Photobleaching of the Photoactive Yellow Protein from *Ectothiorhodospira halophila* Promotes Binding to Lipid Bilayers: Evidence from Surface Plasmon Resonance Spectroscopy

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ABSTRACT   The photoactive yellow protein (PYP) from the phototrophic bacterium *Ectothiorhodospira halophila* is a small, soluble protein that undergoes reversible photobleaching upon blue light irradiation and may function to mediate the negative phototactic response. Based on previous studies of the effects of solvent viscosity and of aliphatic alcohols on PYP photokinetics, we proposed that photobleaching is concomitant with a protein conformational change that exposes a hydrophobic region on the protein surface. In the present investigation, we have used surface plasmon resonance (SPR) spectroscopy to characterize the binding of PYP to lipid bilayers deposited on a thin silver film. SPR spectra demonstrate that the net negatively charged PYP molecule can bind in a saturable manner to electrically neutral, net positively, and net negatively charged bilayers. Illumination with either blue or white light of a PYP solution, which is in contact with the bilayer, at concentrations below saturation results in an increase in the extent of binding, consistent with exposure of a high affinity hydrophobic surface in the photobleached state, a property that may contribute to its biological function. A value for the thickness of the bound PYP layer (23 Å), obtained from theoretical fits to the SPR spectra, is consistent with the structure of the protein determined by x-ray crystallography and indicates that the molecule binds with its long axis parallel to the membrane surface.

INTRODUCTION

Photoactive yellow protein (PYP) is a small (M_r 14,000), water-soluble, negatively charged protein having a visible absorption wavelength maximum of 446 nm ($\epsilon = 48$ mM^{-1} cm^{-1}), which undergoes a light-induced bleach and recovery cycle similar to that of the sensory rhodopsins (Meyer et al., 1987). The protein has been isolated and purified from three families of halophilic phototrophic bacteria (Meyer, 1985; Meyer et al., 1990; T. E. Meyer, unpublished data), although immunological evidence indicates that it may be even more widespread in its occurrence (Hoff et al., 1994). Action spectra indicate that PYP may be involved in mediating a negative phototactic response in *Ectothiorhodospira halophila* (Sprenger et al., 1993).

A 2.4 Å crystal structure determination of PYP has been reported (McRee et al., 1989) that reveals a β sandwich-type folding similar to those of the biliproteins, and fatty acid and retinol binding proteins (Newcomer et al., 1984; Holden et al., 1987; Eads et al., 1993). Amino acid sequence determination and mass spectroscopic analysis indicate that the chromophore has a molecular weight of 147 and is bound to Cys-69, perhaps via a thiol ester linkage (Van Beeumen et al., 1993). Although the chemical structure of the chromophore is presently uncertain, PYP is clearly not related to the retinal-type proteins belonging to the rhodopsin family, despite its photochemical properties.

The kinetic characteristics of the PYP photocycle have been extensively studied (Meyer et al., 1987, 1989, 1991, 1993). The 446 nm absorbance band is partially bleached and red-shifted (to $\approx$495 nm) in less than 10 ns. This is followed by a further bleach ($k \approx 10^4$ s^{-1}) and blue-shift to $\approx$340 nm, and a slower exponential recovery ($k \approx 2$ s^{-1}). The effects of solvent viscosity on the kinetics of the photobleaching process suggest the occurrence of a protein conformational change (Meyer et al., 1989), and the transient uptake and release of a proton has been shown to accompany the bleach and recovery cycle (Meyer et al., 1993). The quantum yield for the photobleaching process is appropriately large for a biologically relevant event (0.64; Meyer et al., 1989); consistent with this, the fluorescence lifetime of PYP is 12 ps (Meyer et al., 1993).

The addition of aliphatic alcohols to PYP solutions has been found to accelerate the photobleaching process and to retard the recovery in a manner that correlates with the hydrophobicity of the alcohol (Meyer et al., 1989). This has been attributed to the exposure of a hydrophobic site on the protein as a consequence of the conformational change that accompanies photobleaching. Although the mechanism by which PYP may mediate the negative phototactic response is unknown at present, this type of structural change suggests that the photobleached form of PYP can interact with the membrane-bound flagellar apparatus. To provide further insights into this aspect of PYP function, we have utilized surface plasmon resonance (SPR) spectroscopy to characterize the binding of PYP to electrically charged and neutral lipid bilayer membranes, in the presence and absence of visible light irradiation.

