DEHYDRATION-INDUCED MOLECULAR STRUCTURAL CHANGES OF PURPLE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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ABSTRACT The nature and extent of dehydration-induced molecular structural changes of the purple membrane of *Halobacterium halobium* have been studied by absorption and circular dichroism spectra in solution and in oriented membrane films. High glycerol concentrations, exhaustive dry nitrogen gas flushing, and exhaustive high-vacuum pumping were employed as dehydrants. The effect of these dehydrants on the spectra were reversible, similar, and additive. Analysis of the spectral changes observed at maximal dehydration revealed: (*a*) at least two additional optical states of the bacteriorhodopsin, one at higher energy and another at lower energy than the characteristic dark- and light-adapted states; (*b*) no change in the dichroic ratio at the visible absorption maximum within experimental error; (*c*) no change in the polarity of the visible monomeric retinylidene circular dichroic bands; (*d*) pronounced reduction in the characteristic excitonic interactions among the retinals in the hexagonal crystalline lattice of the membrane; (*e*) no changes in the native structural anisotropism of the membrane in respect to the orientation of the amino acid aromatic rings of the bacteriorhodopsin; (*f*) no changes in the secondary structure of the bacteriorhodopsin; and (*g*) a net tilting of ~20.5° per segment of the helical polypeptide segments of the bacteriorhodopsin away from the membrane normal. A molecular model of the structural changes of the membrane resulting from water removal consistent with these findings can be constructed. Dehydration results in only subtle localized tertiary structural changes of the protein which do not significantly alter its shape or size. However, there are pronounced global supramolecular structural changes of the membrane. Water removal, which is most likely to be from the lipid headgroups of the membrane, disrupts the interactions responsible for maintaining the native crystalline lattice of the membrane resulting in pronounced randomization of the positions of the proteins in the membrane.

INTRODUCTION

It is clear from the molecular viewpoint that water molecules must play an important role in the structure-function relationships of biological systems. Nevertheless, the extent and nature of this role still remains problematic. The present study pertains to this problem in membranes. The purple membrane (PM) of *Halobacterium halobium* is an ideal model membrane for this kind of study because of the relative simplicity of its structure and function. Furthermore, it is one of the most extensively studied membranes for which a relatively rich literature is currently available (for comprehensive reviews, see references 1–4 and the references cited therein). In addition, there has been considerable success in correlating spectra with structural features of this membrane in this laboratory over the last decade (5–10).

The PM is a distinctive, flat purple disk-shaped membrane component synthesized by the bacterium in response to low oxygen tension and enhanced light illumination. Under these conditions, this organism changes from oxidative phosphorylation as its primary energy mechanism to photophosphorylation based on the chromophore retinal. The PM disks contain a sole transmembrane protein, bacteriorhodopsin (bR), which is a retinal-containing chromoprotein. The bR is the best-characterized example of a transmembrane protein currently available and serves as an archetype for many others. The retinal is bound to the bacterioopsin apoprotein by means of a protonated Schiff-base linkage to a lysine group.

Light absorption by the retinal drives a complex photocycle of the bR in which the chromophoric retinal undergoes a reversible configurational change from the all-*trans* to the 13-*cis* form resulting in deprotonation and reprotonation of the Schiff-base nitrogen. This photocycle is coupled to the vectorial translocation of protons across the bacterial cell membrane which results in the establishment of an outward-directed proton gradient. The potential energy of the resulting gradient drives the anaerobic ATP synthesis of the organism.

The bR consists of a single polypeptide chain of 248 amino acids with a molecular weight of 26,866 D. It comprises 75% of the dry weight of the PM with the remainder being lipids. The secondary structure of the bR

is generally believed to be highly α-helical. However, it may be more α_{II} in form than the classic α_I (11, 12). Although the possibility of significant β-structure has also been recently proposed, this possibility remains controversial (13–15). The bR polypeptide chains traverse the PM bilayer seven times embedding 80% of the amino acids within the hydrophobic bilayers of the PM. The rigid closely packed polypeptide segments, containing about 24–32 amino acids, are oriented nearly perpendicular to the membrane plane. The bR molecules are arranged within the PM as cyclic trimers forming a hexagonal lattice. The degree of the crystalline order of the PM is sufficiently high to promote excitonic resonance among the chromophoric retinals.

The polypeptide segments do not enclose a large transmembrane aqueous channel. However, this laboratory has presented evidence that during the light-mediated bleaching in the presence of hydroxylamine and during the photocycle of the PM, the PM most likely undergoes reversible large scale global structure changes (7, 9). Two important consequences of these changes are the tilting of the polypeptide segments of the bR away from the membrane normal and a loss of the excitonic resonance among the chromophoric retinals. The former suggests that these changes may result in the formation of transmembrane aqueous channels normal to the membrane plane during the bleaching of the PM, which can account for the enhanced proton permeability of the PM due to this process, and in the formation of pulsating transmembrane channels or deformation waves during the photocycle of the PM, which can provide the driving force for the active proton transport process of the PM and can also account for the need of illumination during the bleaching and reduction processes. The latter suggests that the interactions responsible for maintaining the crystalline lattice of the PM are disrupted, indicative of a significant change in the supramolecular structure of the membrane. Therefore, these reversible perturbations of the PM structure result in significant changes in the global conformation of the bR coupled to changes in the supramolecular structure of the PM. In contrast, other reversible perturbations of the PM structure by such agents as pH, dark-light adaptation and polyhydric alcohols result only in subtle conformational changes of the bR with no apparent tilting of the polypeptide segments of the bR away from the membrane normal and with no observable changes of the PM supramolecular structure (5, ,8, 10).

The specific question addressed in the present study is which of these two apparent types of reversible PM structural changes result when water molecules are removed from the PM. A number of studies on the influence of water on the structure and function of the PM have been published over the last decade indicating that modification of the light-dark response (16–18), the photocycle (19–21), M_{412} kinetics (22), and changes in absorption energy (23, 24) result from the dehydration of the membrane. However, the characterizations of the accompanying structural changes have been somewhat limited in regard to nature and extent (25, 26).

The absorption and circular dichroic (CD) spectra of PM were studied in solutions with very high glycerol concentrations (up to 99% by volume) and in oriented films with and without glycerol impregnation and subjected to different degrees of dehydration by dry nitrogen gas flushing and high vacuum pumping. Analysis of the spectra by methods developed by this laboratory revealed that removal of water molecules from the PM results in both subtle localized conformational changes of the bR and significant changes in the supramolecular structure of the PM. Therefore, it is concluded that the water molecule is a very essential component for maintaining the integrity of the membrane structure.

MATERIALS AND METHODS

The PM was isolated according to the method of Becher and Cassim (27) with minor changes. Instead of the sucrose step-gradient purification, purification was achieved by many washings. Although this method sharply reduces PM yield, it results in PM preparations of equal or greater purity without ever exposing the PM to sucrose (10). Whenever it was necessary to store PM preparations, 0.05% sodium azide was added to prevent bacterial alteration of the PM structure. However, PM preparations were never subjected to storage time exceeding a few days before spectral studies.

PM suspensions in solutions with glycerol volume concentrations of 80% and higher were prepared in the following manner. 5 ml of PM aqueous suspension with bR concentrations between 27 and 37 μM was added to 10 mL of spectrophotometric grade glycerol (Mallinckrodt Inc., St. Louis, MO) in a heavy-wall Erlenmeyer suction flask. First the solution was degassed with a hydroaspirator while being constantly stirred. Then a high vacuum was applied exhaustively to the flask, which was equipped with a cold trap, using a Duo Seal model 1402 vacuum pump (Welch Scientific Co., Skokie, IL) while the solution was subjected to constant stirring. Under these conditions, the highest glycerol volume concentration for PM suspensions achieved was 99%. PM suspensions in 80, 85, 90, and 95% glycerol were achieved by progressive dilutions of the 99% glycerol solution. Glycerol contents were determined with a refractometer (Carl Zeiss, Inc., Thornwood, NY). In the bR concentration ranges used, the spectral parameters were linear with bR concentration. Therefore, spectral presentations were normalized to a given bR concentration.

