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Binding of Phytochrome to Liposomes and Protoplasts†

In-Soo Kim and Pill-Soon Song*

ABSTRACT: The physiologically active form of oat phytochrome, Pfr, increases its binding to egg lecithin unilamellar liposomes with increasing ionic strength of the medium while the binding of Pr is almost constant. The preferential binding of Pfr is as much as twice that of Pr at KCl concentrations above 0.2 M, in 0.1 M phosphate buffer (pH 7.2) at 27 °C. The binding of phytochrome to liposomes is also enhanced by ~80% at 27 °C compared to that at 3 °C. Thus, it appears that the binding between Pfr phytochrome and liposomes is hydrophobic in nature whereas the binding of Pr is not predominantly through hydrophobic interactions. The binding of both Pfr and Pr to multilamellar liposomes increases with increasing cholesterol content in the liposomes. The extent of phytochrome's binding is higher in the neutral pH region than above pH 7.5. It takes several hours to reach an equilibrium of binding. The photoreversion of liposome-bound Pfr is inhibited by 40% compared to that of free Pfr, while the phototransformation of liposome-bound Pr to Pfr is promoted by 30%. The rate of dithionite-accelerated dark reversion of liposome-bound Pfr is lower by 50% than that of the free form. These results are consistent with the proposal that the hydrophobic binding site involved results from a vacancy produced by the reorientation or displacement of the Pfr chromophore from the protein. Upon binding to phytochrome, unilamellar liposomes undergo fusion to form larger diameter liposomes. No preferential binding of the Pfr form was found with intact oat protoplasts in vitro.

The mode of action of phytochrome in the red light triggered morphogenic responses of plants remains elusive, in spite of many attempts to elucidate its nature at different levels of study. An attractive hypothesis for the mechanism of phytochrome-mediated photomorphogenesis is based on the proposal that phytochrome, at least in its physiologically active Pfr form, modulates membrane properties by binding to an as yet unidentified receptor [Marmé, 1974; Schäfer, 1975; Schäfer et al., 1976; Pratt, 1978; Rüdiger, 1980 (review)]. A

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large fraction of phytochrome becomes pelletable upon irradiation of either intact tissues or crude extracts with red light (Quail et al., 1973; Pratt & Marmé, 1976; Quail, 1978; Pratt, 1980). Apparently, the induction of pelletability of Pfr in vivo is rapid and can be reversed by far-red irradiation (Pratt & Marmé, 1976; Quail, 1978). Recently, it has been shown that the Pfr[1] form of phytochrome binds more tightly to a membrane fraction enriched with mitochondria than does the Pr form (Cedel & Roux, 1980).

The nature of the interactions between phytochrome and membranes is not well understood, although it is known that the binding is enhanced by divalent cations (Marmé et al., 1974; Cedel & Roux, 1980). Song et al. (1979) proposed that the preferential binding capacity of Pfr, relative to Pr, could be attributable to the development of a hydrophobic surface on the Pfr apoprotein as a result of the reorientation of the chromophore in the Pfr form. Thus, the chromophore in the Pfr form may be substantially more exposed to the medium than the Pr chromophore, resulting in a partial vacancy at the hydrophobic chromophore binding site which could serve as the binding site for the hydrophobic fluorescence probe, 8-anilinonaphthalene-1-sulfonate (ANS) (Hahn et al., 1980; Hahn & Song, 1981).

In an attempt to investigate the differential reactivity of the Pr and Pfr forms of phytochrome, we describe in this report a detailed study of their binding properties using liposomes as a model system. The binding studies described here were undertaken to elucidate the nature of the protein surface of the Pr and Pfr forms of phytochrome with respect to their binding to liposomes. The binding of phytochrome to oat protoplasts is also described.

Materials and Methods

Phytochrome. Undegraded phytochrome was isolated and purified from etiolated oat seedlings (*Avena sativa* cv, Garry oat) by means of both "conventional" (Rice & Briggs, 1973; Song et al., 1979) and "Affi-Gel Blue" affinity chromatographic methods with some modifications (Smith & Daniels, 1981a,b; Song et al., 1981). In the conventional phytochrome isolation, extracts of 2.5 kg of oat seedling tissue were applied to a brushite column (11 × 12 cm), followed by ammonium sulfate fractionation of the eluates with saturated ammonium sulfate solution, pH 7.6. Saturated ammonium sulfate solution (90 mL) (Schwarz/Mann, ultra pure grade; 520 g/L in deionized water, neutralized with Tris) was added to 100 mL of the brushite pool.

Typically, 35 mg of phytochrome was recovered by this procedure; it was then redissolved and desalted by chromatography on a Sephadex G-25 column. This was followed by ion-exchange chromatography on a DEAE-cellulose column (2 × 25 cm, Whatman DE-52) equilibrated with 10 mM bis-Tris–propane (BTP) buffer containing 10 mM KCl, pH 7.4. After washing the column with 100 mL of equilibration buffer, the phytochrome was eluted with a convex gradient of 200 mL of 10 mM BTP-10 mM KCl and 400 mL of 10 mM BTP–250 mM KCl, pH 7.4. The flow rate was 120 mL/h. Eluates containing phytochrome were pooled and fractionated with 80 mL of saturated ammonium sulfate solution per 100 mL of the pooled eluate. The phytochrome was then dissolved and passed through a Bio-Gel A-1.5m (2 × 100 cm, 200–400 mesh; Bio-Rad) column equilibrated with 0.1 M sodium phosphate buffer, 50 mM KCl, and 0.1 mM EDTA, pH 7.8. In the final preparations, ~10 mg of phytochrome with varying degrees of purity (A_{660}/A_{280} = 0.40–0.67) was recovered. The whole procedure was completed in less than 24 h.