In other laboratories, the SPR technique has been applied to the optical properties of thin film coatings (Rothenhauser

et al., 1988), to metal-electrolyte interfaces (Rothenhauser et al., 1988; Abeles, 1979), and to specific recognition reactions at self-assembled monolayers on gold (Pockrand et al., 1977; Hickel and Knoll, 1990; Haussling et al., 1991; Morgan et al., 1992; Schuster et al., 1993; Terrettaz et al., 1993; Swanson et al., 1993); Bondeson et al., 1993). In previous work from this laboratory involving SPR measurements, we have characterized the structural properties of self-assembled solid-supported planar lipid bilayers composed of egg phosphatidylcholine (PC) (Salamon et al., 1994a), which we have previously used in electrochemical measurements (Salamon and Tollin, 1991, 1992; Salamon et al., 1992, 1993), and carried out studies of lipid bilayer-protein association using rhodopsin (Salamon et al., 1994b). The results of these investigations clearly demonstrate the utility of this methodology for obtaining information on the process of protein reconstitution into a lipid bilayer, and on light-induced conformational alterations of reconstituted rhodopsin. Here we apply this technique to demonstrate association between a lipid bilayer and two water-soluble proteins (cytochrome c and PYP). As will be demonstrated below, the results are consistent with an increase in the hydrophobic character of PYP in the photobleached state.

MATERIALS AND METHODS

Photoactive yellow protein from *E. halophila* was obtained as previously described (Meyer, 1985). Using a microliter syringe, small aliquots of a concentrated PYP solution (140 μM) were added to the aqueous compartment of the SPR apparatus, which contained 1.5 ml of 5 mM phosphate buffer, pH 7.0. Horse heart cytochrome c (Type VI) was obtained from Sigma Chemical Co. (St. Louis, MO) and was used in the oxidized form without additional purification.

Lipid film formation and SPR cell design

Self-assembled lipid films were formed from a solution containing 8 mg/ml of egg phosphatidylcholine (PC; Sigma), plus or minus either 30 mol% dioctadecyldimethylammonium chloride (DODAC; American Tokyo Kasei, Inc., Portland, OR), or 25 mol% dihexadecylphosphate (DHP; the potassium salt was generated from the corresponding free acid obtained from Sigma), in squalene/butanol (0.3:10 v/v) (Fluka Chemie AG, Buchs, Switzerland). The measurements with cytochrome c have utilized egg PC bilayers with compositions as described in the legend to Fig. 2. The lipid bilayers were generated on the metal surface using a procedure described previously (Salamon and Tollin, 1993; Salamon et al., 1994a). This method of membrane formation is quite similar to that used to form freely suspended lipid bilayer membranes separating two aqueous solutions (Pethig, 1979; Ivanov, 1988; Tien et al., 1991). It involves spreading a small amount of lipid solution ($\approx$4 μl) via a Hamilton microsyringe across an orifice in a Teflon sheet (4 mm in diameter) that separates the silver film from the aqueous phase (Fig. 1).

SPR measurements were performed using a previously described cell (Salamon et al., 1994a) that contains a right angle glass prism coated with a thin metallic silver film, which is in contact with an aqueous compartment. The silver was vacuum-deposited onto the prism (BK-7 glass) with a deposition rate of 1 nm/s. Before deposition, the prisms were cleaned with reagent grade solvents and blown dry with nitrogen gas. Fig. 1 shows a schematic diagram of the cell, together with the experimental geometry used for the SPR studies.
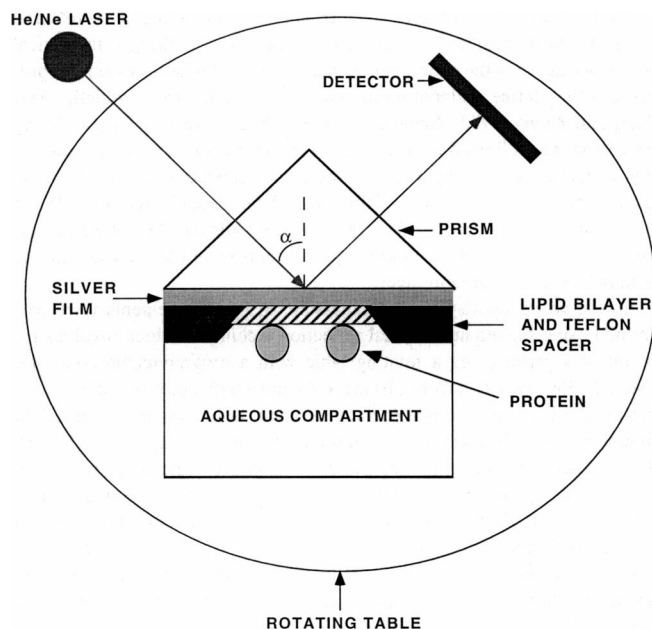

FIGURE 1   Schematic view from the top of the SPR apparatus.