Well-oriented PM films were formed by layering ~1 cc PM solution (OD_{568} = 0.5–1.8) which was previously passed through a 5-μm filter (Gelman GA-1) and degassed, onto optical quality flats of Suprasil-S quartz (Precision Cells, Inc., Hicksville, NY). Solutions were allowed to dry slowly in a desiccator containing either a beaker of Drierite or a saturated $LiCl_2$ solution which maintained the relative humidity at ~20%. Before spectral studies, the films were fully hydrated by incubating them for at least 24 h in a desiccator with a beaker of saturated K_2SO_4 solution which maintained the relative humidity at ~95%. For the visible and near-UV spectral studies, the hydrated film absorbances were in the OD_{568} range of 0.05 to 0.1 and, for the far-UV studies, absorbances were in the OD_{568} range of 0.01 to 0.02.

Glycerol-impregnated films were formed from fully hydrated films by covering the film surface with excess 20% glycerol solution. The excess solution was then poured off, leaving a very thin layer of solution clinging to the film surface. The films were then incubated in a desiccator with a beaker of saturated K_2SO_4 solution for at least 24 h. After 24 h, the K_2SO_4 beaker was exchanged for a beaker of saturated $LiCl_2$ solution to permit the films to dry slowly for 36–48 h. Before spectral studies, the

films were fully hydrated by exposing them to an atmosphere of 80% relative humidity for a very short period of time in a desiccator with a beaker of saturated K_2SO_4 or to room atmosphere depending on the prevailing ambient conditions. Films of suitable quality for spectral measurements, especially CD, are difficult to fabricate. Minor structural imperfections in the films can result in drastic errors in spectral measurements. The criteria used in this laboratory for film quality control has been given in previous publications (7, 9). Linear dichroism measurements of these films at 568 nm yielded values identical to those published by Heyn et al. (28). Because these authors showed by neutron diffraction measurements that the mosaic spread of their films were negligible, it is a reasonable assumption that the mosaic spread of films used in the present study must be likewise. In addition, electron microscope examinations of the PM samples dried on carbon-coated formvar grids showed them to be oriented parallel to the surface.

CD spectra were recorded on a Cary 60 spectropolarimeter with a 6003 CD attachment (Varian Associates, Inc., Palo Alto, CA) or on a J-500A spectropolarimeter (Jasco Inc., Easton, MD). Absorption spectra were recorded on a Cary 118C spectrophotometer with far ultraviolet modification and a scattered transmission accessory (Varian Associates, Inc., Palo Alto, CA). All spectra were measured at 20°C. The PM samples were light adapted unless otherwise indicated.

For solution spectral studies, rectangular and cylindrical optical cells with Suprasil-S quartz windows (Precision Cells, Inc., Hicksville, NY) were used. Pathlengths varied from 10 to 0.1 mm depending upon the spectral region studied. Films, which were formed on rectangular optical quality flats, were studied in specially designed quartz optical cell Dewars (Kontes-Martin, Evanston, IL) (9). These Dewars could be either flushed with dry gas or evacuated with a vacuum pump. Films were also formed on the inside of one of the windows of 20-mm cylindrical optical cells with Suprasil-S quartz windows. The fill holes of the cells were fitted with high-vacuum stopcocks (Kontes Glass Co., Vineland, NJ) so that cells could be evacuated with a vacuum pump. All exhaustive vacuum pumping was achieved by the employment of a cold trap.

Linear dichroism of PM films was studied as previously described in detail (8, 9, 29). For homogenous orientations of the chromophoric retinal, the dichroic ratio, D is given by:

$$D = \frac{A_H}{A_v} = 1 + \frac{1}{n^2}(2\tan^2\phi - 1)\sin^2\alpha,$$

where A_H and A_V are the absorbance of horizontally and vertically polarized light, respectively; n is the refractive index of the PM; ϕ is the angle of the $\pi - \pi^*$ NV_1 transition dipole moment of the retinal with respect to the membrane; and α is the angle between the plane of the optical flat and the incident light. For heterogeneous orientations of the retinal, the average D is given by:

$$\langle D \rangle = 1 + \frac{1}{n^2}\left[2\frac{\sum_i \sin^2\phi_i}{\sum_i \cos^2\phi_i} - 1\right]\sin^2\alpha.$$

In the present case, α was set at 45° and n was assumed to be 1.53 for the visible wavelengths.

RESULTS

Visible Spectra

The visible absorption spectra of light-adapted PM subjected to a number of different perturbing conditions are compared with the native PM spectra in Fig. 1. In Fig. 1 *A*, the absorption of PM in solutions with 85, 95, and 99% glycerol by volume are compared with the absorption in aqueous solution. In a previous publication from this

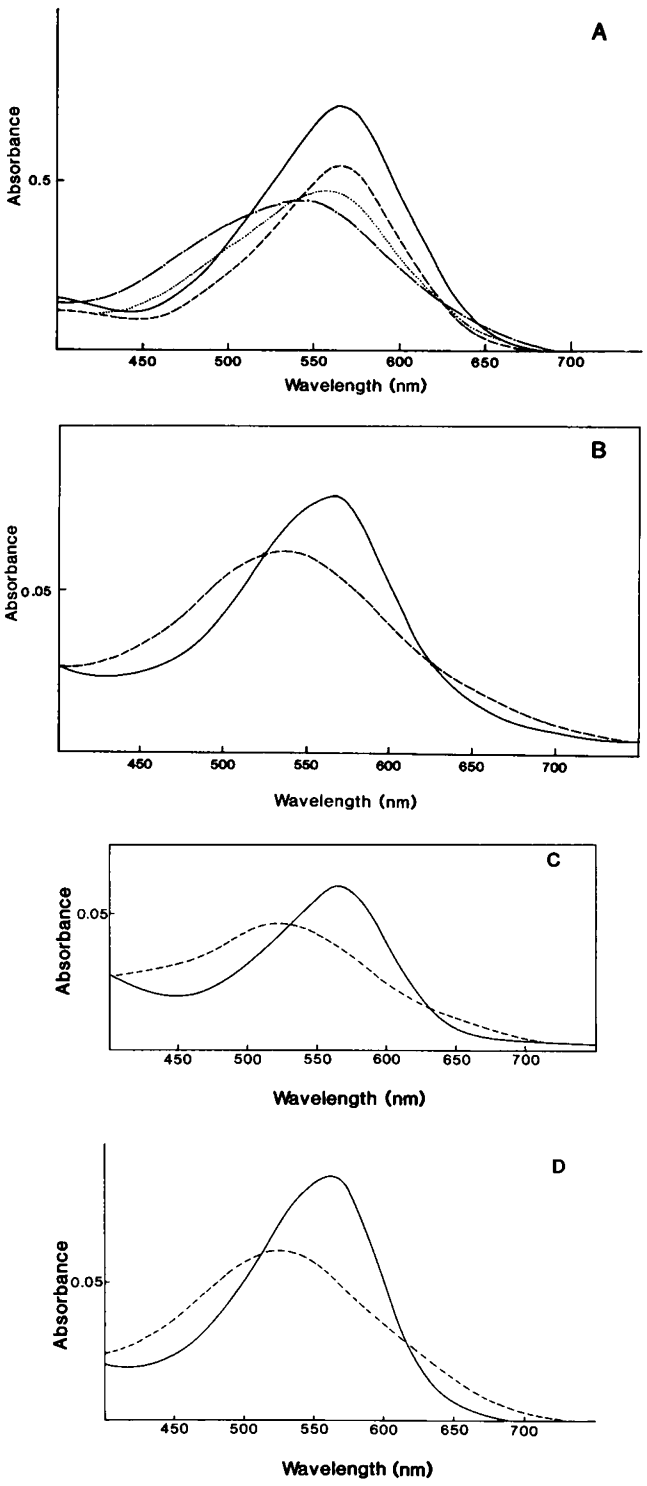

FIGURE 1 Effects of dehydration on visible absorbance of the PM. (*A*) PM in high glycerol solutions, 0% (——), 85% (– – –), 95% (· · ·), and 99% (- · -) by volume (the optical path and the protein concentrations were 1 cm and 11.1 μM, respectively). (*B*) PM film, hydrated (——) and dehydrated with exhaustive dry nitrogen gas flushing (– – –). (*C*) PM film, hydrated (——) and dehydrated with exhaustive high vacuum pumping (– – –). (*D*) Glycerol-impregnated PM film, hydrated (——) and dehydrated with exhaustive dry nitrogen gas flushing (– – –).

