J. Membrane Biol. 46, 239–254 (1979)

Linear Dichroism of Rhodopsin in Air-Water Interface Films

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Received 7 September 1978

Summary. Air-water interface films of purified cattle rhodopsin and defined phospholipids are formed by the osmotic lysis of reconstituted membrane vesicles. The interface films thus formed consist of a phospholipid monolayer containing vesicle membrane fragments. Rhodopsin molecules at the interface are restricted within the membrane fragments where they are spectrophotometrically intact and capable of undergoing photoregeneration and chemical regeneration. Multilayers of up to 8 layers can be built from these interface films. The visible absorption band of rhodopsin in these multilayers is linearly dichroic. Quantitative analysis of the linear dichroism reveals that the dipole moment of transition of the retinal chromophore in rhodopsin forms an angle of $15° \pm 4°$ with the plane of the membrane fragments in the interface film. This orientation of the chromophore relative to the plane of the membrane is essentially the same as that observed in the intact retina. Thus, the orientation of rhodopsin in the interface films is similar to that in the intact disc membranes.

The outer segment of the rod photoreceptors in the vertebrate retina consists of a stack of discs surrounded by a plasma membrane (Cohen, 1963). Each disc is a flattened saccule lying in a plane perpendicular to the long axis of the outer segment. The thickness of each disc is 150 Å and the center-to-center distance between discs is about twice their thickness (300 Å) (Korenbrot, Brown & Cone, 1973). Rhodopsin, the light-absorbing pigment of the rod cell, is confined to the disc and plasma membranes of the outer segment (Jan & Revel, 1974; Basinger, Bok & Hall, 1976) where it constitutes 80–90% of the protein present (Robinson, Heitzman, 1972; Gordon-Walker & Bownds, 1972). The characteristic visible absorption spectrum of rhodopsin arises from the links between its chromophore, an 11-cis 12-*S*-cis isomer of retinal, and

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0022-2631/79/0046-0239 $03.20
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the protein moiéty (Hubbard, Bownds & Yoshizawa, 1965; Honig & Karplus, 1971). The retinal chromophore consists of a β-ionylidene ring bound to a 9-carbon polyene chain with five double bonds. The vector of the dipole moment of electronic transition of the chromophore lies along this conjugated chain and, thus, the chromophore absorbs plane polarized light most strongly when the electric vector of the light is parallel to the long axis of the polyene chain (Honig & Ebrey, 1974). The orientation of the dipole moment of transition of the chromophore has been used in the past as an intrinsic marker to demonstrate that rhodopsin molecules are oriented in the disc membrane (Liebman, 1962; Cone & Brown, 1967) and that they are free to undergo both rotational (Cone, 1972) and translational diffusion (Liebman & Entine, 1974; Poo & Cone, 1974) in the membrane. The study of linear dichroism provides, therefore, a means to determine the orientation of rhodopsin.

Schmidt (1938) first reported that isolated frog rod outer segments appear colored when illuminated with white light polarized in a plane normal to the long axis of the outer segment, but are nearly colorless when the illuminating light is polarized in a plane parallel to the long axis of the outer segment. Denton (1959) confirmed this linear dichroism in isolated retinas. Liebman (1962), Wald, Brown & Gibbons (1963), and Harosi (1975) developed high sensitivity microspectrophotometers, and through their use they determined that the dichroic ratio of the rhodopsin absorption band (absorbance normal to the long axis of the outer segment/absorbance parallel to the long axis) in several vertebrate species is as high as 5:1. Liebman (1962) has quantitatively accounted for this high dichroic ratio by assuming that rhodopsin molecules in the disc membrane are oriented such that the dipole moment of transition of the chromophore forms an angle of 16° with the plane of the disc membrane. Since in the normal eye light passes down the long axis of the outer segment, the orientation of the protein in the plane of the disc membrane and the stacking of the discs place the chromophore in a nearly ideal orientation for optimal absorption of stimulus light.

Photoexcitation of rhodopsin leads to changes in the ion permeability of the outer segment plasma membrane, but the mechanism of this process remains unknown (Hagins, 1972; Montal & Korenbrot, 1976). We have recently reported (Korenbrot & Pramik, 1977) on an experimental approach to studying the function of rhodopsin that investigates the electrochemical properties of air-water interface films consisting of purified rhodopsin and phospholipids. Rhodopsin in these interface films is spectrophotometrically intact and capable of undergoing photoregener-

ation and chemical regeneration with characteristics similar to those exhibited by the pigment molecule in the rod membranes (Korenbrot & Pramik, 1977; Korenbrot, 1977). However, the orientation of rhodopsin in these films was unknown. We report here that the visible absorption band of rhodopsin in these interface films is linearly dichroic, and analysis of this dichroism shows that the orientation of the rhodopsin chromophore in these films is quantitatively indistinguishable from that in the intact disc membrane.