For the Affi-Gel Blue method of phytochrome purification, the ammonium sulfate fraction of the brushite eluate was applied to an Affi-Gel Blue column (2 × 30 cm, 100–200 mesh; Bio-Rad). All manipulations of the column followed the original procedure (Smith & Daniels, 1981a,b) except for the use of lumichrome-free flavin mononucleotide as an eluant (Song et al., 1981). The phytochrome obtained from the Affi-Gel Blue column was subjected to gel filtration as described in the "conventional" procedure. The Affi-Gel purified phytochrome had A_{660}/A_{280} ratios of 0.71–0.83 with a typical yield of ~10 mg. Phytochrome preparations with a A_{660}/A_{280} ratio of 0.83 were not contaminated with any proteins detectable by $NaDodSO_4$–polyacrylamide gel electrophoresis of 20-μg preparations applied. The single protein band corresponded to M_r 120000 (Song et al., 1981).

Iodination of Phytochrome. The Affi-Gel Blue purified phytochrome (A_{660}/A_{280} ~ 0.80) was used for iodination following the method of Georgevich et al. (1977). Enzymatic iodination was initiated by adding 10 μL of 0.1 mM H_2O_2 (Fisher Scientific) to a vial containing 0.2 mCi of $Na^{125}I$ (New England Nuclear), 0.5 mg of phytochrome as Pr, and 5 μg of lactoperoxidase (Sigma) in 1 mL of 0.1 M sodium phosphate, 50 mM KCl, and 0.1 mM EDTA at pH 7.8. Another 10 μL of H_2O_2 was added after 10 min. After an additional 10 min, the mixture was passed through a Bio-Gel A-0.5m column (1.5 × 25 cm; Bio-Rad) equilibrated with 20 mM sodium phosphate and 10 mM KCl at pH 7.2. The iodinated phytochrome retained its photoreversibility, suggesting that the iodination procedure used did not irreversibly damage the phytochrome.

Preparation of Liposomes. Liposomes were prepared in 20 mM sodium phosphate buffer, pH 7.2, containing 100 mM KCl at 20–25 °C, unless otherwise stated. Multilamellar vesicles of lipids were prepared by vortex dispersion as described elsewhere (Juliano & Stamps, 1975). A chloroform solution (Waters Associates) containing 10 mg of egg lecithin (L-α-phosphatidylcholine, Sigma Type V-E) and 1 mg of cholesterol was evaporated to dryness under reduced pressure. Thin films of the lipid mixture were dispersed in 10 mL of buffer with a vortex until the bubbles disappeared. The milky suspension of multilamellar vesicles was centrifuged at 17000*g* for 20 min and resuspended to a concentration of 8 mg of lipid per mL of buffer. For the preparation of unilamellar liposomes, the thin films of lipid mixtures were dispersed in 2 mL of buffer and sonicated under a nitrogen atmosphere until transparent (~2 h with a Bransonic 12 sonicator, 80 W). The sonicated solution was then passed through a Sepharose CL-4B column (1.5 × 30 cm) with a flow rate of 6 mL/h. Multilamellar liposomes eluted in the void volume of the column were discarded. The second peak fractions containing unilamellar liposomes were collected (Figure 3A,B) and concentrated to 3 mg of lipids per mL through Millipore ultrafiltration (Millipore no. PTHK 025 10).

Protoplast Preparation and Binding Study. Oat seedlings were grown in vermiculite for 6 days at 28 °C under fluorescent lighting. Protoplasts released after a cellulysin (Calbiochem) treatment were isolated from oat leaves in the presence of 10 mM arginine by the method of Altman et al. (1977). The protoplast preparation was further purified by discontinous gradient centrifugation (Hughes et al., 1978) with some modification: the protoplast suspension in 0.6 M mannitol was overlaid on 0.6 M sucrose in 1 mM phosphate buffer,

[1] Abbreviations used: Pfr, physiologically active, far-red absorbing form of phytochrome; Pr, red light absorbing form of phytochrome.

pH 5.7, rather than 0.45 M mannitol and 0.45 M sucrose in the nutrient solution. The number of protoplasts in 0.6 M mannitol was counted in a hemocytometer and adjusted to a final concentration of 1.5×10^7 protoplasts per mL. One milliliter of protoplasts in 0.6 M mannitol was mixed with 0.2 mL of ^{125}I-labeled phytochrome (40 μg) and 0.1 mL of 0.6 M mannitol and incubated for 1 h at 300 K. The incubation mixture was centrifuged after dilution with 10 mL of 0.6 M mannitol. The pellets were resuspended by brief sonication in 0.5 mL of water and assayed for radioactivity.

Binding of Phytochrome to Liposomes. For the binding to multilamellar liposomes, 0.5 mL of multilamellar liposomes (4 mg of lipids) was mixed with 0.25 mL of ^{125}I-labeled phytochrome (50 μg of phytochrome) in its Pr or Pfr form and incubated for 1 h at 300 K. The incubation mixture was diluted with 10 mL of 20 mM sodium phosphate and 100 mM KCl, pH 7.2, before centrifugation at 34000*g* for 30 min and washed with the same buffer at 300 K. The vesicle pellets were then resuspended in 0.5 mL of water and subjected to radioactivity measurements.

For the binding to unilamellar liposomes, 1 mL of the conventionally purified phytochrome ($A_{660}/A_{280} > 0.60$; 1.03 mg of phytochrome/mL) was mixed with 1.5 mL of liposomes (4.5 mg of lipids) and incubated for 3 h at 300 K after irradiation with red or far-red light. The pH of the phytochrome solution was adjusted to 7.2 with Tris just before mixing. The incubation mixture was then applied to a Sepharose CL-4B column (1.5 × 30 cm) equilibrated with 50 mM sodium phosphate, pH 7.2, containing an appropriate amount of KCl. The column was eluted with equilibration buffer at a flow rate of 6 mL/h at 276 K.