laboratory, it was shown that glycerol additions up to 80% lead to no change in the wavelength of the 568-nm band and only a slight decrease of ~2% in the absorbance of this band (10). It is apparent that the changes shown in spectra *A* are in sharp contrast to the previous findings. Further increases in glycerol concentration by only 5% (from 80 to 85%) results in a large 22% decrease in the absorbance with still no change in the band wavelength. However, continual increases in glycerol concentration (from 85 to 99%) results in a shift of the band wavelength to ca 540 nm. Although the decrease in absorbance continues with this final glycerol addition, the rate of decrease is much less than that observed between 80 and 85% glycerol addition. It is noteworthy that the greatest wavelength shift occurs between 95 and 99% glycerol, whereas the greatest decrease in absorbance occurs between 80 and 85%. However, when the absorbance of PM in 99% glycerol solution is measured under continuous exhaustive evacuation, the spectrum undergoes no further changes.

A difference absorption spectrum of PM in 99% glycerol and aqueous solutions (not shown) indicates three peaks: two positive ones at ca 470 and 660 nm and a negative one at ca 570 nm with the ratio of the magnitudes of the peaks at 470, 570, and 660 nm being about 1:3:0.2, respectively. The long-wavelength peak is difficult to resolve accurately due to its relatively weak magnitude.

Fig. 1 *B* demonstrates the change in visible absorption spectrum of a light-adapted hydrated PM film before and after exhaustive flushing with dry nitrogen gas. This relatively mild method of dehydration of the PM film results in a shift of 568-nm band maximum to ca 538 nm with a distinct decrease in its absorbance. A difference spectrum (not shown) of the partially dehydrated film curve minus the hydrated one can resolve three peaks: two positive ones at ca 480 and 660 nm and a negative one at ca 570 nm with the ratios of the magnitudes of the peaks at 480, 570, and 660 nm being about 1:2:0.4, respectively. The same results can be obtained when dry oxygen gas flushing is substituted for the nitrogen flushing. Fig. 1 *C* demonstrates the change in the absorption spectrum of a similar film when subjected to a more severe process of dehydration, exhaustive vacuum pumping. In this case, the shift of the 568-nm band maximum is to ca 530 nm. Recently, Hildebrandt and Stockburger (21) have reported a similar shift of the 568-nm band maximum. A difference spectrum (not shown) based on these curves yields results similar to the one based on the curves of Fig. 1 *B*. Time course studies of the dehydration process (spectra not shown) reveals that these spectral changes are continuous and that the saturation time of the process depends on the method used. Furthermore, the dehydrated films can be returned rapidly to their hydrated states by exposing them to prevailing room humidities. However, the rehydrated films are now in the dark-adapted state and absorb maximally at ca 558 nm.

Previously, Lazarev and Terpugov (23) published time-course dehydration spectra of the dark-adapted PM film, showing that after 40 min of vacuum pumping, the maximum of the 558-nm band undergoes a shift to only ca 540 nm. However, in our hands, after subjecting a dark-adapted film to ~15 min of vacuum pumping, a shift from 558 to ca 520 nm was achieved (spectrum not shown). Additional pumping time produced no further changes. In the dehydrated minus hydrated difference spectrum, three peaks could also be resolved with about a 1:3:0.2 magnitude ratio at ca 480, 560, and 660 nm, respectively. Therefore, the long-wavelength peak observed in the difference spectrum of the light-adapted film seems to be present also in the difference spectrum of the dark-adapted film. However, the relative magnitude of the peak appears to be smaller. Lazarev and Terpugov (23) did not resolve this peak in their difference spectrum.

The visible absorption spectra of a light-adapted maximally glycerol-impregnated PM film, before and after dehydration with exhaustive nitrogen gas flushing, are shown in Fig. 1 *D*. Dehydration results in the shift of the 568-nm band to ca 530 nm. Similar results were obtained with the exhaustive vacuum-pumping method of dehydration. Again, when the film is exposed to the prevailing room humidity, the band maximum rapidly shifts to ca 558 nm, the characteristic wavelength of this band in the dark-adapted film spectrum. The time course study of the absorption under continuous nitrogen gas flush indicated that the spectral changes were continuous as in the glycerol-free case (spectra not shown). However, the spectral changes in this case were somewhat more complicated than those of the glycerol-free film. The isosbestic points were not always consistent. This was also the case with glycerol-free films; however, the variations were more pronounced with glycerol-impregnated films.

The dichroic ratio at the band maximum at 568 nm of the hydrated glycerol-free films was previously shown to be ~0.85 ± 0.03 (8, 9). Measurements of this ratio for glycerol-impregnated films yields the same value for both the hydrated and dehydrated states at 568 and 530 nm, respectively.

The visible CD spectra of light-adapted PM subjected to identical perturbing conditions used in Fig. 1 are demonstrated in Fig. 2. The spectra of PM in solutions of very high glycerol content are shown in spectra *A*. Previously, we showed that glycerol additions up to 80% caused the ratio of the ellipticity of the positive lobe to that of the negative one of the characteristic bilobed band centered at ca 574 nm in an aqueous solution to decrease from 1.55 to 0.65 and the crossover wavelength to shift from 574 to 562 nm (10). From 80 to 85% glycerol addition, this ratio continues to decrease from 0.65 to 0.56. However, from 85% glycerol to 99%, it increases from 0.56 back to 1.60. Although the ratio is nearly identical in aqueous and 99% glycerol solutions, the ellipticities of both lobes are three times greater in aqueous solution than in 99% glycerol solution.

