J. Membrane Biol. 86, 229–238 (1985)

The Journal of
Membrane Biology

Effects of Polyhydric Alcohols on the Conformational Stability of the Purple Membrane

James E. Draheim and Joseph Y. Cassim

Laboratory of J.Y. Cassim, Department of Microbiology and Program in Biophysics, College of Biological Sciences, The Ohio State University, Columbus, Ohio 43210

Summary. The effect of different-sized polyhydric alcohols on the absorption and circular dichroic spectra of the purple membrane has been studied over the wavelength region 800 to 185 nm. Analysis of both the solution and the film spectra of the membrane revealed that these solvents induce conformational changes in the sole membrane protein, bacteriorhodopsin. Additional evidence supportive of these changes was obtained from protein fluorescence spectral studies. Although the net secondary structure of the bacteriorhodopsin is not observably altered, there is a reversible change in the protein tertiary structure. This change does not result in any significant change in the membrane crystallinity or the alignment of the protein helical polypeptide segments with respect to the membrane plane. However, it is of sufficient extent to change the protein-induced screw sense of the retinyl-chromophore symmetry and the local environments of the protein aromatic residues. The underlying mechanism appears to be a membrane surface-solvent interaction phenomenon since the spectral perturbation caused by these solvents appears to be independent of their effective sizes. Furthermore, partial enzymatic removal of the hydrophilic portion of the bacteriorhodopsins with papain increased the response of the membrane to this perturbation. An interpretation of these results is that polyhydric alcohols enhance hydrophobic interactions in the purple membrane which induces a more compact conformation of the bacteriorhodopsin. A possible molecular mechanism is presented.

Key Words purple membrane · bacteriorhodopsin · glycerol · circular dichroism · oriented films · polyhydric alcohols

Introduction

Polyhydric alcohols such as glycerol are popular solvents for a number of biological applications. They are especially indispensible for low-temperature studies and for storage of biological matter (Ruwart & Suelter, 1971; Yoshizawa, 1972). However, there is substantial evidence that these solvents can alter the conformational stability of proteins and of assembled protein systems. Glycerol has been shown to stabilize the activity of enzymes and the native structure of proteins (Jarabak, 1972; Myers & Jakoby, 1973) as well as stabilizing the structure of assembled protein systems (Shifrin & Parrott, 1975). For example, high concentrations of glycerol will enhance the self-assembly of purified tubulin to form microtubules (Lee & Timasheff, 1977).

Membranes have been studied in glycerol solution both to facilitate low-temperature spectral measurements (Becher et al., 1978; Kriebel et al., 1979; Kalisky et al., 1981) and to reduce spectral distortions due to light scattering (Brith-Linder & Rosenheck, 1977; Ebrey et al., 1977). However, no attempts have been made as yet to evaluate the effects of this solvent on membrane structure. The present study pertains to this issue. The purple membrane (PM) of *Halobacterium halobium* is an ideal membrane for this kind of study because its structure and function are relatively simple and well studied (for comprehensive reviews *see* Henderson, 1977; Eisenbach & Caplan, 1979; Stoeckenius et al., 1979, 1981; Ottolenghi, 1980; Stoeckenius & Bogomolni, 1982; Dencher, 1983). In addition, there has been considerable success in correlating spectra with structure for this membrane (Becher & Cassim, 1976, 1977; Muccio & Cassim, 1979*a*,*b*; Draheim & Cassim, 1985).

The most important function of the PM is the light-induced proton pumping activity which is essential to the anaerobic ATP synthesis process of this bacterium. The isolated PM consists of disks containing a single rhodopsin-like protein, bacteriorhodopsin (bR), which constitutes 75% of the membrane's dry weight. The remaining portion of the disk consists of various lipids. The bR molecules are organized into an extended hexagonal lattice of $P3$ symmetry (Blaurock, 1975; Henderson & Unwin, 1975). The bR is composed of a single highly alpha-helical polypeptide chain of 248 amino acids with a molecular weight of 26,534 daltons (Khorana et al., 1979; Ovchinnikov et al., 1979). The polypeptide is folded back and forth on itself

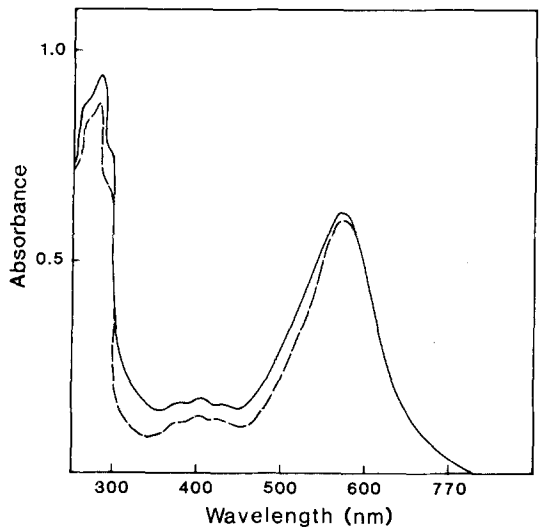

Fig. 1. Visible and near ultraviolet absorption spectra of the purple membrane in aqueous solution (——) and in 80% glycerol solution (----). Optical pathlength was 1 cm and the membrane protein concentration was 12.7 μM

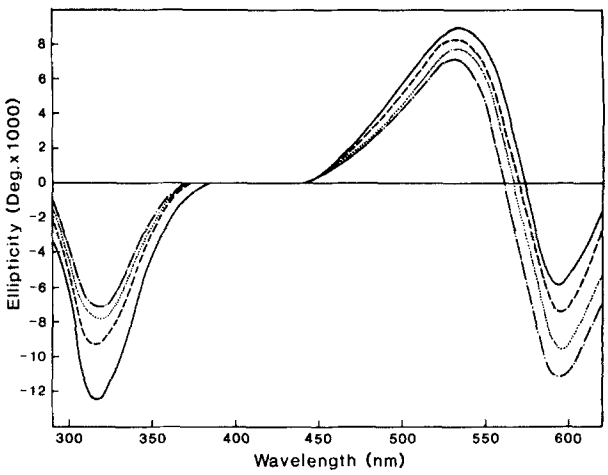

Fig. 2. Visible circular dichroic spectra of the purple membrane in 0% (——), 40% (----), 60% (····) and 80% (-·-·) glycerol solutions. Optical pathlength was 1 cm and the membrane protein concentration was 12.7 μM. The spectra were normalized to $OD_{568} = 1.0$

forming seven rodlike structural segments oriented nearly perpendicular to the plane of the membrane disks (Henderson & Unwin, 1975). Each bR contains a single retinyl chromophore positioned deep within the PM bilayer and oriented about 20 to 24° out of the PM plane (Bogomolni et al., 1977; Heyn et al., 1977; Neugebauer et al., 1977; Korenstein & Hess, 1978; Papadopoulous et al., 1978; Stoeckenius et al., 1979). It is covalently bound to the protein *via* a protonated Schiff base bond with an epsilon-amino group of a lysine residue.

