SURFACE POTENTIAL ON PURPLE MEMBRANES AND ITS SIDEDNESS STUDIED BY A RESONANCE RAMAN DYE PROBE

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ABSTRACT A new technique for the measurement of membrane surface potential is proposed and demonstrated. The method is based on the fact that a positively charged styryl dye molecule aggregates when present at high concentration in the Debye layer near a membrane bearing a negative surface potential. The dye in its aggregated form exhibits marked differences in its resonance Raman spectrum relative to the free dye molecules. This method was used to study the potential on the surfaces of the purple membrane that contains the pigment bacteriorhodopsin. A value of −29.5 mV was found for membranes with bacteriorhodopsin in its relaxed, light-adapted state, and the potential decreased to −34.5 mV when most of the bacteriorhodopsin was converted to the M_{412} intermediate. Because the dye probe does not diffuse through the lipid bilayer, it can be used to probe the potential on the external or internal surface of a vesicle. Thus, we found that the potential on the purple membrane was asymmetric and was localized mainly on the surface that faces the cytoplasm in the cell.

INTRODUCTION

Bacteriorhodopsin is a membrane-bound lipo-protein that is found in the purple patches of the cytoplasmic membrane of *Halobacterium halobium*. This protein is a unique photosynthetic system, because it converts the light energy absorbed in the retinal chromophore attached to it directly into electrochemical energy by pumping protons vectorially across the membrane (Oesterhelt and Stoeckenius, 1973). It was shown by the technique of resonance Raman spectroscopy, that the Schiff-base bond, which links the retinal chromophore to the ϵ-amino group of a lysine group, is protonated in the original bacteriorhodopsin molecule (BR) as well as in the K_{610} and L_{550} intermediates in the photochemical cycle (Lewis et al., 1974; Terner et al., 1979; Stockburger et al., 1979; Pande et al., 1981; Braiman and Mathies, 1982). However, the next intermediates have an unprotonated Schiff base (Lewis et al., 1974; Marcus and Lewis, 1978). Because the kinetics of this deprotonation correspond closely to the formation of the cross-membrane potential (Johnson et al., 1981; Ehrenberg et al., 1984), it was reasonable to assume (Lewis et al., 1978) that the Schiff-base deprotonation is a step, or even the trigger, of the pumping mechanism of protons along a chain of hydrogen bonds that span the membrane (Nagle and Morowitz, 1978).

The vectorial pumping of protons across the membrane is not the only source of pH changes in the surrounding medium. Acidification of the medium is also obtained in suspensions of purple membrane fragments (Oesterhelt and Hess, 1973; Garty et al., 1977), where both sides of the membrane are exposed to the same medium. These Bohr protons are released from protein side chains due to changes in their pK values during the photocycle. The release of these protons as well as the possible exposure of charged protein moieties to the medium cause a change in the membrane surface potential. In previous studies that have used amphiphilic spin probes, it was indeed found that a change in the density of surface charge does occur during the photocycle of bacteriorhodopsin (Tokutomi et al., 1980; Carmeli et al., 1980). However, since these studies were performed on purple membrane fragments, the sidedness of the surface charge could not be determined.

In this report, we intend to present the possibility of using the technique of resonance Raman spectroscopy of dye molecules to probe the surface potential on biological membranes. The advantage in using the dye molecule as a resonance Raman probe of surface potential is that the dye does not permeate the lipid bilayer of liposomes. It can therefore be used to probe either side of the membrane separately and not just the average potential of both sides. We present our results on the application of this technique to purple membrane fragments as well as to liposomes reconstituted with bacteriorhodopsin, so that the surface charge density and potential could be determined for each surface separately.

Materials and Methods

The growing of *Halobacterium halobrium* cells and the isolation of the bacteriorhodopsin-containing purple membrane fragments was per-

formed by a standard procedure (Oesterhelt and Stoeckenius, 1974) by Prof. S.R. Caplan at the Weizmann Institute of Science, Israel. The samples of the fragments' suspension were sonicated before the measurements to prevent aggregation. The concentration of bacteriorhodopsin was 20 μM, if not indicated otherwise.

The dye we used was 2-[4-(*p-N,N*-dimethylanilinyl)-1,3-butadienyl]-3-propyl-benzothiazolium iodide (its formula is shown in Fig. 1). This dye was synthesized by Prof. A. Waggoner, Carnegie-Mellon University, Pittsburgh, PA, and codenamed WW-638. It was added from a concentrated (8 mM) stock solution in ethanol. The photodegradation of the dye in the illuminating laser beam used to excite the resonance Raman spectra was always <5%. The concentration of the dye in the samples was usually 60 μM, unless indicated otherwise.