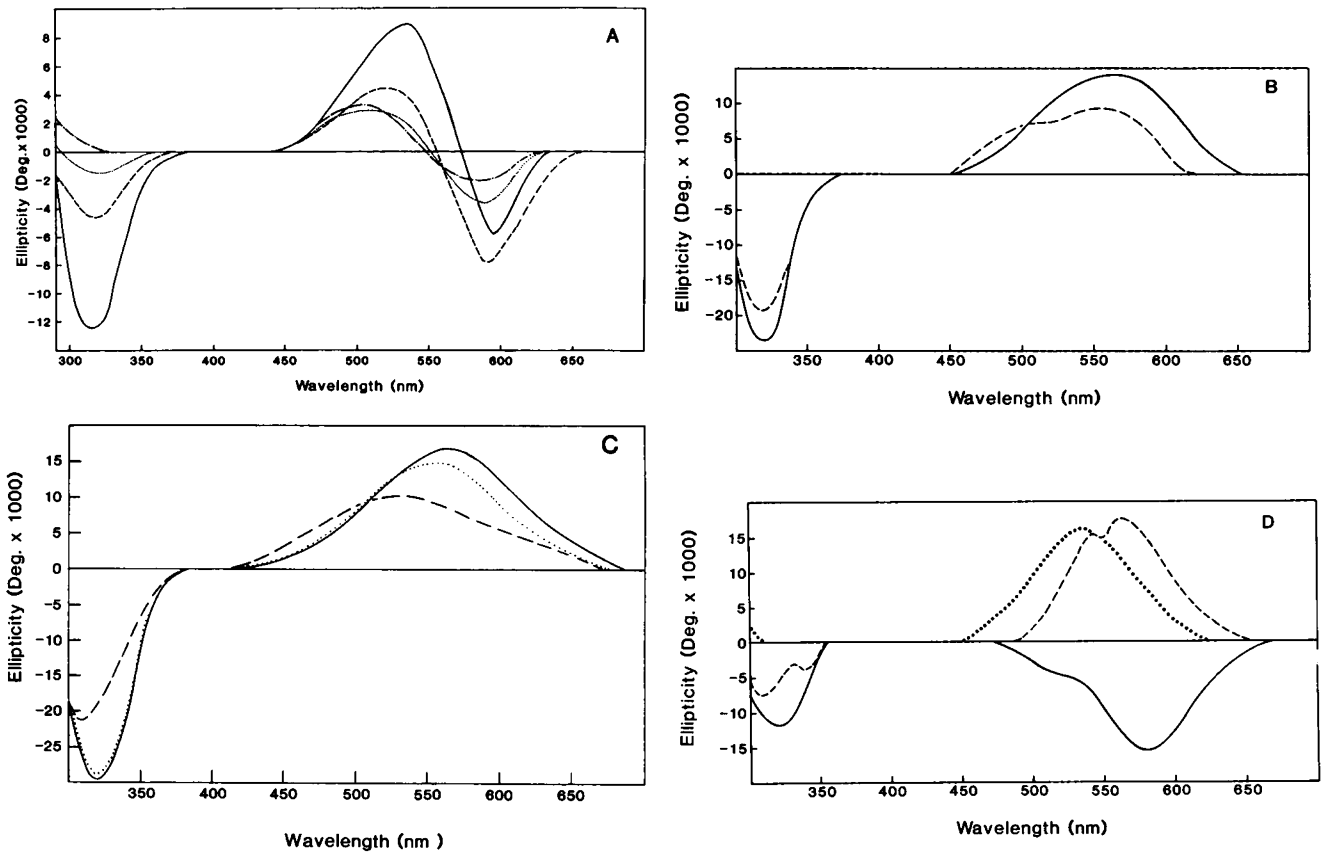

FIGURE 2 Effects of dehydration on visible CD of the PM. (*A*) PM in high-glycerol solutions, 0% (——), 85% (– – –), 95% (· · ·), and 99% (- · -) by volume (the optical path and the protein concentrations were 1 cm and 15.9 μM, respectively). (*B*) PM film, hydrated (——) and dehydrated with exhaustive dry nitrogen gas flushing (– – –). (*C*) PM film, hydrated (——), dehydrated with exhaustive high vacuum pumping (– – –), and rapid rehydration in prevailing room humidity (· · ·). (*D*) PM glycerol-impregnated film, partially dehydrated with dry nitrogen gas flushing (——) and dehydrated with exhaustive dry nitrogen gas flushing (· · ·).

Previously, it was shown that glycerol addition up to 80% resulted in sharp decreases in the ellipticity of the 317-nm CD band and that the rate of decrease was greater with the initial addition of glycerol than with the later ones (10). Two new observations are noteworthy from Fig. 2 *A* regarding glycerol effects on this band: one, that the band completely vanishes in 99% glycerol, and another, that the rate of decrease of the ellipticity of the band is much greater between 85 and 99% glycerol additions than during any other additions. However, in spite of these dynamic changes in ellipticities, the wavelength of this band remains invariant to all glycerol additions.

The changes induced in the film visible CD spectrum of light-adapted PM by exhaustive flushing with dry nitrogen gas are presented in Fig. 2 *B*. The maximum ellipticity of the characteristic single positive Gaussian-shaped band of the hydrated film at 568 nm is reduced by ~33% and the wavelength is shifted by 14 nm to lower wavelengths with a pronounced shoulder evident at ca 510 nm. This partially dehydrated film band appears to be the resultant of two Gaussian bands, one at the wavelength of the original hydrated film band and another at a lower wavelength. The ellipticity of the 317-nm band is only reduced by ~18% with no change in wavelength.

However, when the film is subjected to exhaustive vacuum pumping (Fig. 2 *C*), the maximum is further shifted to ca 530 nm with about a 37% decrease in maximum ellipticity. No shoulder is evident and the resulting band is nearly Gaussian in shape. It is noteworthy that in both processes of dehydration, the polarity of the band remains positive as in the hydrated state. However, in the case of the more severe dehydration, there is not only about a 27% decrease in the ellipticity, but also a ca 10 nm increase in energy of the 317-nm band. The CD of the dehydrated film rapidly reverts to that of the hydrated film when the film is exposed to the prevailing room humidity. However, the resulting CD is that of a typical hydrated dark-adapted film with the long-wavelength band at ca 568-nm and with maximum ellipticity about 10% less than that of the hydrated light-adapted band at 568 nm (5).

The effects of dehydration on the visible CD of glycerol-impregnated film is shown in Fig. 2 *D*. The hydrated glycerol-impregnated film CD is the same as previously published (10). The 568-nm positive band of the hydrated

glycerol-free film (shown in spectra *C*) has been transformed into a negative band at ca 570 nm with a definite shoulder at ca 540 nm in the hydrated glycerol-impregnated film. When the glycerol-impregnated film is subjected to partial dehydration by nitrogen gas flushing, the polarity of this band again reverses to positive with peaks at ca 568 nm and ca 545 nm. However, with exhaustive nitrogen gas flushing, the band becomes nearly Gaussian in shape with a peak positioned at ca 530 nm. The ellipticity of the 317-nm band of the hydrated glycerol-free film is severely depressed in the hydrated glycerol-impregnated film and its wavelength is slightly shifted to ca 320 nm. Upon dehydration, the ellipticity is further depressed and finally vanishes with exhaustive dehydration. The band wavelength shifts to ca 310 nm with a shoulder at ca 340 nm. It is clear from comparison of Fig. 2 *A* and Fig. 2 *D* that the exhaustive dehydrated glycerol-impregnated film CD is similar to that of 99% glycerol solution in regards to this band.

Near Ultraviolet Spectra

The effects of identical perturbing conditions on the near-UV CD of the PM are demonstrated in Fig. 3. The effects of high glycerol concentrations are shown in Fig. 3 *A*. Previous results from lower glycerol concentration studies indicated the wavelength of the prominent positive extremum at ca 262 remains invariant to glycerol additions up to 80% although its ellipticity increases about 57% (10). However, after 80% glycerol addition, as shown in Fig. 3 *A*, its wavelength undergoes gradual shifts to ca 265 nm at 99% addition with the ellipticity continually increasing but at a somewhat greater rate to a 108% change. Both the wavelength and the ellipticity of the prominent negative extremum is invariant to glycerol addition up to 85%. With further additions, the wavelength undergoes a very slight shift from ca 283.2 nm to ca 283.6 nm while the ellipticity rapidly diminishes and becomes positive at 99% glycerol concentration. It is evident that the changes in this spectral