As shown in Figure 3C, some fractions of phytochrome were coeluted with liposomes at the elution volume of 22 mL (fraction I); the void volume was 18 mL. Unilamellar liposomes (fraction II) and free phytochrome (fraction III) were eluted later. The amount of phytochrome recovered in fraction I of Figure 3C was determined to be a liposome-bound phytochrome (see Results). Centrifugation of this fraction at 34000*g* for 30 min at 276 K typically reduced 25% of the absorption of phytochrome and 30% of the absorption at 300 nm of liposome scattering of the solution. Only the supernatant was used for the spectrophotochemical characterization of liposome-bound phytochrome. After saturation of red or far-red light and incubation for 1 h at 300 K in the dark, the concentrated fraction I of Figure 3C was rechromatographed on the Sepharose CL-4B, as described above (Figure 3D).

Spectrophotometric Assays. All spectrophotometric assays were carried out by means of a Cary 118C spectrophotometer at 276 K under green safety light. The difference absorption spectra (Pfr vs. Pr) were recorded with Pfr in a sample beam vs. Pr in a reference beam; both Pfr and Pr were saturated with 660- and 730-nm light, respectively. The irradiation of phytochrome for photoconversion was performed with a Bausch & Lomb microscope illuminator with a 660-nm interference filter (Oriel C572-6600) for red light and a 730-nm infrared cut-off filter (Ealing 26-4457) for far-red light. Phototransformation rates were measured by monitoring the absorbance changes at 660 nm during the continuous irradiation of samples with red or far-red light in situ as described by Jung & Song (1979). Fluence rates of the red and far-red light were 0.19 and 3.04 W/m^2, respectively. Dark reversion rates were determined by the absorbance differences between those at 725 and 660 nm from the time-dependent difference spectra of Pfr vs. Pr. Acceleration of the dark reversion was achieved in the presence of 1 mM sodium dithionite (Pike & Brigss, 1972). The amount of phytochrome was determined spectrophotometrically by using the published extinction coefficient for phytochrome (Tobin & Briggs, 1973).

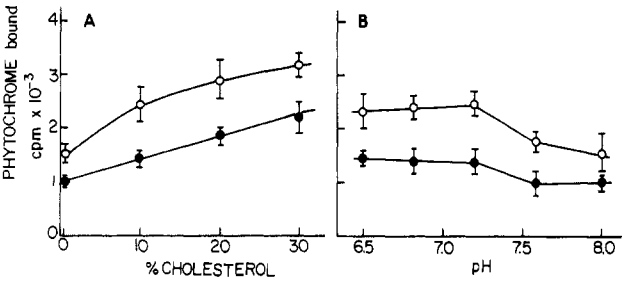

FIGURE 1: (A) Binding of ^{125}I-labeled phytochrome to multilamellar liposomes of egg lecithin supplemented with various concentrations of cholesterol at 300 K. The incubation mixture contained 4 mg of liposome lipids along with an appropriate amount of cholesterol, as indicated, and 50 μg of ^{125}I-labeled phytochrome (specific activity 3.12×10^3 cpm/μg of phytochrome) in 0.75 mL of 20 mM sodium phosphate buffer containing 70 mM KCl, pH 7.2. Incubation and other treatments were done as described under Materials and Methods. The bars represent the error ranges of three measurements, and % represents weight by percentage. Solid circle, Pr; open circle, Pfr. (B) pH profile of the binding of ^{125}I-labeled phytochrome to multilamellar liposomes of egg lecithin and 10% (w/w) cholesterol at 300 K. The reaction mixture contained the same amount of liposome lipids and phytochrome as for (A) in 0.75 mL of 20 mM sodium phosphate buffer containing 100 mM KCl, pH 7.2. The pH of the mixture was adjusted to the desired value with H_3PO_4 or NaOH, before incubation. Dilution and washing of the liposomes were done with buffer of the desired pH. The bars represent the error ranges of two measurements.

Measurements of Radioactivity and Lipid Concentrations. The radioactivity of samples containing ^{125}I-labeled phytochrome was measured by using a Beckman L-200 scintillation counter. One-half milliliter of the sample was added to 10 mL of a scintillation cocktail containing 9 g of PPO and 0.6 g of POPOP in 750 mL of Triton X-114 and 2250 mL of xylene.

Lipid concentrations were calculated from the calibration curve of turbidity at 300 nm vs. phospholipid phosphorus content. For the calibration curve, liposomes were prepared in the same way as described under Preparation of Liposomes except that the sonication buffer contained 10 mM Tris buffer (pH 7.2) instead of phosphate buffer. Multi- and unilamellar liposomes separated by Sepharose CL-4B column chromatography were assayed for absorbance at 300 nm and phosphorus content according to Gomori's method (1942). Liposomes were concentrated through a Millipore filter, when necessary. The phosphorus content of the egg lecithin was 3.92%.

Results

Binding of Phytochrome to Liposomes. The binding of phytochrome to multilamellar liposomes is a function of cholesterol content in the liposomes (Figure 1A) and pH of the medium (Figure 1B). It is clear that cholesterol enhances the binding of both the Pr and Pfr forms of phytochrome with the preferential binding of the Pfr form over the Pr form to the liposomes. However the ratios of the preferential binding have a tendency to decrease with increasing cholesterol content in the liposomes, showing the maximum preferential binding of 60% at 10% (w/w) cholesterol content. The pH effects show that the extent of binding remains almost constant in the neutral pH region (6.5–7.2) but decreases at pH's above 7.5. The error ranges of 10–25% between measurements was considered to be due to the inherent heterogenity of multilamellar liposomes. The kinetics of phytochrome binding to multilamellar liposomes are shown in Figure 2. It requires

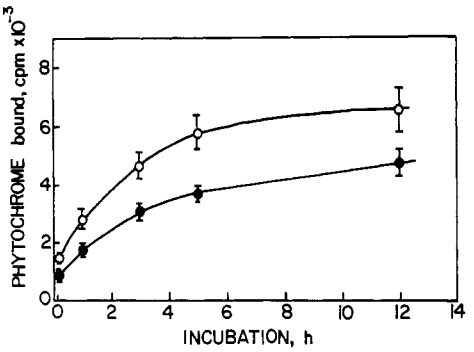

FIGURE 2: Time course of the ^{125}I-labeled phytochrome binding to multilamellar liposomes of egg lecithin and 10% (w/w) cholesterol at 300 K. Portions (0.75 mL) of the incubation mixture were removed, at the time intervals indicated, from the mixture of 4.5 mL containing 24 mg of liposome lipids and 300 μg of phytochrome (specific activity 3.11×10^3 cpm/μg of phytochrome) as Pr or Pfr in 20 mM sodium phosphate buffer containing 70 mM KCl, pH 7.2, and treated as described under Materials and Methods. Solid circle, Pr; open circle, Pfr. The bars represent the error ranges of two measurements.

several hours to reach an equilibrium of binding.