Materials and Methods

Materials

Egg phosphatidyl-choline (egg PC) was extracted from fresh egg yolks and was purified according to Singleton, Gray, Brown and White (1965). Brain phosphatidylserine (brain PS) was extracted from fresh bovine brains and was purified according to Papahadjopoulos and Miller (1967). Divalent ions were removed from the brain PS by its conversion first to an acid form and then to a Na salt. The purified phospholipids appeared as single spots in silica gel TLC plates and were stored in hexane/ethanol (9:1) under nitrogen at −70 °C. The detergent used, tridecyl trimethyl ammonium bromide (TriTAB) was synthesized according to the method of Hong and Hubbell (1973) and was purified by recrystallization from acetone-methanol. All inorganic chemicals were analytical reagent grade. Water used throughout was four times glass distilled, including once from alkaline permanganate and once from sulfuric acid.

Purified delipidated cattle rhodopsin containing less than 1 mole phosphate per mole rhodopsin was prepared by following the methods of Hong and Hubbell (1973) and Applebury *et al.* (1974), as previously described (Korenbrot & Pramik, 1977). Rod outer segments from frozen bovine retinas were purified by sucrose flotations. Rhodopsin was extracted from the outer segments with the use of TriTAB and was then separated from other proteins and from phospholipids by column chromatography in hydroxylapatite. The rhodopsin absorption spectrum in detergent had typical absorbance ratios A278/A498 of 1.7 (range 1.61–1.85) and A400/A498 of 0.19 (range .17–.22). Rhodopsin was incorporated into multilayered membrane vesicles by removal of TriTAB through exhaustive dialysis in the presence of added phospholipids. (Hong & Hubbell, 1973; Korenbrot & Pramik, 1977). In the present experiments, membranes were formed with either egg PC/rhodopsin in the 100:1 phospholipid/protein mole ratio or with an egg PC/brain PS (1:1 weight ratio) mixture to obtain a 115:1 mole ratio of phospholipid to rhodopsin. The multilayered rhodopsin membrane vesicles were harvested by centrifugation and stored in the dark at 4 °C in a standard salt solution consisting of 200 mM NaCl, 10 mM MOPS buffer, pH 6.8, for periods never longer than 2 weeks. The rhodopsin absorption spectrum in these membrane vesicles had typical absorption ratios A278/A498 of 2.1 (range 2 to 2.3) and A400/A498 of 0.27 (range 0.25 to 0.32).

Formation of Interface Films and Multilayers

Interface films containing rhodopsin and lipids were formed by the osmotic method previously described in detail (Korenbrot & Pramik, 1977). A suspension of single bilayer

vesicles in the standard salt solution was prepared by a 30–60 sec bath sonication of the multilayered rhodopsin membrane vesicles. The vesicles were then gently applied onto a clean glass slide partially immersed in an aqueous subphase consisting of 5 mM MOPS, 4×10^{-4} M $CdCl_2$, pH 6.8, and contained in a Teflon Langmuir trough ($10.5 \times 40 \times 1.5$ cm). Films at the air-water interface formed instantaneously as a result of the osmotic lysis of the membrane vesicles on the aqueous surface. The interface films could then be collapsed by a mechanically driven Teflon barrier in the trough. The surface pressure of the film, measured by the Wilhelmy method (Gaines, 1966), was continuously monitored.

Multilayers were formed by transferring the rhodopsin-lipid films from the air-water interface onto a clean hydrophilic glass support. Two glass slides (Scientific Products #2 coverslip, 0.18–0.22 mm thick, cut to 1×2.2 cm), cleaned in warm dichromate-sulfuric acid solution and rinsed extensively with distilled water, were held side by side and slowly inserted and withdrawn (0.5 cm/min) through the interface film. As the film transferred, the surface pressure at the interface was kept constant with the use of a servo-loop which automatically advanced the Teflon barrier in the trough. The success of film transfer was monitored by the loss of surface area. The glass slides emerged wet from the subphase and were allowed to air dry (about 20 min) between successive depositions.

Spectrophotometry

Visible absorption spectra were recorded with a Cary 118-C double-beam Spectrophotometer (Varian Instruments, Sunnyvale, Ca.) equipped with a scattering transmission accessory and interfaced on line to a Nicolet 1072 dedicated signal-averaging computer (Nicolet Instruments, Madison Wisc.). For absorbance measurements, glass slides were held in a plane normal to the measuring beam in specially constructed holders. First the baseline of the spectrophotometer was manually adjusted between 650 and 400 nm with clean glass slides in the sample and reference beams. The slide in the sample beam was then replaced by a slide bearing the rhodopsin multilayer, and spectra were recorded. For the measurements of dichroism, glass slides were placed in precision holders at a fixed angle ($\alpha = 43°$) with respect to the measuring beams. A baseline was first recorded with a light polarizer in position and clean glass slides in both the sample and reference holders. Because of the small signals recorded it was important to use high quality polarizers (Polacoat supplied by Perkin-Elmer). After a baseline had been recorded, the glass slide in the sample beam was replaced by one bearing an unbleached rhodopsin-multilayer. The slide in the reference beam was replaced by a second rhodopsin multilayer slide, prepared side by side with that in the sample holder, but in which rhodopsin molecules had been thoroughly bleached ($> 99\%$). Thus rhodopsin difference spectra were directly recorded. As discussed under *Results*, this was necessary to correct for the light-scattering of the multilayers. The baseline corrections of the recorded spectra were carried out with the on-line computer.