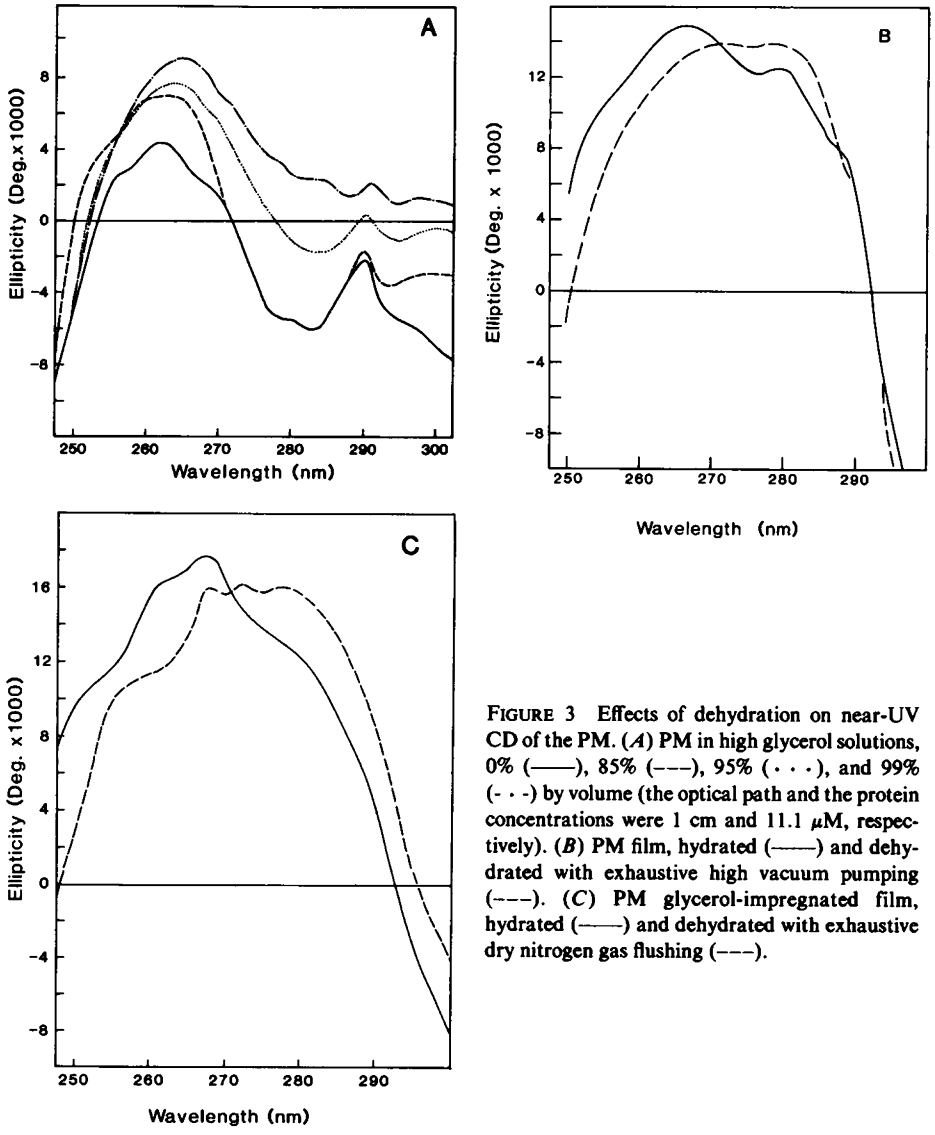

FIGURE 3 Effects of dehydration on near-UV CD of the PM. (*A*) PM in high glycerol solutions, 0% (——), 85% (– – –), 95% (· · ·), and 99% (- · -) by volume (the optical path and the protein concentrations were 1 cm and 11.1 μM, respectively). (*B*) PM film, hydrated (——) and dehydrated with exhaustive high vacuum pumping (– – –). (*C*) PM glycerol-impregnated film, hydrated (——) and dehydrated with exhaustive dry nitrogen gas flushing (– – –).

region of the PM in glycerol solutions are markedly altered after the glycerol concentrations exceed ~80% as was the case with changes in the visible region.

The comparative near-UV film spectra of light-adapted PM in the hydrated and dehydrated states are shown in Fig. 3 *B*. Dehydration was achieved by exhaustive vacuum pumping. The spectral changes are very limited. Upon dehydration, the ellipticities at wavelengths below 270 nm decrease slightly and those at wavelengths above 270 increase slightly. It is noteworthy that the shape of the spectrum is not altered significantly. The minor changes in ellipticity seem to be a reflection of a shift of the entire near-UV CD curve a few nanometers to longer wavelengths upon dehydration. These changes were completely and rapidly reversible, as were the visible spectral changes. Dehydration with exhaustive nitrogen gas flushing produced similar changes but of somewhat lesser magnitudes.

The effects of dehydration on the near-UV CD of the glycerol-impregnated film, demonstrated in Fig. 3 *C*, are very similar to those observed for the glycerol-free film. The only difference was that exhaustive nitrogen gas flushing in this case produced basically the same magnitude of change as the exhaustive vacuum pumping did in the glycerol-free case. Furthermore, in this case, there was no observable spectral change differences between the two methods of dehydration employed, in contrast to the case for the glycerol-free films.

Far Ultraviolet Spectra

The far-UV CD spectra of the PM subject to the same perturbing conditions used in the visible and near-UV spectral studies are demonstrated in Fig. 4. The far-UV solution spectra of the PM in increasing concentration of glycerol are shown in Fig. 4 *A*. The ellipticity of the positive 194-nm band is unaltered by glycerol addition. The ellipticities of the negative bands increase in concert with increasing glycerol concentrations with the ellipticity

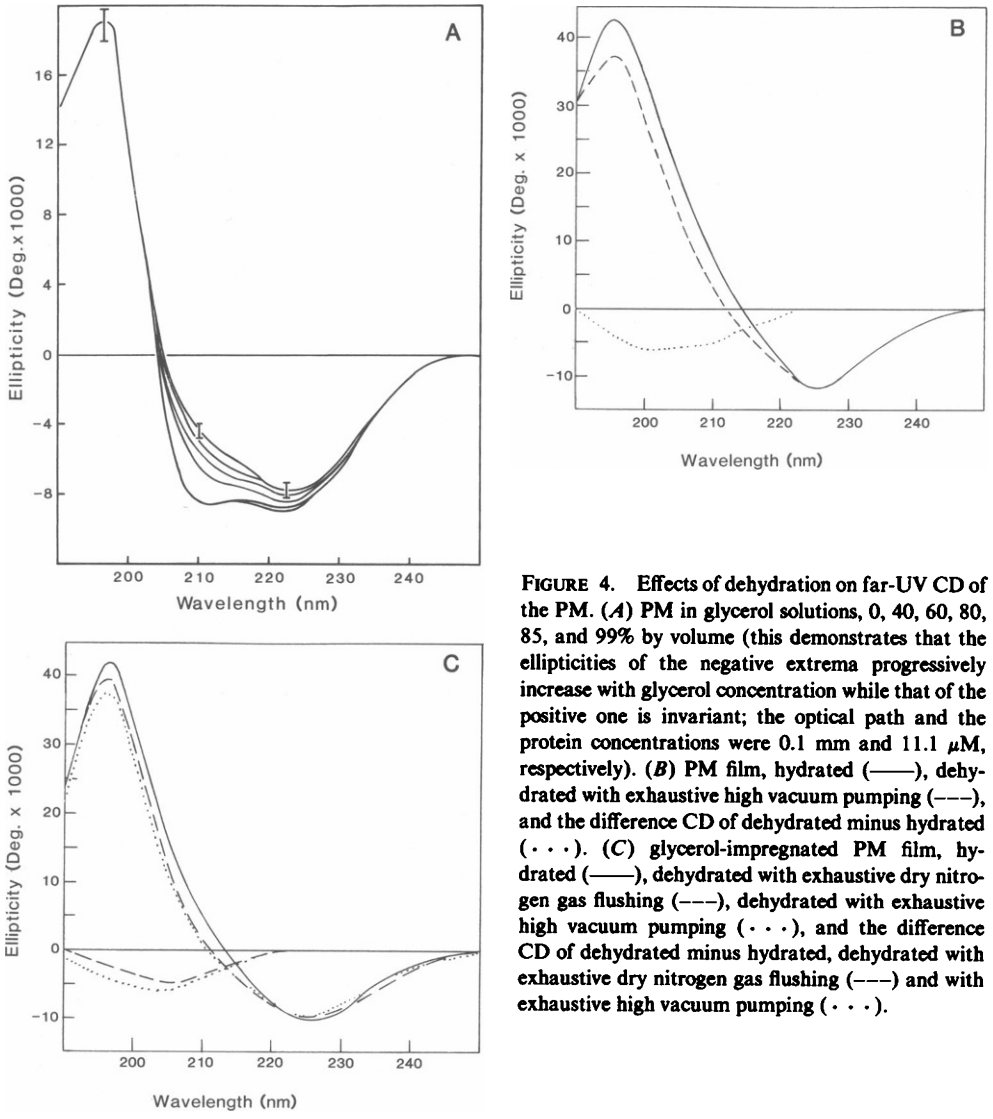

FIGURE 4. Effects of dehydration on far-UV CD of the PM. (*A*) PM in glycerol solutions, 0, 40, 60, 80, 85, and 99% by volume (this demonstrates that the ellipticities of the negative extrema progressively increase with glycerol concentration while that of the positive one is invariant; the optical path and the protein concentrations were 0.1 mm and 11.1 μM, respectively). (*B*) PM film, hydrated (——), dehydrated with exhaustive high vacuum pumping (– – –), and the difference CD of dehydrated minus hydrated (· · ·). (*C*) glycerol-impregnated PM film, hydrated (——), dehydrated with exhaustive dry nitrogen gas flushing (– – –), dehydrated with exhaustive high vacuum pumping (· · ·), and the difference CD of dehydrated minus hydrated, dehydrated with exhaustive dry nitrogen gas flushing (– – –) and with exhaustive high vacuum pumping (· · ·).