Illumination of the sample was accomplished using a fiber optic bundle that entered the SPR cell from the aqueous compartment side. The light source was an Oriel Model 77500 Illuminator, which was used at full intensity with or without a Corning CS 5–59 blue filter (wavelength range 350–500 mnm) or a Schott OG 515 filter (wavelengths >500 nm).

Surface plasmon resonance measurements

A surface plasmon, described as a fluctuation of electron density propagating along a metal surface, can be generated by photons that create an evanescent electromagnetic field in the incident medium, usually on the order of 100 nm in depth. When such a light beam is reflected from the back surface of a thin metallic film (thickness $\approx$ 50 nm), the electromagnetic field polarized parallel to the incident plane excites collective oscillations of the free electrons within the metal (i.e., surface plasmons), which propagate along the interface between the metal and the emergent medium (which may be air or water), with an amplitude that decays exponentially on both sides of that interface. This situation can be described theoretically by Maxwell's equations (Macleod, 1986; Macleod, 1992). A resonance condition exists for the excitation of surface plasmons that is fulfilled by varying the incidence angle (α; see Fig. 1) at a fixed excitation wavelength. Resonance causes the energy of the incident light to be absorbed by the surface plasmon wave, and less light is thereby reflected by the interface between the incident medium and the metal film. At resonance, the reflected energy reaches its minimum. Thus, the measurement of reflectance of the beam as a function of the incident angle produces the SPR curve. Electromagnetic theory shows that the properties of the SPR curve depend on three optical parameters: the refractive index (n), the extinction coefficient (k), and the thickness (t) of the metal film plus the dielectric medium in close contact with it. Therefore, the resonance effect, together with the fact that the electromagnetic field that excites surface plasmons penetrates the interface to the depth of only a fraction of the exciting light wavelength, makes this phenomenon exceedingly sensitive to the optical conditions at the metal surface. Any surface modification in the immediate vicinity of the metal, such as deposition of a lipid bilayer, will change the resonance condition by shifting the position of the minimum and altering the shape of the resonance curve. In our experiment, the incident medium is a glass prism with $n = 1.515$ upon which the layer of metallic silver is deposited. The emergent medium is water that is in contact with the outer metal surface or with a deposited lipid film.

To evaluate the n, k, and t parameters of an adsorbed dielectric material using the SPR method, one can analyze the data by fitting a theoretical resonance curve to the experimental one, using nonlinear least-squares procedures that define a global minimum for the fitting error (Liddell, 1981; Tang and Zheng, 1982; Zhang et al., 1987). In our experiments, the fitting procedure was followed for the bare silver film in contact with an aqueous medium, and the optical parameters thereby calculated were used to obtain the best fit to the SPR curves of the lipid bilayer-coated silver film. When protein molecules were adsorbed onto the lipid bilayer, the optical parameters for the silver and the lipid bilayer systems were used to evaluate the parameters for the protein layer.

In the work reported here, steady-state SPR measurements were performed using the attenuated total reflection technique, which involves rotation of a prism using a rotating table with a programmable controller (Model 855C, Newport Corp., Irvine, CA) and a light detector (silicon solar cell) and a laser light source (helium-neon cw laser; wavelength 6328 Å) mounted on the fixed arm of a goniometer (Salamon et al., 1994a) (Fig. 1). This arrangement allows us to measure the incidence angle with an accuracy of 0.01°. The photocurrent output from the light detector was digitized by a Heath-Zenith SD 4850 digital oscilloscope, and the data were transferred to the hard disk of a computer. The optical arrangement described above allows the SPR spectrum to be measured over a 20° rotation with an acquisition time of 10 s. All measurements were done at room temperature (23 ± 0.5°C).

There are three sources of experimental error that need to be considered in these experiments. First, there is an error in the measurement of the incident angle ($\Delta\alpha$). As indicated above, $\Delta\alpha \approx 0.01°$, which translates into thickness and refractive index errors of 0.5 Å and 0.005, respectively. Second, there is the error that is related to the precision of the theoretical fitting (SD 0.010–0.035), which is relatively small. Probably the largest error in the absolute values of the parameters derives from variations in the lipid bilayer structure. This is reflected in the scattering of the experimental shifts and shape changes of the SPR spectra between different samples. Such scattering produced errors in thickness of $\Delta t = \pm 1$ Å, in refractive index of $\Delta n = \pm 0.02$, and in extinction coefficient of $\Delta k = \pm 0.02$. To characterize the structure of the deposited lipid membrane and the bound protein, it is useful to calculate the mass of the materials forming the surface films, using the optical constants obtained from the SPR measurements. To do this, we have used a Lorentz-Lorenz relationship (Born and Wolf, 1965; Salamon et al., 1994a), in which the mass is expressed in terms of the partial specific volumes of the molecules, the molar refractivity, the thickness of the adsorbed layers, and the refractive indices of the pure materials.