The measurement of the linear dichroism of the rhodopsin-lipid multilayers presented particular experimental difficulties because of the small optical absorbance of the multilayer stack. First, significant bleaching of the sample might be produced by the measuring beam. This is particularly important since the absorption of each of the two planes of polarization was determined in two successive scans of the same slides. By using the narrowest possible slit (0.01 mm) and the fastest possible scan speed (2 nm/sec) consistent with an optimum signal to noise ratio, conditions were found which bleached only 0.3%/scan. Second, because of the small absorbance of the samples, instrumental noise limited the precision of measurement of the absorption spectrum. The signal to noise ratio was improved by a filtering procedure which consisted of scaling a nonnoisy rhodopsin difference spectrum to the multilayer difference spectrum. The nonnoisy difference spectrum was

obtained from a high absorbance nonscattering sample of purified cattle rhodopsin (10 μM in 0.1 M TriTAB). To carry out the filtering procedure, spectra were digitized into a PDP-11 computer (Digital Equipment Corp.) by means of a manual position sensitive tablet (Evans & Sutherland Co.). A linear baseline was then subtracted, if necessary, to make all spectra flat between 660 and 620 nm, where cattle rhodopsin absorbance is negligible. The spectrum of the transparent sample was fit to the multilayer spectrum by an iterative least-squares procedure until successive iterations failed to decrease the error of fit.

Light Exposure

To fully bleach the rhodopsin molecules in a multilayer stack on a glass slide, the slide was continuously exposed for 15 min to light of wavelength longer than 510 nm produced by a 100-W source. While being exposed to light, the slide was kept in a humid environment created by holding it in a beaker containing wet filter paper and covered with Parafilm. Absorbance measurements confirmed that over 99% of the rhodopsin molecules in the multilayers were bleached by this procedure.

Data Analysis

The visible linear dichroism in the rhodopsin-lipid multilayers was analyzed by following the method of analysis of linear dichroism of multilayers containing oriented chromophores developed by Cherry, Hsu and Chapman (1972) for chlorophyll-lipid membranes and by Heyn, Cherry and Muller (1977) for purple membrane multilayers. Since Cherry *et al.* (1972) have shown that the dichroism of shape of lipid bilayers is negligible, any linear dichroism detected in lipid-protein layers must arise from the nonrandom orientation of absorbing chromophores in the layers. Moreover, the treatment of Cherry *et al.* (1972) applies only to the case where no energy transfer occurs between the chromophores. Brown (1972) has demonstrated the absence of energy transfer between rhodopsin chromophores. Figure 1 defines the geometrical features of the experimental design used in these experiments. The polarized light of the measuring beam travels along the X-axis, and its electric vectors lie along the Z axis for vertical polarization and along the Y axis for horizontal polarization. The plane of the rhodopsin-lipid multilayers is positioned at an angle α with respect to the XY plane. In the plane of the rhodopsin-lipid layer the dipole moment of transition of the chromophores forms an angle θ with the *normal* to that plane, N. The index of refraction of the rhodopsin-lipid layer is n.

Under these conditions,

$$D-1=\frac{1}{n^2}(2\cot^2\theta-1)\cos^2\alpha \tag{1}$$

where the dichroic ratio, D, is the ratio of absorbance of vertically polarized over horizontally polarized light.

It must be noted that Eq. (1) is obtained by considering that the chromophores are randomly oriented in the plane of the membrane, i.e., around the normal N. The dipole moment of transition may, therefore, be found in any position along the conical surface making an angle θ with the *normal* to the plane of the layer. The retinal chromophore has indeed been shown to be randomly oriented in the plane of the rhodopsin-containing disc membranes (Hagins & Hennings, 1959; Brown, 1972).