The addition of Pfr phytochrome to unilamellar liposomes showed a signficant increase in turbidity, while Pr gave no visible change. Gel chromatography of the incubation mixture of phytochrome with unilamellar liposomes shows three eluting peaks, which correspond to the relative elution volume (V_e/V_0) of 1.2 (fraction I) and the elution volume of unilamellar liposomes (fraction II) and free phytochrome (fraction III) as presented in Figure 3C. Fraction I contained both phytochrome and cholesterol [tested by the method of Watson (1960)], confirming the presence of liposomes. Before being mixed with phytochrome, the unilamellar liposomes eluted at the same elution volume as for fraction II (Figure 3B). After reaction with phytochrome, liposomes eluted in both fractions I and II. However, a significant amount of phytochrome was not present in fraction II, in which a substantial amount of unilamellar liposomes were eluted (Figure 3C). This result indicates that unilamellar liposomes undergo fusion to produce larger diameter liposomes. The phytochrome eluted in fraction I was concluded to be liposome-bound phytochrome, based on the following evidence. (a) Elution volume of the fraction was behind the void volume ($V_e/V_0 = 1.2$), and the ratio of phytochrome to liposomes (in terms of absorbances) was constant from one tube fraction to the other tube fraction. (b) Rechromatography of the fraction on a Sepharose CL-4B column recovered about 80% of both the original phytochrome and liposomes at the same elution volume as in the first column (Figure 3D). (c) After centrifugation of the fraction, a similar portion of phytochrome and liposomes (75% of the phytochrome absorption and 70% of the 300-nm absorption, mainly due to liposomes) remained in the supernatant. (d) Liposome-bound phytochrome has its own characteristic phototransformation (vide infra). It should be mentioned that phytochrome as Pr or Pfr ($A_{660}/A_{280} > 0.6$) was stable for 3 h in 0.1 M phosphate buffer (pH 7.2) containing 0.1–0.3 M KCl at the concentration of 0.4 mg/mL in terms of spectrophotometrically assayed photoreversibility and turbidity.

The binding of phytochrome to unilamellar liposomes is drastically affected by ionic strength as well as incubation temperature, as seen in Figure 4. By increasing the ionic strength of the medium, the binding of Pfr increased steadily, leveling off at KCl concentrations greater than 200 mM (Figure 4). However, the binding of Pr is not significantly enhanced. In fact, at a lower temperature (276 K) the binding of Pr slowly declines with an increase in KCl concentration.

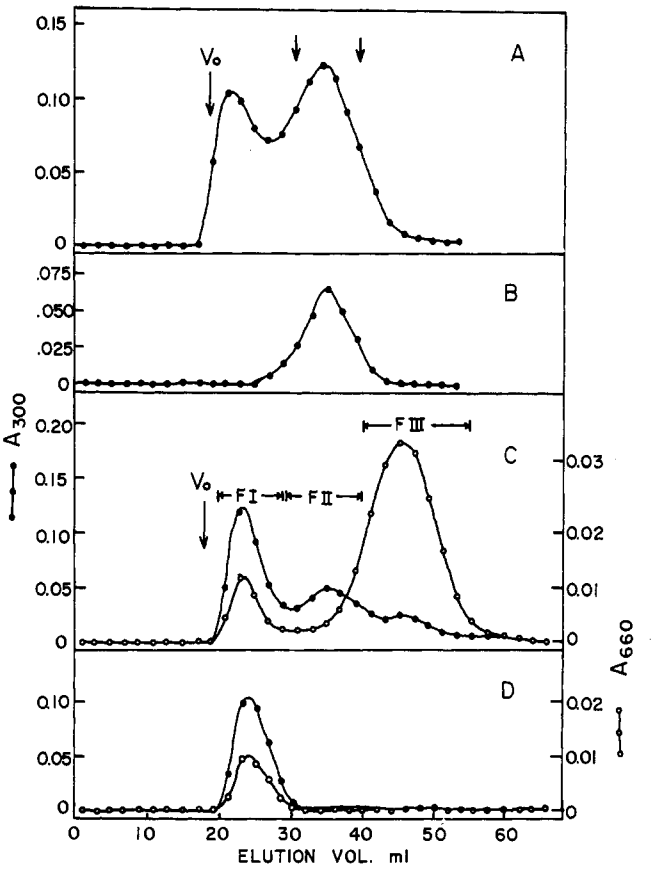

FIGURE 3: Gel filtration elution profiles of Sepharose CL-4B column (1.5 × 30 cm) at 276 K. Each fraction was assayed for absorbance at 300 nm for liposomes and at 660 nm for phytochrome after irradiation with far-red light. (A) Sonicated dispersion of 10 mg of egg lecithin supplemented with 1 mg of cholesterol in 2 mL of 20 mM sodium phosphate buffer containing 200 mM KCl, pH 7.2. (B) Unilamellar liposomes (2 mL) collected and concentrated from the fraction of (A), as indicated by arrows. (C) Incubation mixture of 1 mL of phytochrome solution ($A_{660}/A_{280} = 0.63$; 1.03 mg of phytochrome/mL) as Pfr and 1.5 mL of liposomes (4.5 mg of lipids) collected and concentrated from the second fraction of (A). The mixture contained 52 mM sodium phosphate and 140 mM KCl, pH 7.2; it was applied on the column after incubation for 3 h at 300 K. Void volume (V_0) was calibrated with λ-DNA (Biolabs, Bevorly, MA). (D) Rechromatography of the concentrated fraction I of (C). Equilibration and elution buffer: 20 mM sodium phosphate and 200 mM KCl, pH 7.2, for (A) and (B); 52 mM sodium phosphate and 140 mM KCl, pH 7.2, for (C) and (D).

