phytochrome, the bulk of which is soluble, whereas the readily phosphorylated protein is undetectable in soluble fractions. (*iii*) The two proteins show major differences in light sensitivity. Continuous red light for 6 hr gradually reduces the phytochrome detectable on Western blots to a small fraction of that in the dark control. Blue light also reduces the amount of phytochrome detectable, but at a slower rate, consistent with the lower photostationary equilibrium for the ratio of the far-red-absorbing form of phytochrome to total phytochrome, P_{fr}/P_{tot}, in blue than in red light (27). By contrast, continuous red light is virtually without effect on the amount of phosphorylation detectable at 120 kDa, whereas as little as 10 s of blue light completely eliminates it. Thus it is possible to lose physically detectable phytochrome without comparable loss of detectable phosphorylation and vice versa.

If the phytochrome–plasma membrane association described here is truly hydrophobic, it could be the result of covalent attachment of a fatty acid to the protein (e.g., refs. 28 and 29), as the deduced amino acid sequence, at least for *Avena sativa* (30) and *Cucurbita pepo* (31) phytochrome, does not shown any extensive hydrophobic domains. Alternatively, it could be the product of a different gene coding for more hydrophobic protein. Less can be said about the nature of the association of the readily phosphorylated 120-kDa protein with the plasma membrane. Formally, treatments affecting the level detectable on gels could alter its capacity for phosphorylation, could release it from the membrane, could release a specific kinase from the membrane, or could activate an associated phosphatase.

We conclude that the rapid light-induced change in the phosphorylation detectable at 120 kDa on SDS/PAGE gels could represent an early step in a transduction chain mediated by a blue light photoreceptor. However, until the question of entrapment for the membrane-associated phytochrome is clarified, it is premature to postulate a role for this phytochrome component.

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