Upon addition of a small volume of PYP solution in the dark to the sample cell containing a previously formed lipid bilayer, the SPR spectra were found to change slowly over a period of 20–30 min before reaching a final position. All of the spectra and the associated fitting parameters given below were obtained after equilibrium was reached. In contrast, SPR spectral changes induced by light were immediate (<1 min) and did not change further with time of irradiation.

RESULTS AND DISCUSSION

To demonstrate the ability of our system to detect association between lipid bilayers and water soluble proteins, we have carried out some preliminary measurements with the very well characterized protein cytochrome c, whose association with membranes has been very well studied (Nicholls, 1976; Roberts and Hess, 1977; Brown and Wuthrich, 1977; Gupte and Hackenbrock, 1988; Heimburg et al., 1991; Muga et al., Spooner and Watts, 1992; Cheddar and Tollin, 1992; Snel and Marsh, 1994). Fig. 2 shows typical experimental SPR curves that illustrate cytochrome c binding to silver films coated with an electrically neutral (egg PC; panel A) and a negatively charged (egg PC plus dihexadecylphosphate; panel B) lipid bilayer. The binding of cytochrome c to the
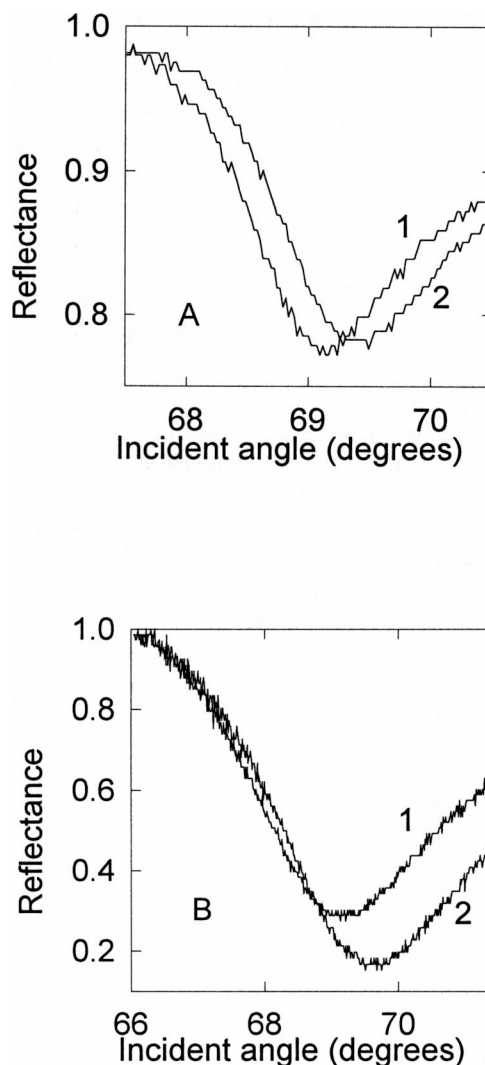

FIGURE 2   Experimental SPR spectra obtained with lipid bilayer made either from a membrane-forming solution containing 10 mg/ml PC (A) or 2.5 mg/ml PC plus 30 mol% DHP (B) deposited on a silver film, in the absence (curve 1) and the presence (curve 2) of 0.1 mM cytochrome c.

lipid bilayers is clearly indicated by a shift of the resonance curves to larger incident angle values. Note also that the thickness obtained from theoretical fits to the data for the protein film adsorbed onto the lipid bilayer is the same in both cases (31 Å, which is in good agreement with the known dimensions of the cytochrome c molecule), whereas the refractive index values are different (1.43 for the neutral PC bilayer, and 1.53 for the negatively charged lipid membrane). The difference in n values is due to differences in the membrane-bound protein mass (lipid to cytochrome mole ratio is approximately 40 and 20 for the neutral and negatively charged bilayers, respectively). Thus, the amount of bound cytochrome c obtained with the neutral egg PC is increased significantly by incorporating negatively charged molecules into the lipid bilayer, as is expected as a consequence of electrostatic interactions between the positively charged cytochrome and the negative lipid surface. From the

electrochemical measurements, we know that such incorporation also improves cytochrome c electron transfer to the silver electrode surface (Salamon and Tollin, 1991).