Thus, at a high ionic strength and higher temperature, the preferential binding to liposomes of Pfr over Pr is magnified, as can be seen in Figure 4.

At maximum binding (in the presence of 0.2–0.3 M KCl; Figure 4), 80 μg of phytochrome and 2 mg of liposome lipids were recovered in fraction I of Figure 3C. On the basis of 3100 lipid molecules per egg lecithin unilamellar liposome supplemented with 10% (w/w) cholesterol (Newman & Huang, 1975), approximately one Pfr molecule is bound per unilamellar liposome. The amount of liposome lipids recovered in fraction I of Figure 3C is in general proportional to that of phytochrome present in that fraction.

Evidence for the apparent irreversible binding of phytochrome, i.e., no significant dissociation of bound phytochrome upon phototransformation with red/far-red light treatment, was obtained by rechromatography of liposome-bound phytochrome. Fraction I of Figure 3C was irradiated with red or far-red light, incubated for 1 h, and subjected to rechromatography (Figure 3D), as described under Materials and Methods. In both cases of irradiation, the recovery of phytochrome in the liposome fraction was about 80% without any

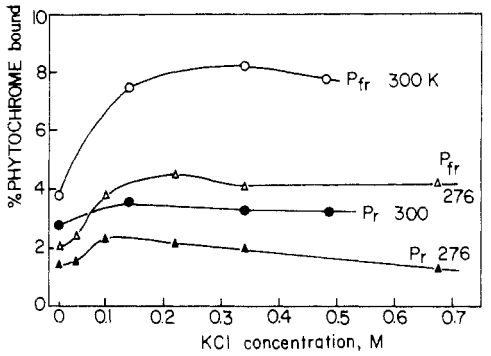

FIGURE 4: Ionic strength and temperature dependence of the binding of phytochrome to unilamellar liposomes containing 10% (w/w) cholesterol. KCl concentrations indicated are the final concentrations in the incubation mixture. A mixture of 1 mL of phytochrome solution ($A_{660}/A_{280} > 0.60$; 1.03 mg of phytochrome per mL) and 1.5 mL of liposomes (4.5 mg of lipids) were incubated for 3 h at 300 K or for 12 h at 276 K. The liposomes were prepared in 20 mM sodium phosphate buffer (pH 7.2) containing an appropriate amount of KCl. The incubation mixture was applied to a Sepharose CL-4B column and eluted as described under Materials and Methods. The numbers in the figure represent the incubation temperature. Percent of phytochrome bound was calculated from the amount of phytochrome eluted in the fraction I of Figure 3C against the total amount of phytochrome mixed with the liposomes.

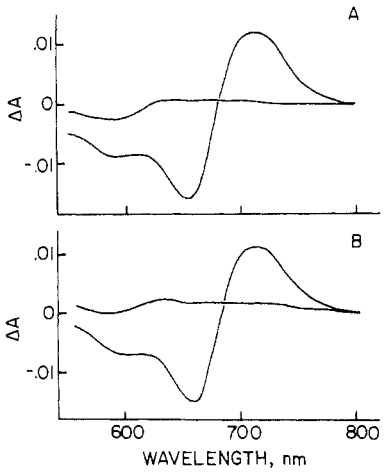

FIGURE 5: Difference absorption spectra of free phytochrome (A) and liposome-bound phytochrome as the Pfr form (B) in 52 mM sodium phosphate and 140 mM KCl (pH 7.2) at 276 K. The spectra were recorded with Pfr in a sample beam vs. Pr in a reference beam.

significant difference between the red and far-red treated samples.

Spectral and Photochemical Properties of Liposome-Bound Phytochrome. The absorption spectrum of liposome-bound phytochrome cannot be clearly resolved, due to its low absorbance and the turbidity of liposomes. However, the spectral properties of liposome-bound phytochrome can be compared with those of free phytochrome based on their difference spectra (Figure 5). There is no significant difference between the absorption maxima of bound and free phytochromes at 276 K. However, the absorbance ratio of 725 nm to 667 nm decreases from 0.72 for the free phytochrome to 0.57 for the bound phytochrome. This behavior is reminescent of the effect of the hydrophobic fluorescence probe, ANS, on Pfr's absorption band maximum, which is reduced in intensity by the binding of ANS (Hahn & Song, 1981). However, Pratt & Cundiff (1975) observed that the presence of cations such as Ca^{2+} and Zn^{2+} also reduced the far-*red* extinction of Pfr.

The phototransformation plots shown in Figure 6 were treated with a linear regression method, yielding the rate constants presented in Table I. The phototransformation of