Kinetic resonance Raman spectra were measured by circulating a suspension of purple membrane fragments from a reservoir using a variable speed pump (MicroPump Corp., Concord, CA, model 120-441-10A). The solution passed through a capillary tube having a 1-mm diam where it was illuminated by the laser beam focused down to 40-μm diam. The average passage time through the illuminated spot could be adjusted between 8 and 200 μs. The reservoir was large enough that the average circulation time was longer than 30 ms to ensure completion of the photocycle between illuminations.

Unilamellar liposomes reconstituted with bacteriorhodopsion, which have a large internal trapping volume into which the dye WW-638 was introduced, were prepared by the method of freezing and thawing of presonicated liposomes (Kasahara and Hinkle, 1977). 10 mg of egg lecithin (Sigma Chemical Co., St. Louis, MO) were suspended in 1 ml of 10-mM water solution of KCl containing the dye WW-638 at a concentration of 60 μm, and sonicated to clarity in a cylindrical bath sonicator (Laboratory Supply Inc., Hicksville, NY). The suspension was frozen by insertion into liquid nitrogen, then left to thaw at room temperature and sonicated for 30 s. Vesicles with a large trapping volume of up to 10 μl/1 mg phospholipid were reported to be obtained by this method (Pick, 1981). The suspension was gel-filtered to remove the extraliposomal dye, on micro-columns of Sephadex G-50 (Pharmacia Fine Chemicals, Div. Pharmacia Inc., Piscataway, NJ, 20–80 μm particle size).

The measurement of resonance Raman spectra was performed on a microcomputer-controlled spectrometer that was described in detail elsewhere (Meiri et al., 1983). We used an Ar^+ laser (Coherent Radiation Inc., Palo Alto, CA) to excite the Raman spectra, a double monochromator (Spex Industries, Inc., Metuchen, NJ, model 1403) to analyze the scattered light, and a cooled photomultiplier (RCA Electro-Optics & Devices, RCA Solid State Div., Lancaster, PA, model C31034-02) followed by an amplifier-discriminator (Pacific Instruments Inc., Concord, CA, model AD-6) for light detection. The photon counts were fed into the Apple II microcomputer (Apple Computer Inc., Cupertino, CA), which also stepped the monochromator. The spectral resolution as well as the step size were usually 1 cm^{-1}. The intensity of the dye's intrinsic fluorescence decreased relative to the resonance Raman intensity as the exciting wavelength shortened, although even with 514.5 nm excitation it did not interfere significantly with the measurement of the Raman spectrum. Absorption spectra were measured on a Cary 17 spectrophotometer (Cary Instruments, Palo Alto, CA).

For resolving neighboring Raman lines and calculating their areas, we used either an IBM Harwell fitting program (IBM Instruments, Inc., IBM Corp., Danbury, CT) or a program in BASIC (Ruckdeschel, 1981), executed on the Apple microcomputer. Both calculations gave results within 3% of each other.

RESULTS AND DISCUSSION

Spectroscopic Properties of the Dye Indicator

The absorption spectra of the dye WW-638 in various solvents are shown in Fig. 1. As can be seen, the location of the absorption maximum shifts to the red upon a decrease in the dielectric constant of the solvent. This is a common behavior for polar chromophores, where the stabilization of the ground state by solvation is more effective than in the vertically excited state (Loew et al., 1979). However, as can be seen in Fig. 1, the absorption spectrum of the dye in a suspension of lecithin liposomes, with or without bacteriorhodopsin reconstituted into them, is very similar to that in water. Therefore, we may conclude that the dye does not segregate itself from the aqueous solution into the low-polarity lipid medium. This conclusion will be important for our later discussion of the effect of membrane surface potential on the resonance Raman spectrum of this dye.

The absorption spectra of aqueous solutions of the dye at various concentrations are shown in Fig. 2. Upon an increase of the concentration from $2 \cdot 10^{-5}$ M to $2 \cdot 10^{-4}$ M, the absorption maximum shifts from 516 to 523 nm. The effect of the increased dye concentration on its resonance Raman spectrum is shown in Fig. 3. At this point it should be stressed that the exact assignment of all the vibrational modes of the studied molecule is not of critical importance as is the understanding of the trends in some of the bands, as discussed below. In the important region of 1,400–1,700 cm^{-1}, we assign the three major bands in the following way (Bellamy, 1975; Panizzi et al., 1971): the split band at 1,430 and 1,446 cm^{-1} is assigned as a breathing mode of the aniline group, conjugated with the