Positively charged bilayers

Fig. 3 shows SPR spectra obtained from a silver film with a net positively charged PC/DODAC bilayer deposited on it, in the presence and absence of a saturating amount of PYP added to the aqueous phase. The binding of PYP to the lipid surface is clearly indicated by the shift of the SPR resonance to larger incident angles. The observed changes in the SPR spectra were found to saturate when the total PYP concentration in the aqueous cell compartment reached approximately 6 μM (Fig. 4). The SPR curve shown in Fig. 3 is that obtained at saturation. Also shown in Fig. 3 are the theoretical fits to these SPR curves, from which the values given in Table 1 for the optical parameters n and t of the protein layer, and the molar ratio of total lipid to bound protein, are obtained (see Materials and Methods). As is evident, the data are fit quite well by the calculated curves. The values for k, which reflect mainly scattering effects in the surface films (Wijekoon et al., 1992; Salamon et al., 1994a, b), are not included in Table 1. These values are rather small, ranging from 0.0 to 0.02 for all of the systems studied here, and do not provide any useful information.

The value obtained for the thickness of the PYP layer (23 Å) is in good agreement with that obtained from x-ray dif-
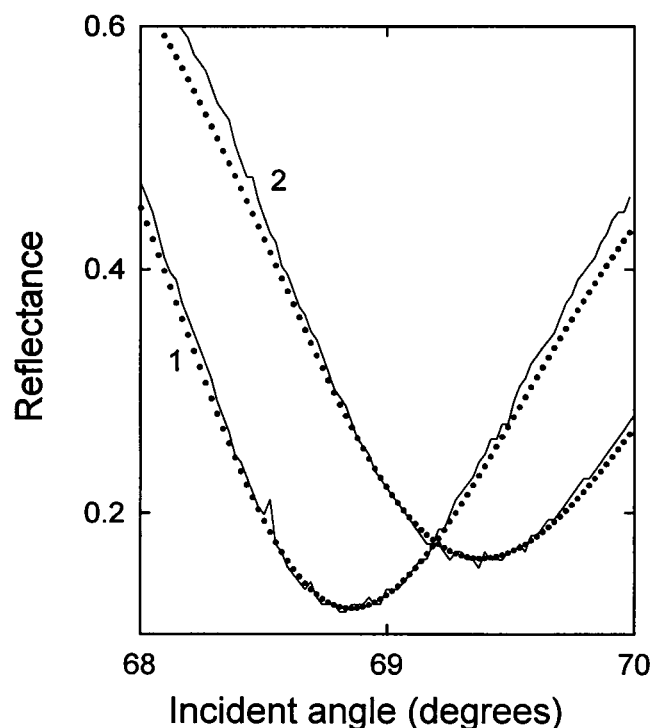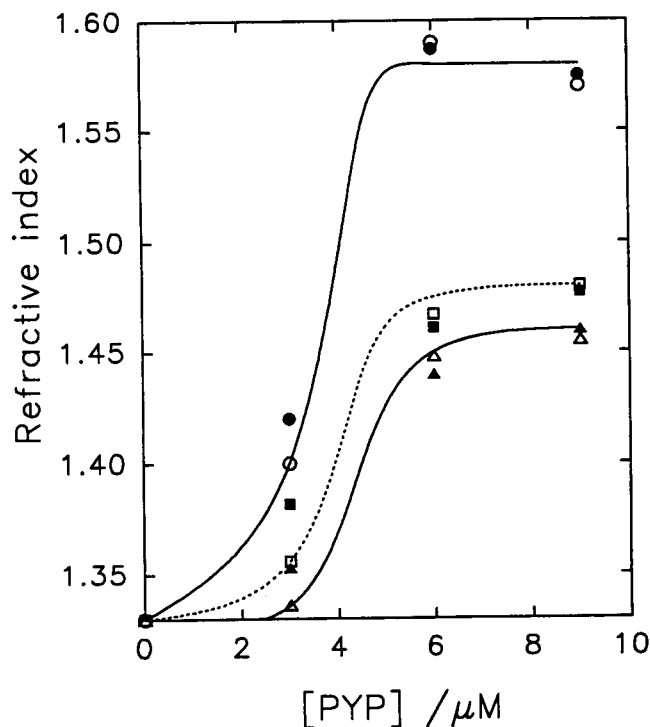

FIGURE 4 Plots of refractive index of the PYP layer, obtained from theoretical fits to the SPR spectra, as a function of the concentration of added protein, in the dark (*open symbols*) and upon white light irradiation (*filled symbols*).