of the 210-nm band increasing at a much faster rate than that of the 223-nm band. At 99% glycerol concentration, where the index of refraction of the solution is ~1.4732 at 589 nm, the ellipticity of the 210-nm band has increased by ~94% whereas that of the 223-nm band has increased by only ~13%. There is also approximately a 2-nm and a 1-nm shift of the crossover wavelength and the 223-nm band, respectively, to shorter wavelengths. These changes, induced by glycerol, were shown to be completely reversible by exhaustive dialysis studies. The comparative far-UV CD spectra of hydrated and dehydrated PM films are given in Fig. 4 *B*. The dehydration of the film is achieved with exhaustive vacuum pumping. In contrast, dehydration with exhaustive dry nitrogen gas flushing results in no observable spectral changes. The spectral changes demonstrated here are completely and rapidly reversible. Dehydration of the film results in a ca 2.5-nm shift of the crossover wavelength to shorter wavelengths. The difference spectrum of the dehydrated minus the hydrated PM is also given in Fig. 4 *B*. Spectral studies of PM films in the far-UV are difficult due to a number of technical problems. However, it is obvious that in spite of these experimental difficulties, the difference spectrum reveals a reasonably well-shaped single Gaussian curve centered at ca 206 nm.

The comparative far-UV spectra of hydrated and dehydrated glycerol-impregnated films is shown in Fig. 4 *C*. The results from both methods of dehydration, exhaustive nitrogen gas flushing and exhaustive high vacuum pumping, are given. Within experimental uncertainty both methods produced the same reversible spectral effects as in the case of the glycerol-free films in which these effects could only be produced by exhaustive vacuum pumping.

DISCUSSION

Visible Spectra

The analysis of the visible absorption and CD of the PM based on quantum mechanical theories of molecular spectra have been given in a number of publications (5–10, 30–34). The absorption spectrum is dominated by an apparent single band which has been assigned to the electric dipole–allowed and magnetic dipole–forbidden $\pi - \pi^*$ (NV_1) transition of the chromoporic retinal of the bR (5). This band is considered to be the superposition of three bands resulting from the cyclic-trimeric degenerate oscillator coupling (exciton model) of the retinals within the hexagonal crystal lattice of the PM which conforms to a P3 space group. According to the formalism of this model and the PM geometry, two of these bands will be degenerate and their transition dipole moments will be polarized in the membrane plane while the third one will be polarized in a direction normal to this plane. Furthermore, most of the oscillatory strength of the transition will be vested in the in-plane bands at the expense of the out-of-plane band. In addition, the induced excitonic transition dipole moments will possess substantial rotational strengths which will give rise to three CD bands, the resultant of which will be a conservative bilobed CD band centered about the wavelength of the absorption band. However, since the characteristic bilobed CD band of the light-adapted PM in solution is not conservative, it is necessary to propose the existence of an additional band to account for this nonconservativeness. Such a band is possible with the formalism of the one-electron mechanism of induced optical activity which utilizes an apoprotein-imposed dissymmetric perturbation to reduce the retinylidene C_{2h} symmetry. This mechanism can not only generate a CD band centered with the excitonic bands but also a companion band at ca 317 nm (where, it has been proposed, a magnetic dipole–allowed $\pi - \pi^*$ transition resides) with the same rotational strength but of opposite sign. However, the 317-nm CD band is considered to be the superposition of at least two bands due to its relatively high rotational strength. This companion band plus another CD band induced by an electric-magnetic resonance coupling mechanism which couples the proposed magnetic dipole–allowed $\pi - \pi^*$ transition at ca 317 nm and an electric dipole–allowed $\pi - \pi^*$ transition of some nearby aromatic amino acid residue planar ring.

Due to the unique polarizations of the exciton bands in regard to the membrane coordinates and the formalisms of the molecular spectral mechanisms, the solution spectrum, in which the measuring light is oriented at random with respect to the membrane, may differ from the film spectrum, in which the light is oriented perpendicular to the membrane plane. Because the absorption spectrum is dominated by the in-plane degenerate bands, the two spectra are expected to be very similar. On the other hand, the excitonic contributions cannot be present in the film CD spectrum and this spectrum must contain only contributions from the one-electron and electric-magnetic resonance coupling mechanisms. Experimental evidence has been in excellent accord with these theoretical predictions (7).

The absorption spectral changes resulting from the effects of very high glycerol concentrations, dry nitrogen gas flushing, and high vacuum pumping are very similar and additive as seen in Fig. 1. Therefore, it is reasonable to assume that effects of these three perturbants are due to the same cause, the removal of water molecules from the PM. It is clear that this dehydration process must result in structural changes in the local retinylidene environments of the apoprotein of the bR in view of the transformation of much of the protein molecules from the bR_{568} or the bR_{558} states absorbing at 568 and 558 nm, respectively, to at least two new states, one absorbing at higher and another at lower energy. The higher energy state has been observed by a number of laboratories and has been labeled bR^{dh}_{530} (17, ,21, 23). The low energy state has never been reported before in the absorbance spectra of the dehydrated PM. It is difficult to determine its exact wavelength but it lies

somewhere between 625 and 675 nm. At present, its nature remains problematic. However, it is interesting to note that a bR_{630} state has been identified in the photocycle of bR^{dh}_{530} by resonance Roman spectroscopy (21). The energy changes are accompanied by a significant decrease in absorbance. Because the dipole strength of the absorption band is attributed solely to excitonic interactions, this decrease in absorbance reflects changes in both the bR conformation and the supramolecular organization of the PM. Excitonic coupling is intimately dependent on the chromophoric positions, orientations, and order in the PM crystalline lattice.

The effects of glycerol on the PM-visible CD spectra are much more complex than the effects on the absorption spectra. Previously, we showed that the decrease in the ellipticity ratio of the CD bilobed band and in the ellipticity of the 317-nm CD band due to glycerol additions up to 80%, which caused no significant change in the absorption spectra, most likely resulted from a polarity reversal of the proposed one-electron-mechanism-induced companion bands at 568 and 317 nm (10). Theoretically, a reversal of the polarities of the companion bands would be indicative of a change in the sign of perturbation potential. This change in perturbation potential can be interpreted as a change in the apoprotein-induced screw sense of the chromophoric retinal. We proposed that glycerol enhanced hydrophobic interactions in the PM, resulting in a more compact conformation of the bR via a subtle tertiary structural change of the bR (10). Change in the screw sense of the retinylidene symmetry implies either a change in the dissymmetric electric field imposed by the apoprotein in the local environment of the retinal or a structural distortion of the planarity of the retinal. Experimentally, it is difficult to differentiate between these two symmetry-reducing perturbations. It is clear from Fig. 2 that the reversal of these molecular events occur with glycerol concentrations >80%. This must be due to the introduction of an additional glycerol-induced factor. This additional factor, which counteracts the screw-sense reversing effect of glycerol, is most likely due to the hygroscopic property of glycerol which becomes prominent at higher glycerol concentrations. It should be noted that the membrane bilayer is relatively permeable to small uncharged polar molecules such as glycerol.

In concert with the absorption band, the CD exciton bands and the nonexciton band associated with them in the characteristic bR bilobed CD band undergoes the same energy shift to ca 540–530 nm under the same experimental conditions, as shown in Fig. 2. The lower energy state seen in the absorption spectra is not obvious in the CD spectra. Either this state is not optically active or the optical activity is too small, due to the low concentration of this state, to be observed. Another important change in the CD spectra due to dehydration is the very sharp reduction of ellipticity of the excitonic bands and associated nonexciton band. These spectral changes are due to the same bR conformational and PM supramolecular organizational changes suggested by the absorption spectral changes. However, it is important to note that in 99% glycerol solution, the dehydration process is only partial because the absorbance energy has only increased to 540 and not to 530 nm as observed in films. Whether the excitonic interaction is completely lost at this absorbance energy remains an unanswerable question at the present time. However, the CD trend seems to indicate that this interaction may most likely continue to drop as this energy increases.