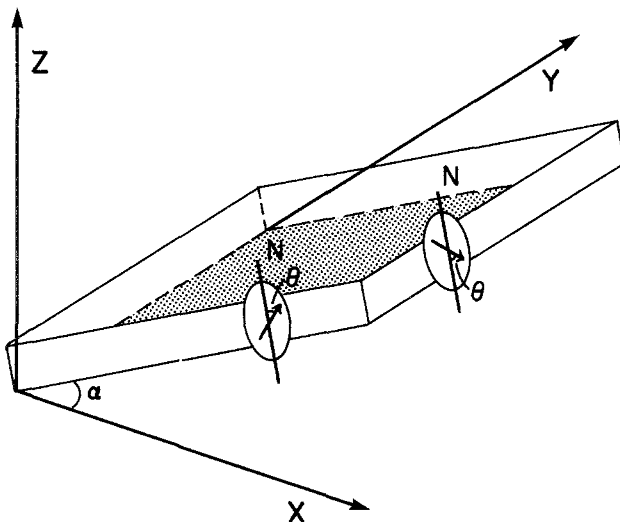

Fig. 1. Geometry of the experimental design. The plane polarized light of the measuring beam travels along the X axis. The electric vector lies along the Z axis for vertically polarized light and along the Y axis for horizontally polarized light. The plane of the rhodopsin-lipid layers forms an angle α with the X-Y plane. Within the plane of the rhodopsin-phospholipid film, the dipole moment of electronic transition of the chromophore (arrow) forms an angle θ with the *normal* to that plane, N

Results and Discussion

The air-water interface films investigated in this report consist of a phospholipid monolayer containing randomly distributed membrane fragments within which rhodopsin is found. The detailed structural features of these films have been described previously (Korenbrot & Pramik, 1977). The presence of membrane fragments at the interface disturbs the organization of the phospholipid monolayer, as evidenced by changes in the surface pressure isotherm. Figure 2 illustrates surface pressure *vs.* area isotherms of films formed by osmotically spreading vesicles consisting of egg PC/brain PS (1:1 weight ratio) and rhodopsin in a 115:1 phospholipid to protein mole ratio. Also illustrated is the isotherm obtained by spreading only the phospholipid mixture. The form of the isotherms indicates that incorporation of the membrane fragments at the interface results in films which are expanded and more compressible than pure phospholipid monolayers. Electron-microscopic observation of these films reveal extensive overlap of the membrane fragments at the interface. As previously reported, a similar overlap is observed in films formed from egg PC/rhodopsin membranes in the 100:1 phospholi-

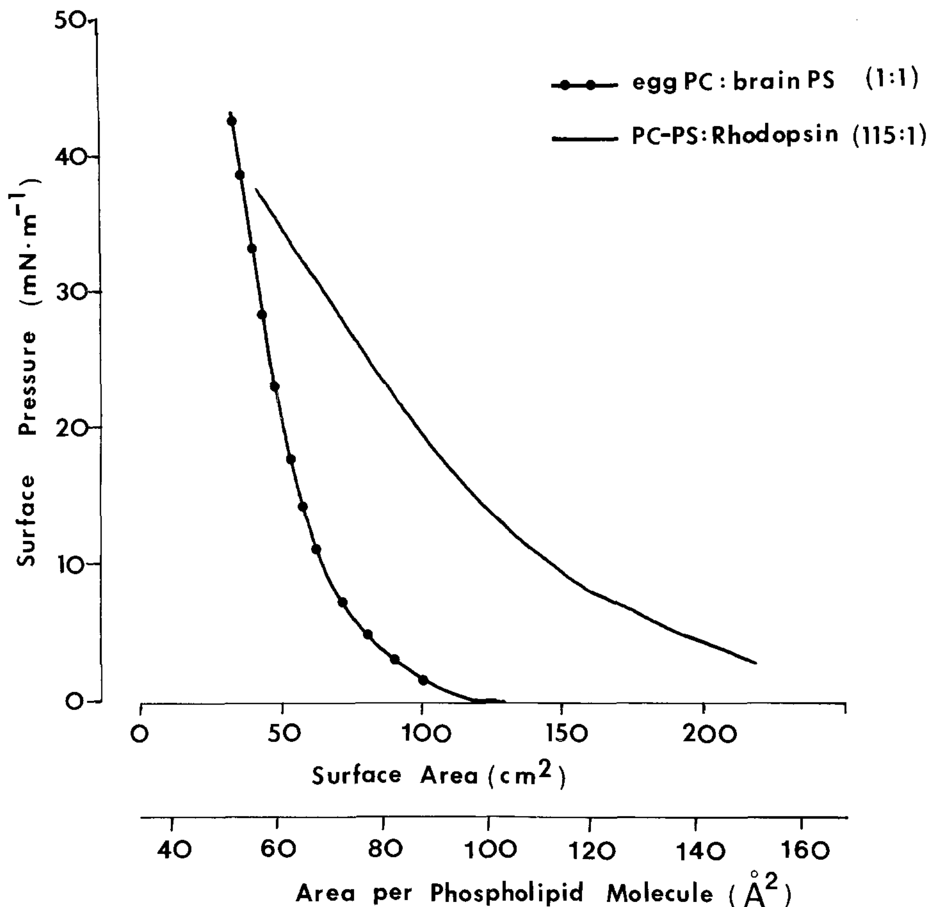

Fig. 2. Surface pressure *vs.* surface area isotherms of egg PC-brains PS, 1:1 by weight mixture, and PC-PS/rhodopsin membrane vesicles. The phospholipid monolayer was formed by spreading 25 μg of lipid from a hexane solution. The calculated area per phospholipid molecule is shown. The PC-PS/rhodopsin interface films was formed by osmotically spreading vesicles of 115:1 phospholipid to protein mole ratio. Isotherms were recorded at 20 °C over a subphase of 5 mM MOPS, 4×10^{-4} M $CdCl_2$, pH 6.8. The isotherms, measured in separate experiments, are redrawn for illustration so as to coincide in area near their collapse pressure