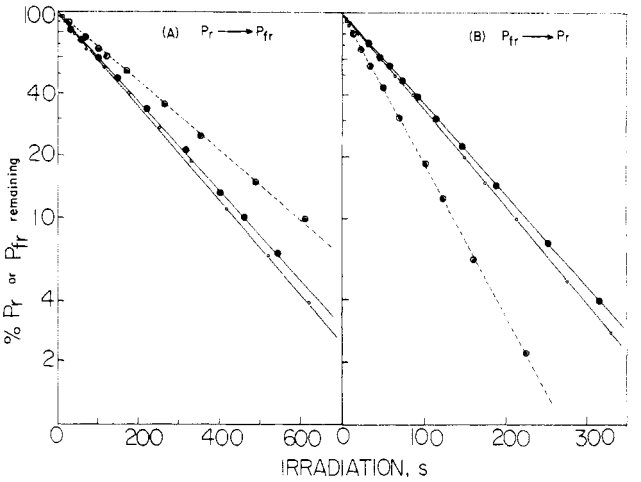

FIGURE 6: (A) Pr to Pfr phototransformation of phytochrome, free (broken line) and bound (unbroken line) forms (●, uncycled Pfr; ○, Pfr produced from Pr in the bound form), at 276 K. The rates were measured by monitoring the absorbance decrease at 660 nm during continuous irradiation with red light. The fluence rate was 0.19 W/m^2 with a 660-nm interference filter. The phytochrome concentration used was 0.16 μM for free phytochrome, 0.10 μM for bound phytochrome as Pr, and 0.14 μM for bound phytochrome as Pfr. The buffer composition was 52 mM sodium phosphate and 140 mM KCl at pH 7.2. (B) The Pfr to Pr phototransformation of free (broken line) and bound (unbroken line) phytochromes (same symbols as for (A) at 276 K. The rates were measured by monitoring the absorbance increase at 660 nm with continuous irradiation with far-red light. The fluence rate was 3.04 W/m^2 with a 730-nm infrared cut-off filter. Phytochrome concentrations and buffer composition were the same as in (A).

Table I: Phototransformation Rates of Free and Liposome-Bound Phytochrome at 276 K[a]

phyto-chrome	Pr → Pfr $k_1 \times 10^3$ (s^{-1})	relative k	Pfr → Pr $k_2 \times 10^2$ (s^{-1})	relative k
free	3.83 (r = 0.999)[b]	1.00	1.656 (r = 1.000)	1.00
bound as Pr	5.18 (r = 0.998)	1.35	1.017 (r = 1.000)	0.61
bound as Pfr	4.94 (r = 1.000)	1.29	1.079 (r = 0.999)	0.65

[a] The absorbance changes at 660 nm during irradiation with red or far-red light, as described in Figure 6, were analyzed by linear regression. [b] r, correlation coefficient.

bound Pr to Pfr is noticeably faster than that of free Pr, whereas the photoreversion from Pfr to Pr is significantly inhibited by the binding of Pfr to liposomes.

Dark Reversion of Liposome-Bound Phytochrome. Parallel to photoreversion, the dark reversion of Pfr is somewhat inhibited upon its binding to liposomes (Figure 7 and Table II). The dithionite acceleration rate of the dark reversion (Mumford & Jenner, 1971; Pike & Briggs, 1972) is lowered by 50% for liposome-bound Pfr in comparison to that for free Pfr (Table II).

Binding of Phytochrome to Protoplasts. The Pfr form of phytochrome preferentially binds to certain subcellular organelles and membranes such as mitochondria (Marmé, 1974; Furuya & Manabe, 1976; Cedel & Roux, 1980). Report on a study of phytochrome binding to protoplasts is not available in the literature. Thus, it was of interest to examine whether Pfr preferentially binds to right-side-out protoplasts, prepared from oat seedling tissue. We were not able to find any significant differences between the binding capacities of Pfr and Pr under the conditions employed, i.e., sodium phosphate concentrations from 5 to 40 mM with or without 7.7 mM

Table II: Dark Reversion Rates of Free and Liposome-Bound Phytochrome in the Presence or Absence of Sodium Dithionite[a]

phytochrome	presence (+) or absence (−) of 1 mM sodium dithionite	fast component $k \times 10^3$ (s^{-1})	fast component dithionite acceleration[d]	slow component $k \times 10^5$ (s^{-1})	slow component dithionite acceleration[d]
free	−	1.69 (r = 0.961)[b]	1.0	1.41 (r = 0.998)	1.0
	+	0.85 (r = 0.985)	0.5	14.96 (r = 0.996)	10.6
bound as Pr	−	1.26 (r = 0.998)	0.8	1.43 (r = 0.992)	1.0
	+	ND[c]		ND	
bound as Pfr	−	1.08 (r = 0.998)	0.6	1.14 (r = 0.994)	0.8
	+	0.70 (r = 0.989)	0.4	7.49 (r = 0.998)	5.3

[a] The fast and slow kinetic components were resolved by the peeling method (Hahn & Song, 1981) from the data in Figure 7. [b] r, correlation coefficient. [c] Not determined. [d] Relative to the dark reversion rate of free Pfr in the absence of dithionite.

Table III: Binding of ^{125}I-Labeled Phytochrome to Oat Protoplasts in 15.4 mM Sodium Phosphate and 0.5 M Mannitol at pH 7.2[a]

phytochrome incubated	cpm due to bound phytochrome: protoplasts without Mg^{2+}	protoplasts with Mg^{2+}[b]	protoplasts Mg^{2+}[b] and KCl[c]
Pr	2600 ± 300	3700 ± 500	3300 ± 400
Pfr	2700 ± 400	3800 ± 300	3200 ± 500

[a] Protoplasts (1.5×10^7 protoplasts; 1 mL) in 0.6 M mannitol was mixed with 0.2 mL of ^{125}I-labeled phytochrome (40 μg of phytochrome; specific activity 7.6×10^3 cpm/μg of phytochrome) and 0.1 mL of 0.6 M mannitol and incubated for 1 h at 300 K; 0.6 M mannitol was replaced by 0.6 M mannitol containing 100 mM $MgCl_2$ or 0.6 M mannitol containing 100 mM $MgCl_2$ plus 1 M KCl, when needed. The incubation mixture was treated as described under Materials and Methods. [b] Final concentration 7.7 mM. [c] Final concentration 77 mM.

$MgCl_2$ (e.g., see Table III). The addition of 77 mM KCl to an incubation mixture containing Mg^{2+} reduced the amount of phytochrome binding by about 10%, but there was no differential binding between Pr and Pfr. Washing the phytochrome-bound protoplasts three times with sodium phosphate buffer did not significantly remove the ^{125}I-labeled phytochrome radioactivity from the protoplasts.

Discussion

The interaction of Pfr phytochrome with liposomes has a hydrophobic character, as evidenced by the effect of ionic strength and temperature on its binding to unilamellar liposomes. The extent of Pfr binding increases with increasing ionic strength as well as increasing temperature. The Pr binding is also not a typical electrostatic interaction, since its binding is not drastically inhibited above a KCl concentration of 0.1 M (Figure 4). The electrostatic interaction between proteins and phospholipids has been shown to be inhibited at 0.1 M ionic strength (Das & Crane, 1964).