There is a relatively small decrease in ellipticity of the 317-nm CD band upon dehydration, as seen in Fig. 2, *B* and *C*. This is expected because a decrease in the nonexcitonic component of the bilobed CD band must result in a decrease in the 317-nm band in view of the one-electron formalism evoked to partially explain the nature of this band. However, the large decrease in this band in high glycerol solutions and glycerol-impregnated films (Fig. 2, *A* and *D*) and its complete absence in the extreme cases (99% glycerol or exhaustive dehydration of glycerol-impregnated film) may be due mostly to another property of glycerol rather than its dehydrant nature. It is possible that the dielectric constant of glycerol may lead to drastic changes in deionizable groups of the proteins which result in the decoupling of the proposed electric-magnetic resonance between the retinal and a nearby aromatic amino residue.

Near Ultraviolet Spectra

It is apparent from Fig. 3 that the spectral changes in high glycerol solutions are relatively more pronounced than those observed in film studies, with and without glycerol. The spectral changes observed in solution are somewhat similar to, but greater in magnitude than, those previously observed when PM was subjected to pH changes from 7 to 12 (8). However, they are quite different from those previously observed when the PM was bleached by light in the presence of hydroxylamine and when bR was in the M_{412} intermediate state of the PM photocycle (6, 7, 9). The induced optical activity in this region is due to the $\pi - \pi^*$ transitions of the aromatic rings of 13 phenylalanines, 11 tryosines, and 7 tryptophans as well as the higher energy $\pi - \pi^*$ transitions of the chromophoric retinal. Because the probability of a significant number of interactions among such a large number of molecules is high, the near-UV CD spectrum is the superposition of a large number of bands. Therefore, no unique analysis is possible. However, because both the 262- and the 283-nm extrema are altered by high glycerol concentrations, subtle change of the bR tertiary structure involving a number of the aromatic amino acid environments is possible. Furthermore, it is evident from the wavelength shifts of the extrema that much of the glycerol-induced changes may be changes in the ionization states of the aromatic amino acids. Also, if some of these amino acids, located near the membrane surface, are exposed to the bulk solvent, the

relatively high index of refraction of glycerol would alter the effective field of the incident light resulting in spectral modifications.

Comparisons of solution and film spectra in Fig. 3 demonstrate that there are major differences between these spectra in the PM. This is due to the fact that the optical activity resulting from the in-plane transition dipole moments of the PM differs from those of the out-of-plane moments. That is, the aromatic rings of some of the amino acids of the bR must lie nearly in the plane of the membrane. Because this structural anisotropism is not altered by the dehydration process, as demonstrated in Fig. 3, *B* and *C*, the resulting tertiary structural changes of the bR induced by dehydration are most likely very localized and subtle. These results are similar to those observed when the PM was subjected to pH perturbation in which there was no change in the structural anisotropism (8). However, they are in contrast to those observed when PM was bleached or during the formation of the M_{412} photointermediate in which there were very drastic changes in structural anisotropism (7, 9).

Far Ultraviolet Spectra

The far-UV CD spectra shown in Fig. 4 clearly indicate that dehydration of the PM by a number of methods does not result in any significant secondary structural alteration of the bR. The resulting ellipticity changes observed in the negative CD bands of the PM upon the progressive addition of glycerol to aqueous solutions, with the complete absence of any change in the positive band, are a classic case of reduction in the dispersion distortions of a CD spectrum resulting from light scattering artifacts (35–39). This optical artifact depends on the difference of the squares of the indices of refraction of the particle and solvent. Since the strong amide absorption band of bR is at ca 193 nm in situ, the maximal difference of the squares of the indices of refraction will be ca 210 nm due to the dispersive nature of the index of refraction. According to theory, the differences should be very much less at 223 nm and negligible at 193 nm. The difference of the squares of the indices of refraction will diminish rapidly with glycerol addition, considering the relatively high index of refraction of glycerol (1.4746 at 589 nm). It is apparent that glycerol eliminates some, if not all, of the flattening of the 210-nm band relative to the 223-nm band of the PM CD in aqueous solution because of the much better matching of the solvent-solute index of refraction in glycerol than in water (the index of refraction of the PM is ~1.53 at 568 nm). It is also noteworthy that the ellipticity of the 223-nm band increased only 13%, indicating that dispersion distortions due to light-scattering artifacts are not significant at this wavelength in contrast to that at 210 nm, as previously suggested (40). Also, some, if not most, of this increase may be due to solvent effects per se on the amide $n - \pi^*$ transition responsible for this band and not on light-scattering artifacts (41). It is clear that these results are not in accord with the conclusions reached by a number of laboratories that the light scattering is not significant in the normal CD spectrum of PM if large acceptance angles are used during measurements (14, 42, 43). Glaeser and co-workers attribute most of the relative depression of the 210-nm band to the presence of appreciable amounts of β-sheet secondary structure (13, 42), whereas Wallace and co-workers attribute it to absorption flattening effects (15, 43).

It is apparent that there are marked differences between the far-UV solution CD spectrum and hydrated film CD spectrum in Fig. 4. These differences are due to the differences in the orientations of the dipole moments of the amide transitions responsible for the CD in this region. The 210-nm CD band of the PM is due mainly to the 206-nm amide rotatory band which arises from transition dipoles polarized in a direction parallel to the axis of the α-helical polypeptide segments of the bR. Rotatory bands responsible for the 223- and 193-nm CD bands arise from transition dipoles polarized perpendicular to the helical axis. Therefore, for light incident along a direction parallel to the helical axis, which is the case in the film studies, interactions between the electric field of the light and the transition dipole moments of the 206-nm rotatory band are not possible. This results in the absence of the 210-nm CD band in the film spectrum and the longer-wavelength displacement of the crossover wavelength relative to the position in the solution spectrum. In addition, relative ellipticities of the 223- and 193-nm bands are also expected to be different in these two types of spectra. For a more detailed theoretical discussion of this phenomenon, the reader is referred to our previous publications and references therein (7, 9, 44).

Dehydration of the film results in a reversible ca 2.5-nm shift of the crossover wavelength to shorter wavelengths as shown in Fig. 4, *B* and *C*. Similar spectral results have been observed previously in this laboratory during the light-mediated bleaching of the PM in the presence of hydroxylamine and during the formation of the M_{412} photointermediate of the PM photocycle (7, 9). Identical to the findings of the present study, it was apparent in those studies also that no significant changes in the secondary structure of the bR were indicated. The only possible explanation with firm theoretical basis for this change is that there has been a net tilting of the polypeptide segments away from the membrane normal with no appreciable change in the secondary structure of the bR as a consequence of the removal of water molecules from the membrane. This action will add a portion of the 206-nm amide rotatory band to the film spectrum resulting in the observed shift of the crossover wavelength. The amount of this shift will depend on the amount of the portion added which depends on the degree of net tilting of the polypeptide segments. If

sufficient tilting has occurred, a definite shoulder will appear on the 223-nm CD band. When the axis of the α-helical segments become randomly oriented with respect to the direction of the light propagation, the net tilt angle per segment, α, will be: $\sin^2 \alpha = \frac{2}{3}$ or $\alpha = 54.8°$ in view of the symmetry of the α-helix. Treatment of PM films with acetone or ethanol by adding drop amounts of these compounds to the films and allowing them to completely evaporate provides the means of obtaining randomized films of PM. Polarized Fourier transform infrared spectroscopy of such a treated PM film indicates that the net tilt angle per segment of the bRs is ~54.8° in the modified PM film (12). The far-UV CD spectrum of this film is identical to the aqueous solution CD spectrum of PM except the ellipticity of the 210-nm band is greater and crossover wavelength is shifted slightly to shorter wavelengths as seen in the CD of PM in high glycerol solutions shown in Fig. 4 *A*. These results are expected because the mismatching of solvent-solute index of refraction should be minimal in PM films as in PM glycerol solutions. At any rate, the main point is that the net secondary structure of the bR has not been altered by this treatment. Additional support for this point was obtained by studying the comparative far-UV CD spectra of PM in aqueous solution before and after this treatment.