Evidence is presented which suggests that the effects of different sized polyhydric alcohols on the PM absorption, circular dichroic, and fluorescence spectra are due to a membrane surface-solvent interaction phenomenon. These solvent-induced spectral effects can be correlated with tertiary structural changes of the bR protein. A possible molecular mechanism for this phenomenon is discussed.

Materials and Methods

The PM was isolated according to the method of Becher and Cassim (1975) with minor modifications. The sucrose step-gradient purification step was eliminated and many washing steps were incorporated into the purification process. Although this modified procedure sharply reduced the PM yield, it produced preparations of equal or greater purity without exposing the PM to sucrose. Polyhydric alcohols of the highest possible purity were purchased from Matheson, Coleman & Bell (Norwood, Ohio) and J.T. Baker (Phillipsburg, New Jersey) and were used immediately after receiving without further purification. The PM was suspended in various solvent mixtures by dilution until the bR final concentration was about 16 μM. Absorption and CD spectra were recorded with a Cary 118c spectrophotometer with far ultraviolet modification and a scattered transmission accessory and with a Cary 60 spectropolarimeter with the 6003 CD attachment, respectively (Varian Associates, Instrument Division, Palo Alto, California), as previously described (Becher & Cassim, 1976; Muccio & Cassim, 1979*a*,*b*). Fluorescence emission spectra were recorded with a 1902 Fluorolog Spectrofluorometer (Spex Industries, Metuchen, New Jersey) using 5-nm bandpass excitation slit widths and 10-nm bandpass emission slit widths. The fluorescence emission spectra were corrected for Raman scattering from the various solvent systems.

These studies were made with light-adapted PM (containing all-*trans* retinal) at neutral pH and at ambient temperatures only. Similar results were obtained with dark-adapted PM (containing equimolar mixture of 13-*cis* and all-*trans* retinal). Oriented PM films were formed on optical quality flats of Suprasil-S quartz (Precision Cells, Inc., Hicksville, New York) according to the method of Muccio and Cassim (1979*a*). In order to impregnate a PM film with glycerol a very thin layer of 20% glycerol solution was first placed over the film. This film was subsequently incubated at high humidity (*ca.* 95%) for at least 48 hr. After incubation the PM film was slowly redried in a dessicator until the color of the film edge changed from purple to pale red. The film was then exposed to the prevailing room humidity until the edge reverted to its characteristic purple color. This film was subsequently sealed inside a special film-plate holder for spectral analysis.

Partial digestion of the PM was achieved, in a reaction solution containing 50 mM cystein, 20 mM EDTA, 30 mM citrate buffer and 0.1% (vol/vol) mercaptoethanol at pH 5.5 using a papain/PM ratio of 1/100 (wt/wt) for 2 hr according to the method of Ovchinnikov et al. (1980). The papain was removed from the PM sample prior to any spectral measurements by three centrifugation washings. The papain (type IV) was purchased from Sigma Co. (St. Louis, Missouri). The PM preparations for the

Table 1. Effects of glycerol on the visible circular-dichroic parameters of the purple membrane

Percent glycerol by volume	Biphasic band					317-nm band	
	Ratio of positive lobe to negative lobe		Percent change in ratio		Crossover wavelength in nm	Ellipticity in millidegrees	Percent change in ellipticity
	Ellipticity, ϕ	Rotational strength, R	ϕ	R			
0	1.55	3.43	0	0	574	−12.4	0
40	1.14	2.52	−26.5	−26.5	570	−9.2	−25.8
60	0.83	1.71	−46.5	−50.2	567	−7.8	−37.1
80	0.65	0.83	−58.1	−75.8	562	−7.1	−42.7

control spectra for the papain-digested PM studies were treated identically with the reaction solution except for the exclusion of the papain.

Results

Visible Region

The absorption spectra of PM suspended in 0% (aqueous solution control) and 80% glycerol is shown in Fig. 1. There is a 3% decrease in extinction at 568 nm in the 80% glycerol spectrum relative to the 0% spectrum. Suspending the PM in glycerol concentrations greater than 80% caused a blue-shift in the spectrum which will be the topic of a subsequent publication. The intense peak at 568 nm has been assigned to the strong (π-π^*) NV_1 retinyl transition (Becher & Cassim, 1976).

The visible CD spectra of PM suspended in increasing concentrations of glycerol is shown in Fig. 2. The 0% CD spectrum of PM displays a large negative CD band at 317 nm. It also exhibits a nonconservative biphasic CD band system centered at *ca.* 568 nm with a predominant positive lobe. The 317-nm negative CD band has been attributed to a magnetic-dipole-allowed weak π-π^* retinyl transition (Muccio & Cassim, 1979*a*). The CD band system centered at *ca.* 568 nm is most likely due to the sum of at least two components (Heyn et al., 1975; Bauer et al., 1976; Becher & Cassim, 1976; Ebrey et al., 1977; Muccio & Cassim, 1979*a*). One component is a single positive Gaussian CD band centered at 568 nm resulting from asymmetric interactions between the retinyl chromophores and the apoproteins. This CD band is often referred to as the monomer CD. The other component is a conservative set of overlapping CD bands centered at 568 nm. These bands are induced by weak excitonic coupling among the retinyl chromophores in the PM crystal lattice and are, therefore, strongly dependent on the lattice order (Muccio & Cassim, 1981). Increasing the glycerol concentration induces a monotonic decrease in ellipticity at 317 nm. In the CD band system centered at *ca.* 568 nm there is also a monotonic decrease in the ratio of the ellipticity of the positive lobe to that of the negative lobe upon increasing the glycerol concentration. This results in a sharp reduction in the nonconservative nature of these CD bands at first. Nevertheless, further increases in the glycerol concentration result in a return to the nonconservative nature of these bands, however, now with a predominant negative lobe. These spectral changes are summarized in Table 1. Furthermore, these solvent-induced spectral changes are reversible.