The effect of cholesterol incorporated within the liposomes on their permeability is dependent upon the type of liposome.

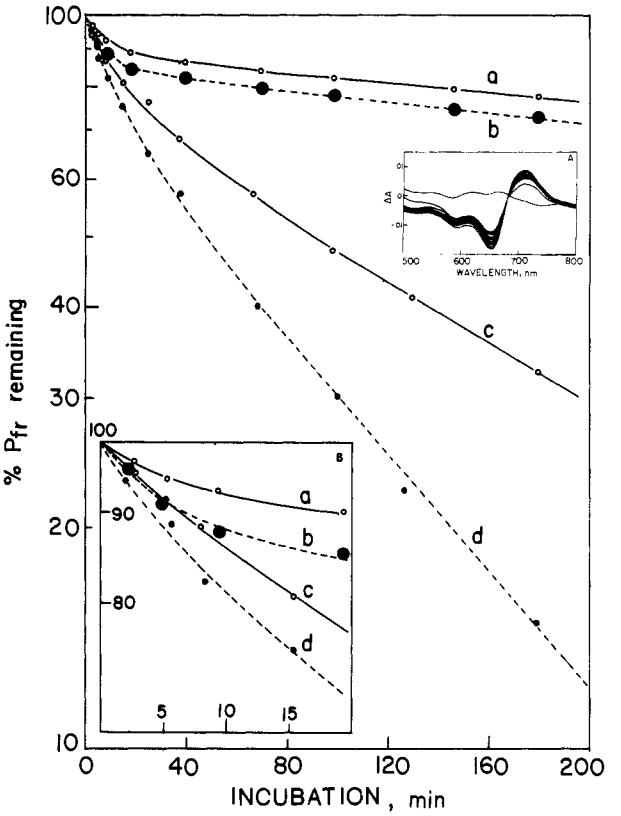

FIGURE 7: Dark reversion of free (broken lines) and bound (unbroken lines) phytochromes in the absence (A and B) and presence (C and D) of 1 mM sodium dithionite at 276 K. The rates were determined by the absorbance changes from the time course of the difference spectra (inset A) with Pfr in a sample beam vs. Pr in a reference beam. The concentration of free phytochrome was 0.12 μM. The concentrations of phytochrome bound as Pr or Pfr were 0.08 μM and 0.12 μM, respectively, in 52 mM sodium phosphate and 140 mM KCl, pH 7.2.

In the small (30–50-nm diameter) unilamellar liposome, the presence of cholesterol increases its permeability to hydrophobic molecules, whereas its permeability to hydrophilic molecules is decreased (LaBelle & Racker, 1977). However, cholesterol decreases the permeability of multilamellar liposomes to both hydrophobic and hydrophilic molecules (DeGier et al., 1970; Demel et al., 1972). From the data shown in Figure 1A, the ratio of preferential binding of Pfr to multilamellar liposomes decreases to some degree above a cholesterol content of 10% (w/w). This is also indicative of the hydrophobic nature of Pfr binding to liposomes. However, it can also be seen that the binding of both Pr and Pfr is enhanced with increasing cholesterol content in the liposomes.

The increased binding capacity of phytochrome with cholesterol may be explained in terms of the binding of phytochrome to the cholesterol phase of the lipid bilayer, since cholesterol itself is known to bind to both Pr and Pfr (Roth-Bejerano & Kendrick, 1979). However, these authors did not find significant interactions between phytochrome and lecithin, contrary to our result. Georgevich et al. (1976) had reported that only the Pfr form could bind to a liposome made of lecithin and cholesterol.

It is well-known that cholesterol introduces an additional ordering of the hydrocarbon side chains to the lipid bilayer (Levine & Wilkins, 1971; Lapper et al., 1972). To what extent the rigidity of the bilayer augmented by cholesterol enhances the phytochrome binding remains to be studied.

Cholesterol-egg lecithin liposomes are essentially neutral in the pH range 6.5–7.2, where phytochrome appears to bind to the hydrophobic phase of the bilayer. However, at higher

pH's this binding decreases, apparently as a result of unfavorable charge interactions between the phytochrome protein (p*I* 5.8–6.4; Hunt & Pratt, 1979) and the liposome lipid polar heads (especially phosphate groups). This implies that phytochrome–liposome binding requires an initial electrostatic interaction before the subsequent hydrophobic stabilization. This type of interaction is also seen in the interaction of some water-soluble proteins with phospholipid bilayers (Kimelberg & Papahadjopoulos, 1971a,b).

The photoreversion and dark reversion of liposome-bound Pfr are significantly slower than those of free Pfr (Tables I and II), while the phototransformation of bound Pr to Pfr is enhanced. An interesting implication of this observation is that photostationary concentrations of Pfr should be different for bound and free phytochrome. Furthermore, the rate of dithionite-accelerated dark reversion of the liposome-bound Pfr is lower by as much as 50% than that of free Pfr (Table II). This trend is similar to the accelerating and inhibitory effects of the hydrophobic fluorescence probe, 8-anilino-1-naphthalenesulfonate, on the phototransformation and photoreversion of Pr and Pfr, respectively (Hahn & Song, 1981).

It was also found that tryptophan fluorescence in liposome-bound Pfr exhibits an increase in intensity, relative to a free Pfr solution (unpublished results). This is also consistent with the postulate of a hydrophobic interaction between the Pfr phytochrome and liposome bilayer, as it has been suggested that tryptophan residue(s) are located at the chromophore binding site (Song et al., 1979; Song, 1981).

These observations may suggest that the chromophore binding site, which becomes at least partially exposed upon phototranformation to Pfr, is occupied by the hydrophobic liposome bilayer. As a consequence, the binding facilitates the phototransformation of Pr to Pfr, retards its reversion, and makes it difficult for dithionite to reach its action site(s), which may be buried upon binding.