The net tilt angle per segment, α, of the bR in the PM under any condition can be estimated from the relationship, $\sin^2 \alpha = 2R_\alpha/3R_0$, where R_α is the magnitude of the 206-nm rotatory band at tilt angle α and R_0 is the magnitude for $\alpha = 54.8°$ (45, 46). In the present case R_α is determined from the difference spectra of the dehydrated minus the hydrated PM shown in Fig. 4, *B* and *C*. R_0 can be determined from the difference spectrum of a PM film before and after organic solvent treatment because such treatments do not alter the thickness of the film as determined by absorbance studies. The estimated α from glycerol-free film spectra, in which tilting could only be produced by exhaustive vacuum pumping, and from glycerol-impregnated film spectra, in which tilting was produced by both exhaustive nitrogen gas flushing and exhaustive high vacuum pumping, was 20.6°, 19.2°, and 21.6°, respectively. It is apparent that the tilt angles are identically about 20.5° within experimental uncertainty and independent of the method of dehydration used as long as a critical degree of dehydration has been achieved. This estimate of α is based on the assumption that the helical segments of bR are perpendicular to the PM plane in the native case. Electron microscopy modeling has indicated an average tilt angle of ~11° (47, 48). On the other hand, infrared linear dichroism studies of PM films have indicated an average tilt angle as high as 33° (49, 50). However, these high values were based on calculations using the helix geometric parameters of the α_I-helix. If those more appropriate for the α_{II}-helix are used, this angle becomes insignificant because in this type of helix, the amide plane is significantly tilted in respect to the helix axis (11, 51). Also, assuming an initial tilt angle of 10° instead of 0°, would result in only about a 4% difference in the determination of R_0, which is well within experimental errors.

It is clear from the far-UV results, as from the results in the near-UV and visible spectral studies, that the dehydration effects of glycerol due to its hygroscopic property are additive to those of nitrogen gas flushing and vacuum pumping. It is also noteworthy that even in dehydrated glycerol studies (viscosity of glycerol is 1,490 cp at 20°C), the tilting process is identical to that observed in glycerol-free studies.

The magnitude of tilting observed on dehydration of the PM is similar to the one (~24.3°) previously determined in this laboratory by far-UV film CD analysis for the light-mediated bleaching of PM in the presence of hydroxylamine (7, 45). Infrared linear dichroism studies of PM films have supported this bleaching induced tilting (12, 50). In fact, if parameters more appropriate for the α_{II}-helix are used in the calculation, the magnitude of the tilting obtained by the two methods is identical within experimental uncertainty (12). Tilting has also been observed during the formation of the M_{412} photointermediate (~5–15°) using this type of far-UV CD analysis (9, 52). However, in this case, infrared spectral and x-ray studies have failed to corroborate the CD findings (53–56). It should be borne in mind that the CD analysis of the tilting phenomenon stands on firm theoretical grounds. One problem may be that in the various studies of the M_{412}, quite different methods are used to trap this transient state. Recently, a number of different M_{412} states have been cited in the literature (57), for example, M^{dh}_{412} in the photocycle of the dehydrated PM (21). In some of these studies, the amount of conversion to this state was very limited. As seen in the dehydration studies, tilting does not occur until a critical amount of bR_{568} has been converted to the high energy dehydrated state. That is, this tilting process seems to be a cooperative one. The tilting in the case of the M_{412} state may also be a cooperative process.

Linear Dichroic Studies

Tilting of the polypeptide segments in the membrane bilayer can result in the disordering of the native ordered retinylidene positions in the PM or in reordering of the original position due to the disruption of the interactions responsible for maintaining the native crystalline lattice of the PM. Although both models of tilting will give the same far-UV CD results, they should give different linear dichroic results in the visible absorbance spectra. For the bR_{568} state, the dichroic ratio at band maximum is ~0.85. If the positions of the retinal are randomized by this tilting and this randomization results in a change in the magnitudes of the retinylidene out-of-plane angles from a nondispersed value of 20° to dispersed values of −10 to 50° in

intervals of 0.1°, a simple calculation (see Materials and Methods) shows the resulting dichroic ratio would become 0.88. However, if there was no dispersion in the magnitude of this angle, so that this angle was evenly distributed between -10 and 50° only due to the tilting, then the resulting dichroic ratio would become 0.98. Therefore, the calculated change in the dichroic ratio for the randomized model is ~4% which is within the experimental certainty of ±4% for such measurements; whereas, the change is ~15% for the reordered model. Because this ratio is the same for bR_{568} at 568 nm and bR_{530} at 530 nm, it is clear that the randomized model is in better accord with the experimental results. If the retinylidene positions are randomized in the PM due to the loss of the membrane crystallinity as suggested by the linear dichroic results, the excitonic interaction among retinals must vanish upon dehydration. It has been observed that tilting and complete loss of excitonic interactions are coupled during the bleaching of the PM and during the formation of M_{412} photointermediate of the PM photocycle. However, excitonic effects can only be detected in solution studies. The highest energy dehydration state in glycerol is ca 540 nm. At this energy, no observable tilting is evident in film studies. Tilting becomes observable only at ca 530 nm. Whether the excitonic interaction is completely lost at this energy remains an unanswerable question at the present time. However, the CD trends in the visible spectra seem to indicate that the excitonic interactions should continue to drop as this energy increases.

CONCLUSIONS

Analysis of spectral changes resulting from the removal of water molecules from the PM by methods developed by this laboratory over the last decade indicates that this perturbation results in only subtle localized conformational changes of the bRs and, in contrast, extensive changes in the supramolecular structure of the PM. It is clear that the dehydration process must result in conformational changes in the local retinylidene environment of the apoprotein of the bR. The tertiary structural changes of the bR due to these conformational changes in the retinylidene local environment are most likely due to charge distribution alterations resulting from ionization state changes of the amino acid residues. The scope of the tertiary structural changes must be limited because dehydration did not alter the native anisotropism of the PM structure in respect to the orientations of the aromatic amino acid rings of the bRs. Furthermore, this is in accord with the suggestion that water removal is most likely mainly from around the lipid headgroups (26, 58) because bR overall physical form seems to be unaltered by this process.

The evidence presented, a 20.5° net tilting angle change of the polypeptide segments and a sharp reduction in the excitonic interactions between retinals coupled to an invariability of the visible dichroic ratio, suggests pronounced changes in the crystalline supramolecular structure of the PM during drastic dehydration. Previously such a supramolecular change had been observed when PM was subjected to light-hydroxylamine-induced bleaching and M_{412} intermediate formation. These perturbations, in common with the dehydration one, result in completely reversible structural changes. However, these perturbations result in dynamic delocalized tertiary structural changes of the bR which alter the physical form of the bR molecule, whereas the dehydration one does not appear to do so. Nevertheless, it is apparent that whether the protein molecular forms are altered or the water molecules are removed, the membrane supramolecular organization undergoes similar extensive changes. In view of the requirements for crystalline structure, it is not suprising that the PM native crystallinity would be critically dependent on the forms of the bR molecule as well as the amount of water molecules. However, the suggested molecular model for the dehydration process should be somewhat different from that for the previous processes. In the dehydration case, removal of the water molecules eventually results in a disruption of the interactions responsible for maintaining the native crystalline lattice of the PM, which seems to be dependent on the presence of a critical amount of water molecules, and leads to a randomization of the bR positions in the PM relative to the membrane plane due to rotations of the bR molecules. This results in the net tilting of the bR polypeptide segments relative to the membrane plane without the relative segmental positions within a bR molecule being altered (that is, no global tertiary structural changes of the bR). In the previous cases, the perturbations brought about a change in the relative segmental positions within the individual bR molecules (that is, the molecules were transformed from a closed conformation to an open one due to significant global tertiary structural changes of the bR) which resulted in the alteration of the native bR form. This then resulted in randomization of the bR positions in the PM and a net tilting of the segments relative to the membrane plane due to disruption of the interactions responsible for maintaining the crystalline supramolecular structure of the PM. It is clear that the key experimental evidence essential to both models is the proof for the loss of excitonic interaction among retinals and the net tilting for polypeptide segments during these perturbations. Such evidence has been provided in previous publications from this laboratory and in the present study.

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