The initial interpretation for the effect of glycerol on the PM CD was given by Brith-Linder and Rosenheck (1977) and Ebrey et al. (1977). They attributed this glycerol effect to the reduction in the light-scattering distortions induced by the particulate nature of the PM. Effective reduction of the differential light scattering was assumed to be achieved in 67% glycerol or 50% sucrose solutions through a solute-solvent refractive index matching mechanism. According to this interpretation, the true monomeric CD band in these solutions should display a relatively weak rotational strength compared to its apparent rotational strength in aqueous solution. Therefore, the nonconservative nature of these CD bands is attributed mainly to light-scattering distortions rather than due to the rotational strength and polarity of the monomeric CD band. However, these authors finished their survey at 67% glycerol addition in which the rotational strengths of the two lobes were apparently nearly identical. Later it became apparent that the subsequent additions of more glycerol (up to 80%) continually increases the rotational strength of the negative lobe relative to that of the positive one (Hsiao

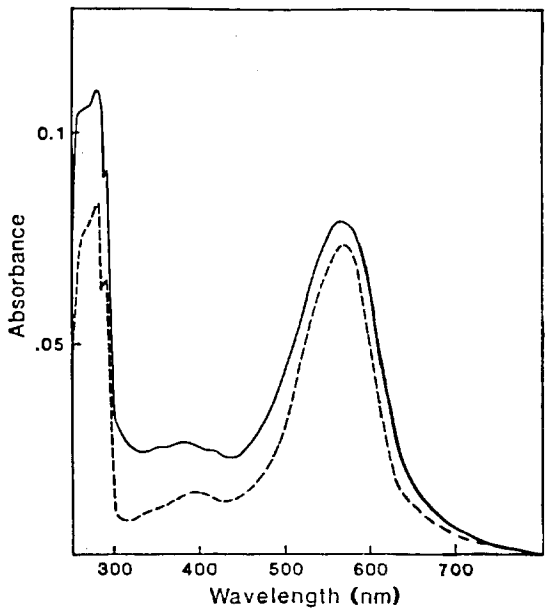

Fig. 3. Visible and near ultraviolet absorption spectra of a hydrated purple membrane film, untreated (——) and impregnated with glycerol (– – – –)

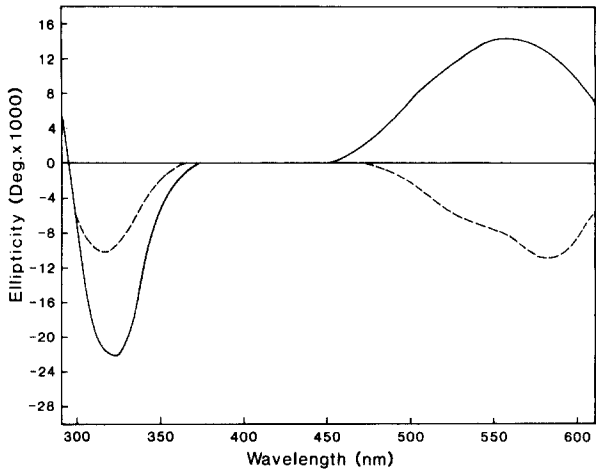

Fig. 4. Visible circular dichroic spectra of a hydrated purple membrane film, untreated (——) and impregnated with glycerol (– – – –)

et al., 1978). This must imply that there is a glycerol-induced change in the sign of the rotational strength of the monomeric CD band. It does not seem plausible that scattering artifacts such as differential light scattering and absorption flattening can be an essential factor for this dynamic spectral alteration. Considering that Jap and co-workers (1983) have found that differential light scattering is not a significant factor in the far-UV CD spectrum based on fluorescence-detected CD measurements, light scattering of this type should be even less of a significant factor in the CD spectra in the near-UV and visible regions. Furthermore, one would not expect scattering artifacts to greatly affect the 317-nm CD band since this band is due to an electric-dipole forbidden transition (Becher & Cassim, 1976). In addition, spectral changes resulting from changes in the effective field due to the enhancement of the solvent index of refraction with increasing concentrations of glycerol are expected to be minimal since the retinals, the amide groups, and most of the aromatic amino acid residues which are the sources of the PM spectrum are buried within the PM hydrophobic interior. Also it is not possible for concentration-obscuring scattering artifacts to reverse the polarity of a CD band.

To determine if glycerol reverses the polarity of the PM monomeric CD band, a glycerol impregnated PM film was formed. In Fig. 3 the absorption spectra of PM films, both control and impregnated with glycerol, are compared. Glycerol reduces the extinction of a PM film at 568 nm by about 5%. However, the wavelength maximum of the absorption spectrum does not change upon the addition of glycerol. Figure 4 compares the visible CD spectra of PM films both control and impregnated with glycerol. Impregnation of the PM film does not significantly change the light-scattering properties of the film in the optically transparent regions (such as 800 nm). In a PM film the optical activity due to excitonic coupling among the retinyl chromophores in the PM crystal lattice must vanish because of the p3 symmetry of the bR organization (Muccio & Cassim, 1979*a*). Thus only the positive monomeric CD band remains in the control spectrum together with a large negative CD band at 320 nm. However, it is obvious that the negative-monomeric CD band induced by glycerol is not a simple scaled mirror image of that of the control spectrum. The band is red-shifted in respect to that of the control spectrum by *ca.* 12 nm and a shoulder is also evident at *ca.* 520 nm. This band is apparently not a single band but the superposition of two bands. The data presented are based on a set of eight films which were maximally impregnated with glycerol. Control of the glycerol concentration in the film is very difficult. In a subsequent publication it will be shown that glycerol concentrations greater than 80% will result in the dehydration of the PM and that dehydration of the PM by a number of methods results in such wavelength changes in the CD spectra. In the present study, the important point is that upon impregnation with glycerol there is a change in the polarity of the monomeric CD band. Furthermore, this phenomenon appears to operate in a continuous manner, since partial glycerol impregnations result