pid/protein mole ratio (Korenbrot & Pramik, 1977). The rhodopsin molecules within the membrane fragments at the interface maintain a structure which, by spectrophotometric criteria, is indistinguishable from that in the intact disc membrane (Korenbrot & Pramik, 1977).

The rhodopsin-lipid interface films transfer to hydrophilic glass slides when the slides are slowly moved through the air-water interface. When the transfer of film is carried out at a constant surface pressure of

38 $mN \cdot m^{-1}$ (5 $mN \cdot m^{-1}$ less than collapse pressure), the egg PC/rhodopsin (100:1) films transfer onto the slide on the upward and downward strokes through the interface only on the first trip. On the second and third trip films transfer only on the upward stroke, and successive trips result in the release of film from the support. Since the original report of Blodgett and Langmuir (1937) on the formation of multilayers from fatty acid monolayers, it has been repeatedly confirmed (Gaines, 1966) that multilayers are more likely to form if short-range forces are brought into play, for example, by incorporating negatively charged molecules into the interface films in the presence of divalent cations in the aqueous subphase. We therefore attempted to form multilayers from egg PC/brain PS (1:1) rhodopsin films (115:1 phospholipid/protein) in the presence of $CdCl_2$ in the aqueous subphase. In contrast to the egg PC/rhodopsin films, the PC-PS/rhodopsin films at 38 $mN \cdot m^{-1}$ transfer onto the glass support exclusively on the upward stroke through the interface on the first four trips. On the fifth transfer through the interface, material begins to release from the glass support. Thus, a total of only 8 layers of PC/PS-rhodopsin films could be reliably built on the two surfaces of a single glass slide.

The rhodopsin multilayer stacks scatter light. To minimize spectral distortions due to this light scattering, rhodopsin difference spectra were recorded directly by measuring the absorbance of a multilayer using as a reference a second multilayer formed side by side with the sample one, but in which all rhodopsin molecules were bleached. A typical multilayer difference absorption spectrum is shown in Fig. 3*A*. The spectrum shown is that of a PC-PS/rhodopsin multilayer (115:1 mole ratio). A single broad absorption band is seen with a peak at 498 nm. This is the characteristic absorption spectrum of cattle rhodopsin. The very small absorbance of the multilayer results in a noisy spectrum due to instrumental limitation. The signal to noise ratio was improved by a computerized procedure which fit a nonnoisy difference spectrum to the multi-layer data (*see Methods*). Figure 3*B* is an illustration of the computerized output showing the original data (Fig. 3*A*) and the best possible fit to it. Also illustrated in Fig. 3*B* is the difference between the scaled transparent difference spectrum and the multilayer spectrum. This difference can be seen to vary randomly about zero. In general, we took data to be acceptable for further analysis when this difference varied randomly about zero within the limits of $\pm 6 \times 10^{-5}$ OD. (This corresponds approximately to 5% of the peak absorbance in the spectra). In addition to the computerized fit, we have also found that a Dartnall