TABLE 1 SPR parameters for PYP adsorbed to lipid bilayers

	t (Å)	n	Lipid:PYPmole ratio
PC/DODAC			
3 μM PYP			
Dark	23	1.38	45:1
Light	23	1.42	26:1
9 μM PYP			
Dark	23	1.57	15:1
PC/DHP			
3 μM PYP			
Dark	22	1.37	120:1
Light	23	1.38	52:1
9 μM PYP			
Dark	22	1.48	24:1
PC			
3 μM PYP			
Dark	21	1.34	180:1
Light	22	1.35	80:1
9 μM PYP			
Dark	22	1.45	28:1

FIGURE 3 SPR spectra (———) obtained with a PC/DODAC bilayer deposited on a silver film, in the absence (curve 1) and the presence (curve 2) of 9 μM PYP. Also shown (●) are the theoretical fits to the data (see text).

fraction for the minor axis of the ellipsoidal PYP molecule (25 Å; McRee et al., 1989). Based on the t and n values, we calculate a mole ratio of lipid to PYP at saturation of 15:1; this would correspond to a DODAC:PYP ratio of 4.5:1, which is in good agreement with the net negative charge of PYP (-5, assuming protonation of the two histidine residues). These parameters suggest that the PYP molecule associates with the bilayer along its long axis (which is con-

sistent with the charge distribution on the protein surface; McRee et al., 1989), and that it is close-packed on the surface of the membrane.

Illumination with white light of a sample containing 3 μM PYP causes a small, but reproducible, shift in the SPR curve to larger incident angles (Fig. 5), corresponding to an increase in n (Fig. 4 and Table 1) and, thus, a corresponding increase in the number of bound PYP molecules. Blue light (350–500 nm) irradiation also produces a similar, although smaller, shift in the SPR spectrum, whereas yellow light (>500 nm) is ineffective. With samples containing saturating amounts of PYP, no additional PYP binding was observed upon irradiation (Fig. 4). No significant return of the SPR spectrum to the pre-illumination condition was observed over a 15 min time interval after cessation of irradiation. We will return to this below.

Negatively charged bilayers

Saturable binding of PYP to negatively charged bilayers was also observed (Figs. 4 and 6, Table 1), although the extent of binding was significantly smaller than with the positively charged bilayer. This is consistent with the overall negative charge of PYP. That it is able to bind at all to a negative surface is most likely a consequence of the asymmetric charge distribution in the protein (McRee et al., 1989), in which clustering of negatively and positively charged residues occurs on opposite sides of the ellipsoidal molecule. The fact that the same thickness value for the protein layer
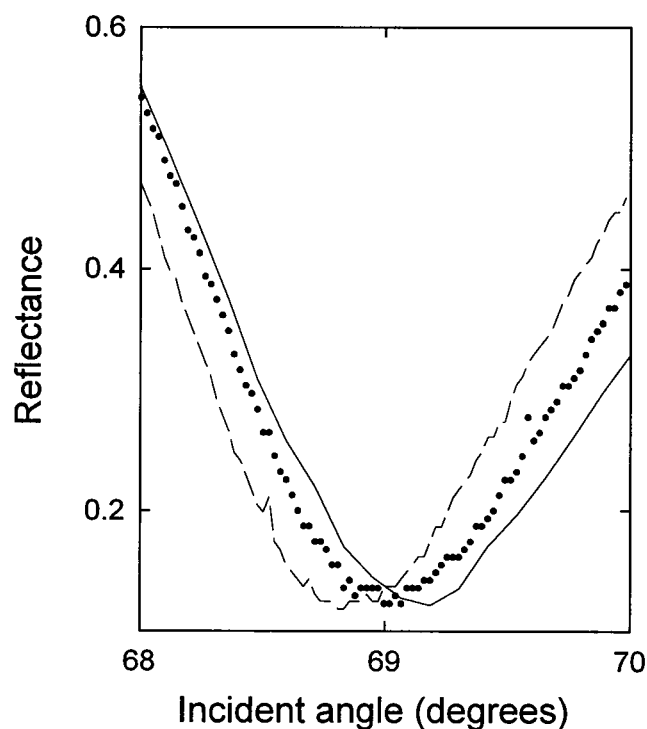