Table 2. Effects of different-sized polyhydric alcohols on the visible circular-dichroic parameters of the purple membrane

Polyhydric alcohol	Molecular weight (daltons)	Percent by volume	Biphasic band		317-nm band	
			Ratio of positive lobe to negative lobe (ellipticity)	Percent change in ratio[a]	Ellipticity in millidegrees	Percent change in ellipticity[a]
Ethylene glycol	62	70	1.00	−35.5	−10.7	−13.7
Propylene glycol	76	70	0.84	−46.0	−7.4	−40.3
Tetramethylene glycol	90	70	0.78	−50.0	−10.2	−17.7
Triethylene glycol	150	70	0.69	−55.5	−8.5	−31.5
Polyethylene Glycol 200	200	70	0.71	−54.2	−9.0	−27.4
Polyethylene Glycol 300	300	70	0.59	−62.1	−8.2	−33.9
Sucrose	342	25	0.90	−42.0	−10.0	−19.4
Sucrose	342	50	0.77	−50.3	−7.4	−40.3
Ficoll 400	400,000	30	0.95	−38.7	−10.8	−12.9
Ficoll 400	400,000	40	0.82	−47.1	−9.6	−22.6

[a] Relative to value in aqueous solution (1.55 and −12.4 millidegree, respectively).

in polarities ranging from positive to negative including zero. Moreover, by hydrating maximally glycerol-impregnated films, a single-negative band at *ca.* 568 nm can be realized with reduced ellipticity. There is also a 52% decrease in the ellipticity of the CD band at 320 nm together with a blue shift to 314 nm. Clearly, this change in polarity cannot be attributed to a differential light-scattering phenomenon but rather to a conformational change in the bR. These results must imply that the screw-sense of the apoprotein-induced reduction of the retinyl chromophore symmetry has been changed by the glycerol.

This type of reversible change had been previously observed during low pH perturbation of the PM (Muccio & Cassim, 1979*b*), although in that case there was also a change in the wavelength of the chromophoric retinyl (π-π^*) NV_1 transition. However, there has been no indication of any change in the PM crystallinity due to either perturbation since in both cases the excitonic interactions among the chromphoric retinals are unaltered. Due to the fact that excitons are collective excitations, their propagation is intrinsically dependent on the order of membrane supramolecular organization. Loss of the regularity of the arrangement of the chromophoric molecules in the membrane space lattice, even due to very subtle changes of the chromophoric positions, can result in decoupling of these interactions. Therefore, excitons can serve as very sensitive probes for changes in membrane crystallinity which are beyond the resolving capabilities of the diffraction methodologies.

In order to establish if the above phenomenon was specific to glycerol or a general polyhydric alcohol solvent effect, the PM was suspended in a number of different-sized polyhydric alcohols. The PM was suspended in aqueous solutions of polyethylene glycol 300, polyethylene glycol 200, triethylene glycol, tetramethylene glycol, propylene glycol, and ethylene glycol (all at 70% by volume). The PM was also suspended in 25% sucrose, 50% sucrose, 30% ficoll, and 40% ficoll. In all these solutions the wavelength of the main visible absorption band remained invariant. Furthermore, visible CD spectrum of the PM in each solvent system was similar to that in 60% glycerol. There was a decrease in ellipticity at 317 nm and a decrease in the ratio of the ellipticity of the positive to the negative lobe centered at *ca.* 568 nm. The spectral changes induced by these polyhydric alcohols are summarized in Table 2. It is apparent that the effect of glycerol on the PM appears to be part of a general phenomenon. It should be noted that the effective sizes of the polyhydric alcohols used in this study varied over a wide range (molecular weight range, 62 to 400,000 daltons). Large molecules induced spectral changes in the PM similar to those induced by smaller molecules. It is very unlikely that molecules the size of polyethylene glycol 300 can penetrate the PM bilayer and directly perturb the retinyl chromophore. Since molecules the size of glycerol and ethylene glycol induce spectral changes similar to those induced by polyethylene glycol 300 and ficoll, it is likely that the effects of these molecules may also be transmitted by a general nonpenetrating process. Therefore, the effect of these polyhydric alcohols on the PM structure must most likely oper-

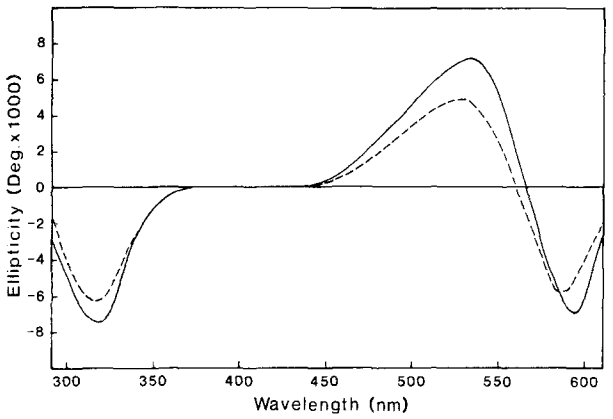

Fig. 5. Visible circular dichroic spectra of the purple membrane in 60% glycerol solution, unmodified (——) and partially digested with papain (----). Optical pathlength was 1 cm and the membrane protein concentration was 15.9 μM

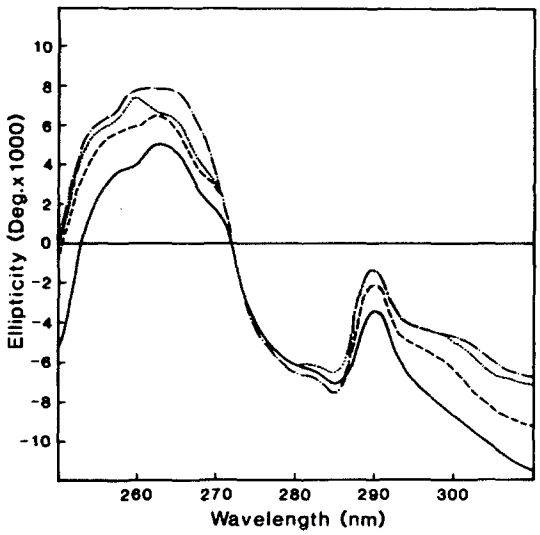

Fig. 6. Near ultraviolet circular dichroic spectra of the purple membrane in 0% (——), 40% (----), 60% (····) and 80% (-·-·) glycerol solutions. Optical pathlength was 1 cm and the membrane protein concentration was 12.7 μM. The spectra were normalized to $OD_{568} = 1.0$

ate through a membrane surface-solvent interaction mechanism rather than through a membrane bilayer-penetrating mechanism.