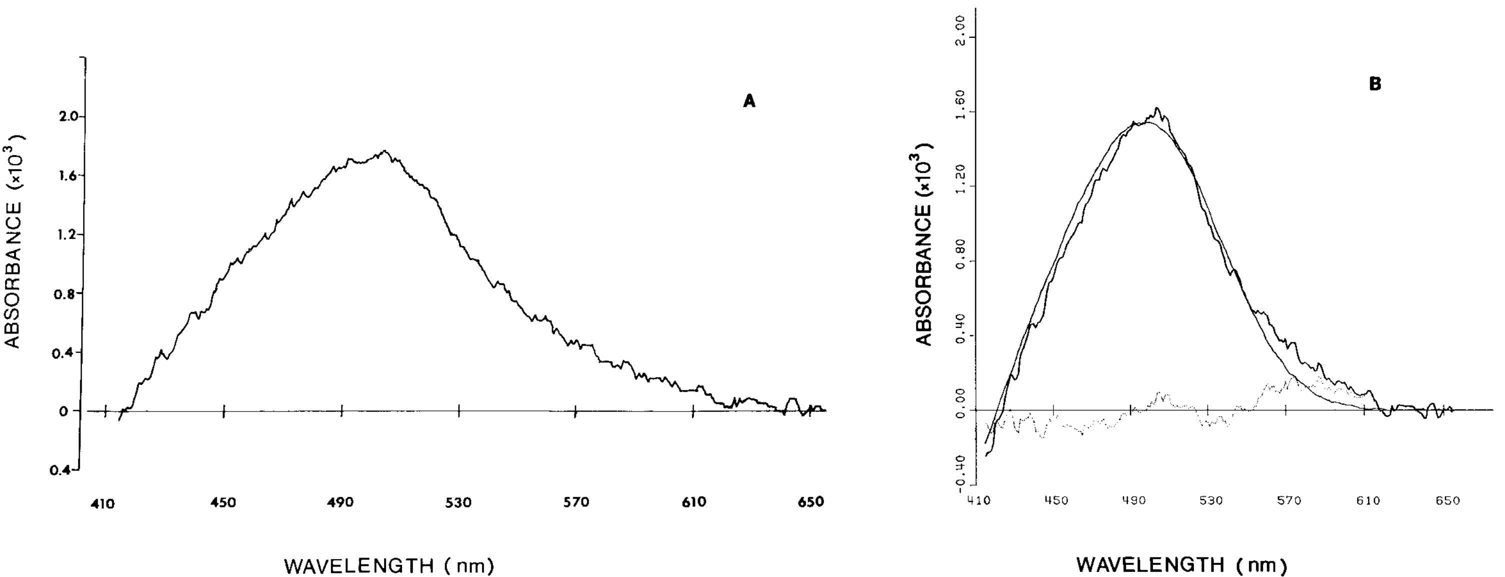

Fig. 3. Absorption difference spectrum of PC-PS/rhodopsin multilayers. Shown are spectra recorded from a multilayer consisting of 8 layers of 115:1 phospholipid/rhodopsin interface film. (*A*): The direct difference spectra obtained by measuring the absorbance of an unbleached rhodopsin multilayer against a reference consisting of a second rhodopsin multilayer, prepared side by side with the sample one, but in which over 99% of the rhodopsin molecules are bleached. (*B*): The same data as in *A* with a linear baseline correction to obtain a flat baseline between 660 and 620 nm. Superposed on the original multilayer data is a smooth line which represents the best fit of a nonnoisy rhodopsin difference spectrum to the multilayer data (*see* text for details). The dotted line about zero is the difference between the multilayer spectrum and the best fit spectrum

monogram with 498 nm λ max fits the difference spectra in the multilayers for all points above 480 nm. Below about 480 nm, the Dartnall monogram, a function generated empirically from absolute, not difference spectra, is not expected to fit difference spectra (Knowles & Dartnall, 1977). Accurate difference spectra of the multilayers could thus be obtained which fully correct for distortion due to light scattering and minimize errors due to random noise.

A difference spectrum measured with plane polarized light in PC-PS/rhodopsin multilayers is illustrated in Fig. 4. The glass slides were positioned at 43° with respect to the measuring beam (α angle in Fig. 1). Each spectrum exhibits a single broad band with a λ_{max} at 498 nm. The continuous line superimposed on the spectra was fit by the computerized graphic system. As shown in Fig. 4, the spectrum measured with horizontally polarized light (electric vector along $0Y$ in Fig. 1) exhibits higher absorbance than the spectrum measured with vertically polarized light (electric vector along $0Z$ in Fig. 1). In four different samples of PC-PS/rhodopsin the dichroic ratio D (absorbance vertically polarized/absorbance horizontally polarized) had an average value of 0.796 ± 0.017 ($\pm$SD, range$=0.772$ to 0.810). The PC rhodopsin multilayers did not have enough absorbance to obtain reliable results. The linear dichroism of the PC-PS/rhodopsin interface films reveals that in these films the retinal chromophore in the rhodopsin membranes is highly oriented.

From the measured dichroic ratio, the orientation of the dipole moment of transition of the retinal chromophore in the multilayers can be quantitatively determined through Eq. (1). The angle between the layers and the measuring beam, α, was taken to be that between the supporting glass slide band and the beam. The actual angular distribution of the individual layers with respect to the plane of the glass support, defined by the mosaic spread, could not be experimentally determined in our case because only eight layers were deposited on the glass. However, the first order lamellar X-ray diffraction of multilayers formed from 80 to 100 interface films consisting of a phospholipid monolayer containing fragments of purple membrane, which we have previously described (Hwang, Korenbrot & Stoeckenius, 1977) and whose structure is nearly the same as that of the rhodopsin films studied here, exhibit a mosaic spread of 4° (Bogomolni *et al.*, 1977). Also, multilayers formed by drying purple membrane fragments and exhibiting an absorbance as large as 0.6 OD at 568 nm exhibit mosaic spreads of about 7° (Heyn *et al.*, 1977). Thus, the mosaic spread of the rhodopsin membrane multilayers with respect to the plane of the glass slide can be expected to