FIGURE 5   SPR spectra obtained with a PC/DODAC bilayer deposited on a silver film, in the absence (– – – –) and the presence (● ● ● ●) of 3 μM PYP, and upon white light illumination (——).
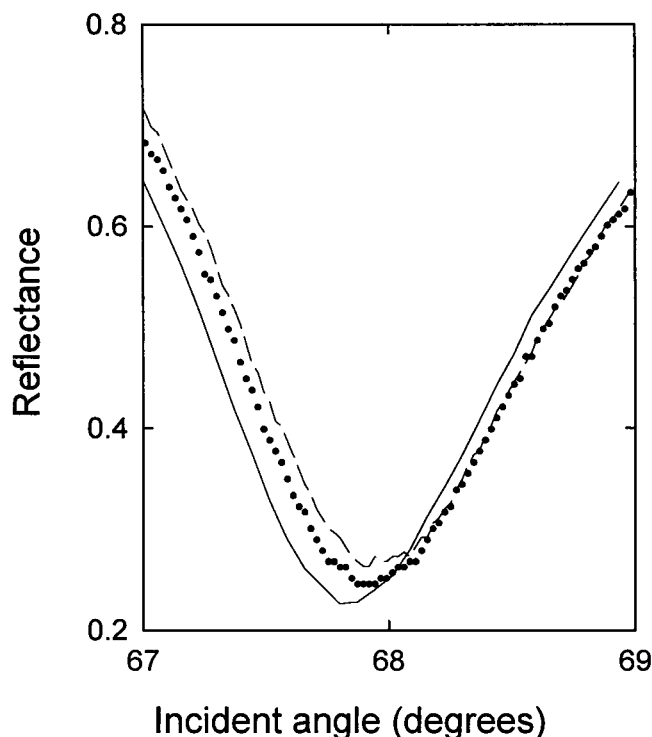

FIGURE 6   SPR spectra obtained with a PC/DHP bilayer deposited on a silver film, in the absence (– – – –) and the presence (● ● ● ●) of 3 μM PYP, and upon white light illumination (——).

is obtained for this system (Table 1) is consistent with this distribution of charged residues.

A striking difference between the SPR results obtained with the negatively and positively charged bilayers is that in the former system, adding PYP shifts the SPR spectra to *smaller* incident angles (Fig. 6). Inasmuch as protein binding must increase the index of refraction (i.e., the mass density) in the metal-water interface region, the only way to accommodate this type of shift, which implies an overall decrease in the index of refraction, is to assume that the protein lowers the mass density in the lipid layer via an attractive interaction between its positively charged surface and the negative head groups of the DHP. This would result in an apparent overall decrease in the index of refraction of the proteolipid film, as a consequence of the fact that the lipid layer is closer to the metal surface than is the protein layer and, thus, influences the SPR spectrum more strongly. Using this assumption, the theoretical fits to the SPR spectra (Table 1) yield values for the n and t parameters of the PYP layer that are closely similar to those obtained with DODAC. This provides strong support for this type of model. Again, white light irradiation of the PYP solution (below saturation) causes an increase in the amount of PYP bound to the lipid surface (Figs. 4 and 6, Table 1). No such increase is observed in the presence of saturating amounts of PYP. As with the PC/DODAC film, turning off the light does not result in a return of the extent of PYP binding to the pre-illumination level, at least over a period of several minutes.

Electrically neutral bilayers

Fig. 7 shows SPR spectra obtained for an electrically neutral (i.e., zwitterionic) PC bilayer in the absence and presence of a nonsaturating amount of PYP (3 μM). The corresponding parameters are listed in Table 1. As with PC/DODAC and PC/DHP, a thickness of 23 Å was obtained for the PYP layer, although, as expected, the amount of bound protein was considerably less than in the case of the positively charged bilayer (by a factor of ~4). Again, irradiation of the sample at PYP levels below saturation with either blue light or white light appreciably increased the extent of PYP binding (Fig. 7 and Table 1), resulting in an approximately twofold change in the lipid:protein ratio at the maximum light intensity. As indicated by the data in Table 1, the amount of bound protein under these conditions was still significantly smaller than that obtained with the PC/DODAC or PC/DHP bilayers. As with the other films, removal of light excitation does not result in a decrease in the amount of PYP bound to the membrane on a several minute time scale.