If the effect of these polyhydric alcohols on the PM is due to a membrane surface-solvent interaction phenomenon, then a physical modification of the surface of the PM should change the response of the PM to these solvents. The visible CD spectra of the PM in which the hydrophilic portion of the bR is partially removed with papain and the unmodified PM are compared in 60% glycerol in Fig. 5. In aqueous solution the spectrum of the papain-digested PM is very similar to that of the unmodified PM. Therefore, this alteration of the surface of the PM does not result in any detectable conformational changes of the protein. However, as shown in Fig. 5, the combined perturbation of the PM by papain digestion and 60% glycerol results in about 19% decrease in the ratio of the ellipticities of the positive and negative lobes of the biphasic band of the PM relative to that of the unmodified PM with the same glycerol addition. In addition, there is a decrease of about 20% in the negative ellipticity of the 317-nm band. According to the data presented in Table 1 these are the changes expected for a higher glycerol concentration. Clearly this modification of the PM surface appears to enhance the response of the PM to glycerol.

Near-UV Region

A change in the tertiary structure of the bR, induced by the presence of polyhydric alcohols, should affect the environment of the aromatic residues and/or the resonance interaction between the aromatic residues of the protein. The absorption spectra of PM suspended in 0 and 80% glycerol is shown in Fig. 1. There is an 8% decrease in extinction of the 80% glycerol spectrum at 280 nm relative to the 0% spectrum. The near-UV CD spectra of PM suspended in increasing concentrations of glycerol is shown in Fig. 6. The increase in ellipticity at 260 nm in concert with glycerol concentrations is consistent with solvent-induced changes in the resonance interactions between the aromatic residues of the bR protein and/or between the retinyl chromophore and one or more aromatic residues. Another possibility is that glycerol induces solvent effects which may alter the pK's of some of the aromatic residues, especially those located near the PM surface. The change in ellipticity in Fig. 6 occurs primarily in the 250 to 270-nm region which is usually associated with transitions due to the phenylalanyl residues. In this region there is an increase in the anisotropy ratio upon suspension of the PM in 80% glycerol since there is a 70% increase in ellipticity and a relatively small 8% decrease in extinction. An increase in the anisotropy ratio may in part be attributed to an increase in the ordered or folded state of the bR protein. In Fig. 6 there is less change in the CD spectrum in the 270 to 290-nm region which is usually associated with transitions due to the tyrosyl and tryptophanyl residues. The near-UV CD spectra of the PM suspended in 70% polyethylene glycol 300, 70% polyethylene glycol 200, or 70% triethylene glycol (*none shown*) were similar to the 60% glycerol spectrum. Furthermore, similar to the

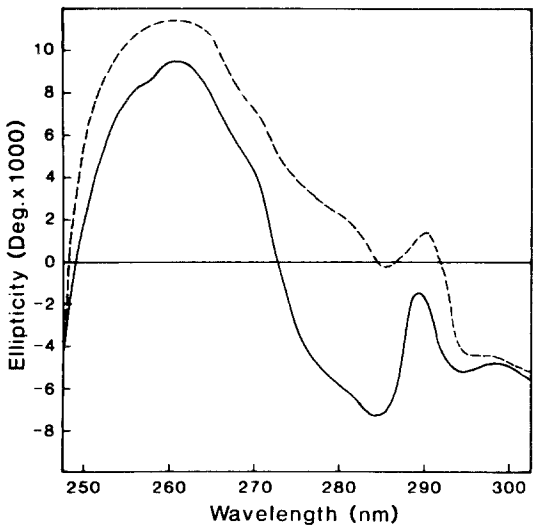

Fig. 7. Near ultraviolet circular dichroic spectra of the purple membrane in 60% glycerol, unmodified (——) and partially digested with papain (– – – –). Optical pathlength was 1 cm and the membrane concentration was 15.9 μM. The spectra were normalized to $OD_{568} = 1.0$

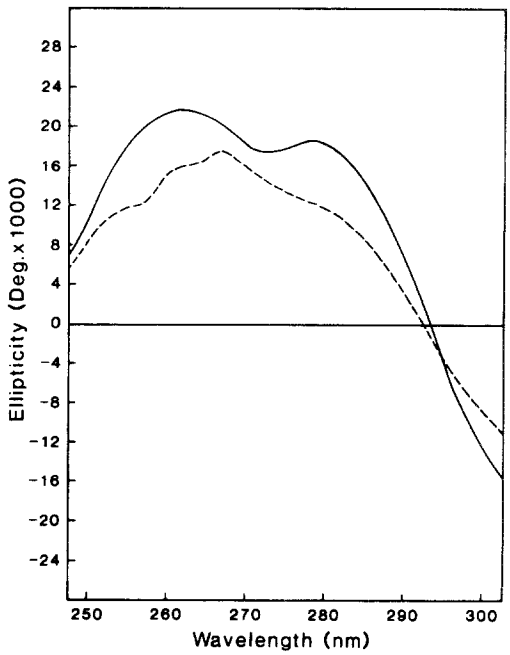

Fig. 8. Near ultraviolet circular dichroic spectra of a hydrated purple membrane film, untreated (——) and impregnated with glycerol (– – – –)

spectral changes in the visible region noted above, the changes in the near-UV were also reversible.