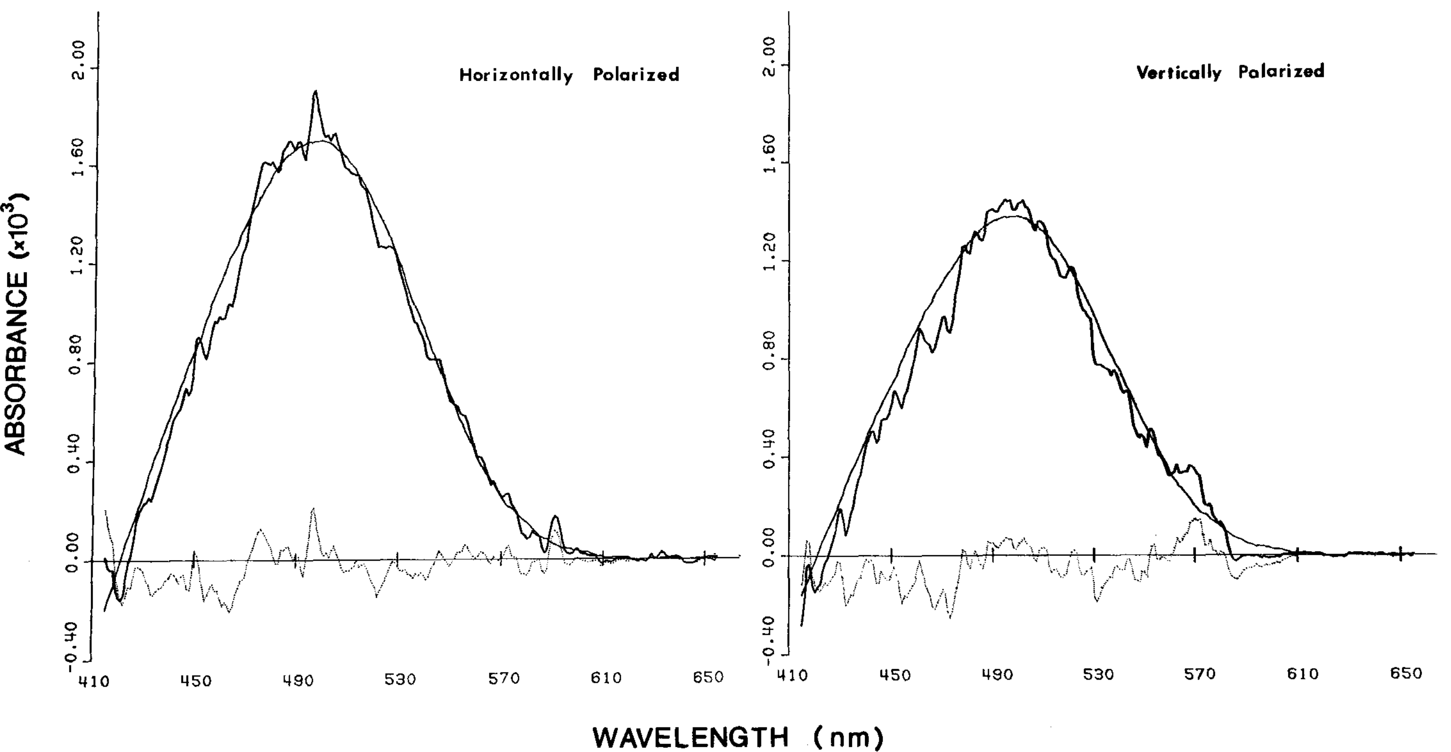

Fig. 4. Linearly polarized absorption difference spectrum of PC-PS/rhodopsin multilayers. Shown are difference spectra recorded from a multilayer consisting of 8 layers of 115:1 phospholipid/rhodopsin interface films. The spectral data shown is plotted by the computer, together with the best fit function and the difference between the original data and the best fit function. There is a higher absorbance of horizontally polarized than of vertically polarized light. The absorbance ratios of the data shown yield a dichroic ratio $D=0.81$, which corresponds to an angle of 17° between the retinal chromophore and the plane of the membrane

be less than about 5°. To estimate the index of refraction of the rhodopsin-lipid membrane, we took advantage of the fact that the chemical composition and index of refraction of rod outer segments is accurately known. The index of refraction of the outer segments, n_{os}, is given by (Liebman, 1975):

$$n_{os} - n_w = fs\ (n_s - n_w) \qquad (2)$$

where n_w and n_s are, respectively, the index of refraction of the water and of the solid contents of the outer segment and *fs* (volume solids/volume outer segment) is the fractional volume occupied by solids in the outer segment. Since the refractive index increment for solids of the composition of those in the outer segment is given by (Blaurock & Wilkins, 1969):