CONCLUSIONS

We attribute the strong binding of PYP to a positively charged PC/DODAC bilayer, and the weaker binding to a negatively charged PC/DHP bilayer, to the electrostatic interactions that occur between the membrane surface and oppositely charged regions of the protein. Consistent with this is the fact that a smaller amount of protein is bound to the electrically neutral membranes. That any PYP at all binds to

the neutral PC bilayer may reflect the much weaker interactions that could occur between the protein surface charges and the positively charged choline moieties of the PC. The latter portion of the lipid head group presumably extends further from the membrane surface than does the negatively charged phosphate moiety, and charge neutralization occurs via nearest neighbor interactions. These could be partially broken by the protein, leading to a weak binding interaction.

The most striking observation is the relatively large light-dependent increase in the extent of PYP binding to the lipid bilayers, concomitant with a stabilization of the bound protein well beyond the time expected for recovery of PYP in an aqueous medium from the photobleached state (several seconds at most). Although we have not measured an action spectrum for this increase in binding, the fact that blue light (but not yellow light) is effective is consistent with the blue light-induced initiation of the photocyclic absorbance changes obtained in solution studies of PYP (Meyer et al., 1987). It is also important to note that, at the light intensities used here ($\approx 10^{16}$ photons cm^{-2} s^{-1}), as well as the low protein concentration in the aqueous medium (3 μM), only a very small amount of PYP would be expected to undergo photobleaching during illumination. Thus, the fact that a change in the SPR spectrum is observed at all implies that the photobleached state must have a much higher affinity for the lipid bilayer than does the dark conformation. Presumably, at saturating concentrations of PYP, the molecules are packed closely enough on the membrane surface that additional binding is sterically precluded.

These results are consistent with the earlier measurements of the effects of aliphatic alcohols on PYP photokinetics (Meyer et al., 1989), which indicated that photobleaching increased the exposure of hydrophobic surfaces in the protein, and that the lifetime of this altered conformation was lengthened by a nonpolar medium. Thus, the light-induced increase in the binding of PYP to the lipid bilayer, and the stabilization of the bound state, could have a similar origin, i.e., the exposure of hydrophobic side chains that could insert into the hydrocarbon phase of the bilayer. Indeed, this property might provide a basis for the biological activity of PYP, by allowing it to become associated with a membrane (or a hydrophobic region of a membrane-bound receptor) and thereby influence flagellar activity during exposure to high light intensities. This requires further study.

It is noteworthy that, despite the probable change in protein conformation that initiation of the PYP photocycle produces (Meyer et al., 1989), the SPR results can be interpreted solely on the basis of changes in the protein mass associated with the membrane surface, without any significant change in the dimensions of the protein layer (i.e., changes in n and not in t). This could be a consequence of several possible factors. i) The conformational changes are too small to be resolved in the present experiments. This is unlikely, inasmuch as our resolution in the t parameter is estimated to be ± 1 Å, as noted above. ii) The conformational transition only results in an appreciable change in the dimensions of the long
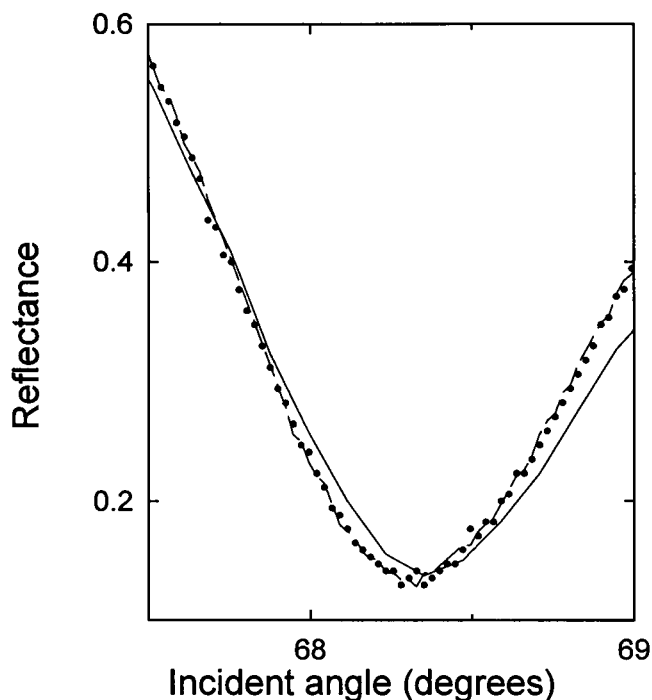

FIGURE 7   SPR spectra obtained with a PC bilayer deposited on a silver film in the absence (– – – –) and the presence (● ● ● ●) of 3 μM PYP, and upon white light illumination (———).

axis of the PYP molecule. If this were the case, such a modification would not be reflected in the t value, because of the mode of binding of PYP to the membrane surface (see above). This deserves further study.

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