The near-UV CD spectra of PM partially digested with papain and unmodified, both suspended in 60% glycerol are demonstrated in Fig. 7. In aqueous solutions there is no significant difference between the near-UV CD spectra of the papain modified and the unmodified PM indicating that partial digestion with papain alone does not induce detectable changes in the conformation of the bR. It appears that modification of the PM with papain alters the PM surface and subsequently enhances the effect of the glycerol perturbation on the bR structure. Therefore, these results are in accord with the results of similar studies in the visible region.

The absorption spectra of PM films, both control and impregnated with glycerol, are compared in Fig. 3. Impregnation with glycerol causes a decrease in the extinction of a PM film at *ca.* 280 nm of about 22%. The near-UV CD spectra of PM films, both control and impregnated with glycerol, are compared in Fig. 8. There are many spectral shifts upon glycerol impregnation together with a general 20% decrease in ellipticity throughout the near-UV region. Impregnation with glycerol decreases the in-plane CD component in both the phenylalanyl region and the tyrosyl and tryptophanyl region of the spectrum. Either the in-plane resonance interactions are reduced, or the transition dipole moments of some of the aromatic residues become aligned more out of the membrane plane. Since, in solution, glycerol does not greatly affect the PM ellipticity in the tyrosyl and tryptophanyl region, it is unlikely that glycerol alters the resonance interactions of the PM tyrosyl and tryptophanyl residues to any great extent. The decrease in the extinction and the ellipticity of a PM film in the 270 to 290-nm region may be due to a small glycerol-induced change in the orientations of the tyrosyl and tryptophanyl residues. Since glycerol does affect the resonance in the phenylalanine region it is difficult to speculate as to the possible changes in the orientations of the phenylalanyl residues.

In order to provide additional evidence for these conformational changes the fluorescence of PM in 0 and 80% glycerol were measured (*none shown*). The excitation monochrometer was set at 285 nm and the emission spectra was recorded over the 300 to 400-nm region. Under these conditions both the tyrosyl and the tryptophanyl residues are excited. In aqueous solution the fluorescence emission spectra of PM exhibited a single peak at 330 nm. Upon suspension of the PM in 80% glycerol the location of this peak was unchanged. However, there was a 20% increase in the relative fluorescence intensity for the PM in 80% glycerol relative to that in an aqueous solution. These results are in general agreement with those of Plotkin and Sherman (1984). This change in quantum yield due to glycerol addition is in accord with the interpretation of the CD spectra that the glycerol induces changes in the environments of the tyrosyl and tryptophanyl

residues. However, this change can also be due to a simple polarity effect. Although it is not possible to distinguish between these two possibilities, the fluorescence result adds strength to the CD interpretations.

Far-UV Region

The far-UV CD spectrum of the PM was measured (*not shown*) to monitor changes in the net secondary structure of the bR of the PM upon suspension in these various solvents. In all cases measured there was little or no change in the ellipticities in this region which could be correlated with significant changes in the net secondary structure of the bR. Furthermore, glycerol impregnation of an oriented PM film did not change the PM film far-UV CD spectrum. This indicates that glycerol does not affect the alignment of the helical polypeptide segments of the bR relative to the plane of the PM (Muccio & Cassim, 1979*a*; Papadopoulos & Cassim, 1981; Draheim & Cassim, 1985). Although there is no indication of a change in the net secondary structure of the bR, significant changes are possible in the tertiary structure of the bR without observably altering its far-UV CD spectrum (Becher & Cassim, 1977). Furthermore, modification of the PM with papain resulted in no significant changes in the secondary structure of the bR without or with glycerol addition.

Discussion

From the results presented it appears that polyhydric alcohols perturb the tertiary structure of the bR without altering its secondary structure. Furthermore, the conformational change is of a subtle nature since the alignment of the protein helical polypeptide segments and the membrane crystallinity are not significantly altered. The effect of these alcohols on the PM must operate through a membrane surface-solvent interaction mechanism since the effect was independent of the effective sizes of the solvent molecules over the extreme range studied. Subtle changes on the surface of the PM appear to perturb the asymmetric environments of the retinyl chromophores which are located within the membrane bilayer. Such phenomena may play an important role in many biological mechanisms.

The glycerol perturbation is large enough to reverse the polarity of the PM monomeric CD band. Simultaneously, this perturbation reduces the ellipticity of the 317-nm CD band. These results are consistent with an explanation for the retinal-induced optical activity of the PM in aqueous solutions at neutral pH previously suggested by Muccio and Cassim (1979*a*). The explanation is as follows: 1) weak excitonic coupling among the retinyl chromophores in the PM crystal lattice gives rise to a conservative biphasic set of overlapping CD bands centered at 568 nm; 2) a dissymmetric-field induced mixing of the electric-dipole allowed (π-π^*) NV_1 transition at 568 nm with the magnetic-dipole allowed higher energy π-π^* transition of the retinyl chromophore at 317 nm can account for the positive monomeric CD band at 568 nm and part of the negative 317-nm CD band; 3) an electric-magnetic resonance coupling between the magnetic-dipole allowed π-π^* transition at 317 nm and an electric-dipole allowed π-π^* transition of some nearby aromatic amino acid residue planar ring (possibly tryptophanyl) can account for some of the positive near-UV CD bands and the remaining portion of the negative 317-nm CD band. Due to the formalism of the transition mixing mechanism, since glycerol reverses the polarity of the monomeric CD band, it must also reverse the polarity of its companion band which is one of the components of the 317-nm CD band. However, in solution glycerol does not appear to greatly affect the ellipticity of the tyrosyl-tryptophanyl region. Thus the negative component of the 317-nm CD band due to the electric-magnetic resonance interaction should not change. Therefore, the sum of these two 317-nm CD band components, which are of opposite polarity, should exhibit a diminished ellipticity when the PM is suspended in polyhydric alcohols.