The present study provides strong evidence in support of the model that the physiological activity of the Pfr form of phytochrome, in contrast to the physiologically inactive Pr form, is associated with the development of a hydrophobic surface area where binding, to an as yet unidentified Pfr receptor or membrane, may take place (Song et al., 1979; Song, 1980; Hahn & Song, 1981).

The fusion of unilamellar liposomes to larger diameter liposomes upon binding to phytochrome is of interest because membrane fusion in vivo is an important event in a wide range of cellular and subcellular activities (Poste & Allison, 1973; review). This phenomenon remains to be studied in detail even though several agents such as calcium ion (Papahadjopoulos et al., 1975), *n*-alkyl bromides (Mason & Miller, 1979), and inactivated Sendai virus (Bächi et al., 1973) are known to induce vesicle fusion in vitro.

In contrast to the observed differential binding of Pfr vs. Pr to liposomes, no significant differences in binding to Pfr and Pr to protoplasts were found (Table III). Although Mg^{2+} ions enhance the binding, both Pr and Pfr bind equally well to the protoplasts, within experimental error. The 77 mM KCl lowers the binding capacity of phytochrome (Table III), in contrast to the binding of phytochrome, particularly Pfr, to liposomes (cf. Figure 4). These results suggest that the binding of phytochrome to the exterior membrane of protoplasts may involve interacting sites different from those in binding to the interior membrane of protoplasts and to the liposome bilayer. At present, it is not known whether the Pfr form preferentially binds to the interior membrane of protoplasts. Marmé (1974) has suggested that phytochrome is localized in the plasma membrane. If so, it may be possible to demonstrate a differential binding of Pfr vs. Pr to the interior membrane ("wrong-side-out" vesicles) of protoplasts. Work is in progress to test this hypothesis.

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Phospholipid Topography of the Photosynthetic Membrane of *Rhodopseudomonas sphaeroides*†

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ABSTRACT: The topography of phospholipids in the photosynthetic membranes of *Rhodopseudomonas sphaeroides* was investigated by using purified chromatophores and spheroplast-derived vesicles (SDVs). Chromatophores are closed vesicles oriented inside out with respect to the cytoplasmic membrane (cytoplasmic side out) and obtained from French-pressed cell lysates. SDVs are oriented right side out (periplasmic side out) and are obtained after osmotic lysis of lysozyme-treated cells. Phosphatidylethanolamine (PE) comprised ~62% and phosphatidylglycerol (PG) comprised ~33% of the total phospholipid of both vesicle preparations. The relatively membrane impermeable reagent trinitrobenzenesulfonate (TNBS) at 3 mM concentration and 5 °C modified chromatophore and SDV PE with kinetics indicating the occurrence of fast- and slow-reacting pools of PE. The fast-reacting pools comprised 33% and 55% of the total PE of chromatophores and SDVs, respectively. The slow-reacting pools comprised 61% and 32% of the total PE of chromatophores and SDVs, respectively. Phospholipase A_2 treatment of chromatophores (1 unit/mg of vesicle protein) for 1 h at 37 °C resulted in hydrolysis of 73% and 77% of the total PG and PE, respectively. Similar enzyme treatment of SDVs resulted in 14% and 60% hydrolysis of the total PG and PE, respectively. Phospholipase A_2 treatment inhibited 60% of the succinate dehydrogenase activity of chromatophores but only 8% of the activity of SDVs, indicating the membrane impermeability of phospholipase A_2. Incubation of chromatophores for 10 min with 3 mM TNBS at 5 °C and then treatment with phospholipase A_2 for 10 min and 1 h resulted in the hydrolysis of 10% and 61%, respectively, of unmodified PE. The results indicate asymmetric distributions of PE polar head groups (32–33% cytoplasmic side, 55–61% periplasmic side) and PG (73% cytoplasmic side, 14% periplasmic side) across the membrane. Also, a rapid and unidirectional transbilayer movement of PE polar head groups from the periplasmic to cytoplasmic surfaces of the membrane appears to occur during phospholipase A_2 hydrolysis on the chromatophore surfaces.

When grown anaerobically with light, the bacterium *Rhodopseudomonas sphaeroides* synthesizes a photosynthetic energy-transducing membrane system. The photosynthetic membrane is comprised of vesicular intracellular invaginations of the cytoplasmic membrane (Niederman & Gibson, 1978). Preparations of closed membrane vesicles representative of this membrane system are derived from French-pressed cell lysates (commonly termed chromatophores) (Gorchein et al., 1968; Fraker & Kaplan, 1971) or from extracts prepared by osmotic lysis of spheroplasts (Hellingwerf et al., 1975; Matsuura & Nishimura, 1977; Lommen & Takemoto, 1978). By several criteria, chromatophores are known to be oriented similarly to the in vivo intracellular vesicles and therefore inside out with respect to the cytoplasmic membrane (cytoplasmic surface out) (Scholes et al., 1969; Prince et al., 1975; Matsuura & Nishimura, 1977). In contrast, spheroplast-derived vesicles (SDVs) are oriented right side out with respect to the cytoplasmic membrane (periplasmic surface out) (Hellingwerf et al., 1975; Matsuura & Nishimura, 1977; Michels & Konings, 1978; Lommen & Takemoto, 1978; Takemoto & Bachmann, 1979). Chromatophores and SDVs can be respectively greater than 95% and 80% uniformly oriented (Lommen & Takemoto, 1978; Elferink et al., 1979; Takemoto & Bachmann, 1979). The availability of opposite and uniformly oriented vesicles with complementary exposed surfaces provides opportunities to investigate the transmembrane topography of the components comprising this membrane.

Approximately 25% of the *R. sphaeroides* photosynthetic membrane is phospholipid (Fraker & Kaplan, 1971). The phospholipids are known to assume key roles in the biosynthesis of the membrane (Lascelles & Szilagyi, 1965; Leuking et al.,

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