$$n_{os} - n_w = 0.0016\ \mathrm{M} \qquad (3)$$

where M is in g/cc, and since the index of refraction of the outer segment is accurately known to be 1.40 (Sidman, 1957; Blaurock & Wilkins, 1969), it can be calculated that the solid content of the outer segment is 44 g/cc. Using the known specific densities of lipid (0.95 cm^3/gm) (Blaurock & Wilkins, 1969), rhodopsin (0.85 cm^3/g) (Hong & Hubbell, 1972), and protein (0.75 cm^3/g) (Blaurock & Wilkins, 1969) and the known lipid and protein weight composition of an outer segment (40% lipid, 60% protein, of which 85% is rhodopsin (Daemen, 1973)), it can be calculated that the fractional volume occupied by solids in the intact outer segment (*fs*) is 0.39. With this and the other values stated above, Eq. (2) yields an index of refraction of $n = 1.50$ for the rod disc membrane. Since the rhodopsin membrane fragments in the interface film have a chemical composotion very similar to that of the intact disc membrane, we will assume that they also have the same index of refraction, 1.50. This value of refractive index is similar to that of other membranes. In any event, the calculated value of θ is only weakly dependent on the value of n (Heyn *et al.*, 1977). Analysis of errors in the quality of the recorded spectra, the expected small spread in the orientation of the layers on the solid support, and the uncertainties in the value of the index of refraction indicate that the determination of the angle of orientation of the dipole moment of transition is reliable to about $\pm 4°$. The average dichroic ratio measured yields a value for $\theta = 75°4' \pm 3°30'$. This corresponds to an average value of $14°56' \pm 3°28'$ ($\pm$SD range 9° to 17°34') for the angle between the dipole moment of transition of the chromophore and the plane of the rhodopsin membrane in the interface film.

The angle that the dipole moment of transition of the chromophore in rhodopsin forms with the plane of the interface film agrees well within the accuracy of the measurement with the angle formed by the chromophore and the plane of the disc membrane (16°) as determined by Liebman (1962, 1975) in the intact retina. That is, rhodopsin molecules in the interface films have the same orientation as that in the intact disc membranes as determined by the position of its chromophore. However, as pointed out above, the data presented here reveal the orientation of the chromophore on the surface of a cone and do not distinguish the position of the protein with respect to membrane sidedness. In intact disc membranes all rhodopsin molecules are positioned such that, for example, specific concanavalin A binding sites are accessible only from the intradisc membrane surface (Rohlich, 1976), whereas certain proteolytic sites, sensitive to papain and thermolysin, are accessible only from the extradisc membrane surface (Saari, 1974; Pober & Stryer, 1975). In contrast, in the reconstituted rhodopsin membranes from which the interface films described here are formed, Hubbell *et al.* (1977) have shown that only approximately 60–70% of the molecules are positioned with their proteolytic sites towards the outside surface of the vesicles. Therefore, the rhodopsin molecules in the films at the air-water interface are probably positioned with only a slight, if any, preference with respect to sidedness, but they are oriented in a manner indistinguishable from that in the native disc membranes.

The use of the chromophore as a reliable indicator of the orientation of rhodopsin in the membrane assumes that retinal maintains a rigid orientation with respect to the opsin molecule. Two independent lines of evidence support this assumption: First, Cone and Brown (1967) have shown that heating photoreceptor membranes results in a parallel loss of linear dichroism and of the early receptor potential. The early receptor potential is a fast photovoltage generated by charge displacements in the rhodopsin molecule which can be recorded only as long as the protein molecules are highly oriented in the membrane. Secondly, and more strongly, Baroin *et al.* (1977) and Kusumi *et al.* (1978) have independently reported that the rotational diffusion of rhodopsin in the disc membrane, measured by directly monitoring the motion of the protein moiety, has exactly the same rotational relaxation time (20 μsec at 20 °C) as that reported by Cone (1972) who measured protein rotation through the motion of the chromophore. Thus, in rhodopsin, the retinal chromophore is essentially immobile with respect to the protein and its orientation faithfully indicates the orientation of the protein.

The study of the linear dichroism of membrane multilayers in general permits the study of orientation of chromophores in the plane of these membranes. Bogomolni *et al.* (1977) and Heyn *et al.* (1977) have used multilayers of purple membrane to determine the orientation of the retinal chromophore of bacteriorhodopsin. Erecinska, Wilson, and Blasie (1978) have reported on the orientation of the chromophores of cytochrome-*c*-oxidase and other redox carriers in the mitochondrial membranes through a study of the linear dichroism of mitochondrial membrane multilayers. Oriented membrane multilayers will be of further use in determining structural features of membrane proteins, for example, through the measurement of dichroism in the infrared range (Rothschild & Clark, 1978). The linear dichroism of the rhodopsin multilayers described here and the quantiative conclusions on the orientation of retinal and rhodopsin in the air-water interface films serve to further validate the use of these films as a model system to study the functional and structural properties of rhodopsin.

We thank G. Gold for helpful discussion. Juan I. Korenbrot is an Alfred P. Sloan Research Fellow. The computation work was carried out in the UCSF computer graphics laboratory supported by NIH grant RR-1081. This research was supported by NIH grant EY-01586.

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