With respect to its electronic transitions the planar retinyl chromophore in the all-*trans* configuration has an idealized C_{2h} symmetry. In order for the retinyl chromophore to exhibit optical activity the apoprotein must reduce the retinyl symmetry since the electric and magnetic dipole moments of a transition are orthogonal in the C_{2h} point group. This can be accomplished by applying either a dissymmetric static electric field or a physical twist to the chromophore. Either mechanism can induce optical activity through a transition mixing mechanism induced by this perturbation potential. The sign of the CD band due to this induced optical activity can be changed by reversing the sign of the net static charge distribution surrounding the chromophore or by reversing the screw sense of the physical twist imposed on the chromophore. Therefore, the symmetry of the retinyl chromophore can serve as an excellent probe for the membrane surface conditions since it is very sensitive to critical surface perturbations.

The effects of glycerol on numerous biological systems have been previously reported by Gekko and Timasheff (1981*a*,*b*). These authors have pro-

posed a mechanism which may also adequately explain the effect of glycerol on the PM. According to this mechanism glycerol stabilizes the structure of numerous proteins by enhancing the solvation layer around the protein. All proteins studied to date were found to be preferentially hydrated in aqueous solutions upon addition of glycerol (Lee & Lee, 1981). Thus the presence of glycerol in an aqueous solution could enhance hydrophobic interactions in the bR protein and raise its chemical potential. This process is thermodynamically unfavorable. To a first approximation, this effect can be assumed to be distributed over the entire protein-solvent interface. Any reduction in the protein-solvent interface would be thermodynamically favorable. Therefore, the presence of glycerol would tend to induce a more folded-compact conformation of the bR. Such a conformational deformation could result in the alteration of the local environments of the retinyl chromophore and some of the protein aromatic residues. This molecular mechanism is consistent with the fact that partial enzymatic removal of the hydrophilic portion of the bR appears to enhance the effect of glycerol on the structure of this protein. Furthermore, it is noteworthy that when the PM is transformed to its M_{412} optical state the CD due to the (π-π^*) NV_1 transition, now at 412 nm, becomes invariant to glycerol addition (Draheim & Cassim, 1985). This invariance, of course, cannot be easily explained by a light-scattering artifact-reducing interpretation of the glycerol effect. On the other hand, considering the observation that during the transformation to the M_{412} optical state the conformation of the bR is changed from a relatively compact closed one to a less-compact open one (Draheim & Cassim, 1985), it is apparent that this invariance can be more readily explained by our proposed model. Conformational deformation due to surface modification is expected to be more effective in the alteration of the chromophore local environment in a more compact form of the bR than in a less compact one.

In the past it has generally been assumed that polyhydric alcohols are inert solvents with respect to membrane structure. In view of the findings of the present study, analysis of data from such studies based on this assumption may require reevaluation. Recently, the chromophoric kinetics during the photocycle of the PM have been studied in 30% ficoll solutions (Ahl & Cone, 1984) and the kinetics of the photocycle in glycerol solutions with glycerol concentrations as high as 80% (Beece et al., 1981; Eisenstein, 1982). Effects of these solvents on the kinetics have been interpreted as due mainly to solvent viscosity changes. Such apparent oversimplification could lead to invalid conclusions since the increase in the compactness of the bR conformation in these solvents may be a key feature in the mechanisms of these phenomena.

References

Ahl, P., Cone, R.A. 1984. Light activates rotations of bacteriorhodopsin in the purple membrane. *Biophys. J.* **45:**1039–1049

Bauer, P.-J., Dencher, N.A., Heyn, M.P. 1976. Evidence for chromophore-chromophore interactions in purple membrane from reconstitution experiments of chromophore-free membrane. *Biophys. Struct. Mechan.* **2:**79–82

Becher, B., Cassim, J.Y. 1975. Improved isolation procedures for the purple membrane of *Halobacterium halobium*. *Prep. Biochem.* **5:**161–178

Becher, B., Cassim, J.Y. 1976. Effects of light adaptation on the purple membrane of *Halobacterium halobium*. *Biophys. J.* **16:**1183–1200

Becher, B., Cassim, J.Y. 1977. Effects of bleaching and regeneration on the purple membrane structure of *Halobacterium halobium*. *Biophys. J.* **19:**285–297

Becher, B., Tokunaga, F., Ebrey, T.G. 1978. Ultraviolet and visible spectra of the purple membrane and its photocycle intermediates. *Biochemistry* **17:**2293–2300

Beece, D., Brownie, S.F., Czege, J., Eisenstein, L., Frauenfelder, H., Good, D., Marden, M.C., Marque, J., Ormos, P., Reinisch, L., Yue, K.T. 1981. The effect of viscosity on the photocycle of bacteriorhodopsin. *Photochem. Photobiol.* **33:**517–522

Blaurock, A.E. 1975. Bacteriorhodopsin: A transmembrane pump containing α-helix. *J. Mol. Biol.* **93:**139–158

Bogomolni, R.A., Huang, S.-B., Tseng, T.W., King, G.I., Stoeckenius, W. 1977. Orientation of the bacteriorhodopsin transition dipole. *Biophys. J.* **17:**98a

Brith-Linder, M., Rosenheck, K. 1977. The circular dichroism of bacteriorhodopsin: Asymmetry and light scattering distortions. *FEBS Lett.* **76:**41–44

Dencher, N.A. 1983. The five retinal protein pigments of *Halobacteria:* Bacteriorhodopsin, halorhodopsin, P565, P370 and slow-cycling rhodopsin. *Photochem. Photobiol.* **38:**753–767

Draheim, J.E., Cassim, J.Y. 1985. Large scale global structural changes of the purple membrane during the photocycle. *Biophys. J.* **46:**497–507

Ebrey, T.G., Becker, B., Mao, B., Kilbride, P., Honig, B. 1977. Exciton interaction and chromophore orientation in the purple membrane. *J. Mol. Biol.* **112:**377–397

Eisenbach, M., Caplan, S.R. 1979. The light-driven pump of *Halobacterium halobium:* Mechanism and function. *Curr. Top. Membr. Trans.* **12:**165–248

Eisenstein, L. 1982. Effects of viscosity on the photocycle of bacteriorhodopsin. *Methods Enzymol.* **88:**297–305

Gekko, K., Timasheff, S.N. 1981*a*. Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol-water mixtures. *Biochemistry* **20:**4667–4676

Gekko, K., Timasheff, S.N. 1981*b*. Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry* **20:**4677–4686

Henderson, R. 1977. The purple membrane from *Halobacterium halobium*. *Rev. Biophys. Bioeng.* **6:**87–109

Henderson, R., Unwin, P.N.T. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature (London)* **257:**28–32

Heyn, M.P., Bauer, P.-J., Dencher, N.A. 1975. Natural CD label to probe the structure of the purple membrane from *Halobacterium halobium* by means of exciton coupling effects. *Biochem. Biophys. Res. Commun.* **67:**897–903

Heyn, M.P., Cherry, R.J., Muller, U. 1977. Transient and linear dichroism studies on bacteriorhodopsin: Determination of the orientation of the 568 nm all-*trans* retinal chromophore. *J. Mol. Biol.* **117:**607–620

Hsiao, T.L., Papadopoulos, G.K., Cassim, J.Y. 1978. Effects of sucrose and glycerol on the purple membrane structure of *Halobacterium halobium*. *Biophys. J.* **18:**182a

Jap, B.K., Maestre, M.F., Hayward, S.B., Glaeser, R.M. 1983. Peptide-chain secondary structure of bacteriorhodopsin. *Biophys. J.* **43:**81–89

Jarabak, J. 1972. Human placental 15-hydroxyprostaglandin dehydrogenase. *Proc. Natl. Acad. Sci. USA* **69:**533–534

Kalisky, O., Feitelson, J., Ottolenghi, M. 1981. Photochemistry and fluorescence of bacteriorhodopsin excited in its 280 nm absorption band. *Biochemistry* **20:**205–209

Khorana, H.G., Gerber, G.E., Herlihy, W.C., Gray, C.P., Anderegg, R.J., Nihei, K., Biemann, K. 1979. Amino acid sequence of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA* **76:**5046–5050

Korenstein, R., Hess, B. 1978. Immobilization of bacteriorhodopsin and orientation of its transition moment in purple membrane. *FEBS Lett.* **89:**15–20

Kriebel, A.N., Gillbro, T., Wild, U.P. 1979. A low temperature investigation of the intermediates of the photocycle of light-adapted bacteriorhodopsin. Optical absorption and fluorescence measurements. *Biochim. Biophys. Acta* **546:**106–120

Lee, J.C., Lee, L.Y. 1981. Preferential solvent interactions between proteins and polyethylene glycols. *J. Biol. Chem.* **256:**625–631

Lee, J.C., Timasheff, S.N. 1977. *In vitro* reconstitution of calf brain microtubules: Effects of solution variables. *Biochemistry* **16:**1754–1764

Muccio, D.D., Cassim, J.Y. 1979*a*. Interpretation of the absorption and circular dichroism spectra of oriented purple membrane films. *Biophys. J.* **26:**427–440

Muccio, D.D., Cassim, J.Y. 1979*b*. Interpretation of the effects of pH on the spectra of the purple membrane. *J. Mol. Biol.* **135:**595–609

Muccio, D.D., Cassim, J.Y. 1981. Analysis of the spectroscopic properties of bacteriorhodopsin in the purple membrane by a crystalline exciton model. *Biophys. J.* **33:**103a

Myers, J.S., Jakoby, W.B. 1973. Effect of polyhydric alcohols on the parameters of enzymes. *Biochem. Biophys. Res. Commun.* **51:**631–636

Neugebauer, D.C., Blaurock, A.E., Worcester, D.L. 1977. Magnetic orientation of purple membrane demonstrated by optical measurements and neutron scattering. *FEBS Lett.* **78:**31–35

Ottolenghi, M. 1980. The photochemistry of rhodopsins. *Adv. Photochem.* **12:**97–200

Ovchinnikov, Y.A., Abdulaev, N.G., Feigina, M.Y., Kiselev, A.V., Lobanov, N.A. 1979. The structural analysis of the functioning bacteriorhodopsin: An overview. *FEBS Lett.* **100:**219–224

Ovchinnikov, Y.A., Abdulaev, N.G., Tsetlin, V.I., Zakis, V.I. 1980. The structural analysis of the functioning bacteriorhodopsin: An overview. *Bioorg. Chem. (USSR)* **6:**1427–1429

Papadopoulos, G.K., Cassim, J.Y. 1981. Interpretations of the solution and oriented film spectra of brown membrane of *Halobacterium halobium*. *Photochem. Photobiol.* **33:**455–466

Papadopoulos, G.K., Hsiao, T.L., Cassim, J.Y. 1978. Determination of the retinal/protein molar ratio for the purple membranes of *Halobacterium halobium* and *Halobacterium cuterubrium*. *Biochem. Biophys. Res. Commun.* **81:**127–132

Plotkin, B.J., Sherman, W.V. 1984. Spectral heterogeneity in protein fluorescence of bacteriorhodopsin: Evidence for intraprotein aqueous regions. *Biochemistry* **23:**5353–5360

Ruwart, M.J., Suelter, C.H. 1971. Activation of yeast pyruvate kinase by natural and artificial cryoprotectants. *J. Biol. Chem.* **246:**5990–5993

Shifrin, S., Parrott, C.L. 1975. Influence of glycerol and other polyhydric alcohols on the quaternary structure of an oligomeric protein. *Arch. Biochem. Biophys.* **166:**426–432

Stoeckenius, W., Bogomolni, R.A. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* **51:**587–616

Stoeckenius, W., Lozier, R.H., Bogomolni, R.A. 1979. Bacteriorhodopsin and the purple membrane of halobacteria. *Biochim. Biophys. Acta* **505:**215–278

Stoeckenius, W., Lozier, R.H., Bogomolni, R.A. 1981. Bacteriorhodopsin photocycle and stoichiometry. *In:* Chemiosmotic Proton Circuits in Biological Membranes. V.P. Skulachev and P.C. Hinkle, editors. pp. 283–309. Addison-Wesley, Reading, Mass.

Yoshizawa, T. 1972. The behavior of visual pigments at low temperatures. *In:* Handbook of Sensory Physiology. H.J.A. Dartnall, editor. Vol. 7, pp. 146–179. Springer, Heidelberg

Received 20 November 1984; revised 1 April 1985