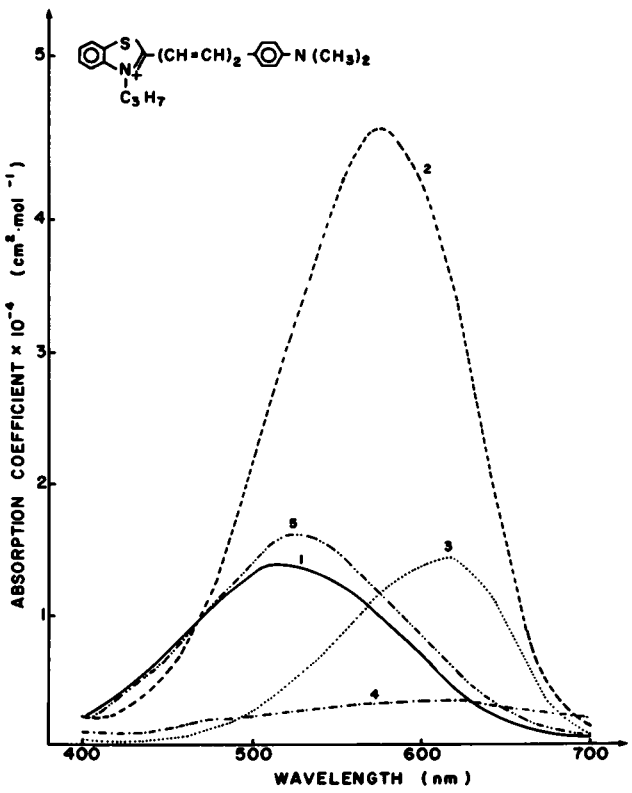

FIGURE 1 Absorption spectra of WW-638 in water (*1*), ethanol (*2*), chloroform (*3*), benzene (*4*), and in an aqueous suspension of lecithin vesicles (1 mg/ml), pH = 6.0 (*5*).

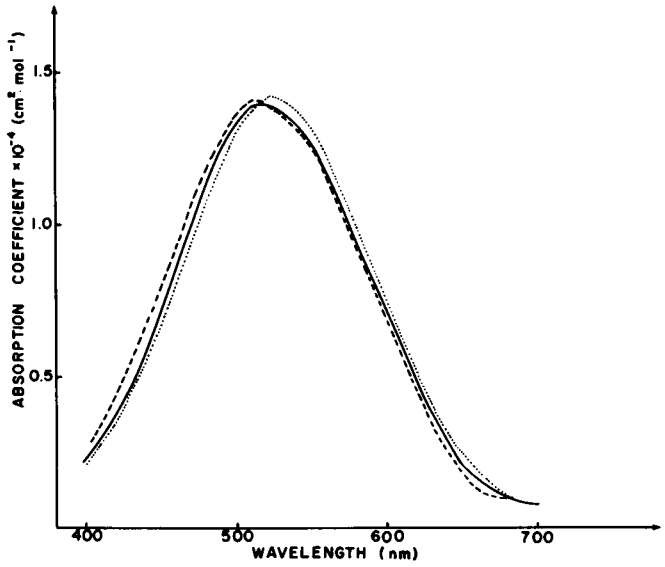

FIGURE 2 Absorption spectra of WW-638 in water, at pH = 6.0. The concentration of the dye is 20 μM (--), 60 μM (——), and 200 μM (·····).

polyenic chain; the band at 1,490 cm^{-1} arises from the benzothiazolic benzene ring, and indeed is totally absent in the spectrum of an almost identical dye which lacks the benzene ring of the benzothiazole group (dye NK-365, from the Japanese Research Institute for Photosensitizing Dyes Co., Okayama, Japan); and the strong and broad band at 1,573 cm^{-1}, which is probably an overlap of a ring mode of the benzothiazole group, a stretching mode of the conjugated polyenic system and a contribution of the amine deformation mode. Additional vibrational modes that are less important in this study appear at 1,018, 1,135, 1,159, and 1,286 cm^{-1}. The important point to be observed in Fig. 3 is that the band at 1,430 cm^{-1}, which appears as a shoulder at a dye concentration of 40 μM, grows in intensity relative to the other bands upon an increase in dye concentration and becomes stronger than the 1,446 cm^{-1} band at a 380 μM concentration. Based on the absorption shift of the dye upon increased concentration and the concomitant increase of the ratio of the Raman bands at 1,430 and 1,446 cm^{-1}, we conclude that the dye tends to aggregate when present at increased concentration, and that the aggregation is reflected in an enhancement of the Raman band at 1,430 cm^{-1}. A similar concentration dependence of both the absorption and Raman spectra was observed with the dye quinaldine red (Koyama et al., 1979). There the shift in the absorption was attributed to exciton interaction between aggregated dye molecules while the changes in the Raman spectrum were assigned to an interaction with a counter ion. In the case of WW-638, we cannot differentiate between the effect on the Raman intensities of an exciton interaction which affects the excited state only (Kasha, 1963), and a real perturbation of the molecular structure in the ground state. However, the ratio of intensities at 1,430 cm^{-1} and 1,446 cm^{-1} correlates with a concentration-dependent interaction.

As a corroboration to the assignment of the two Raman bands at 1,430 and 1,446 cm^{-1} to different species whose slightly different electronic absorption bands contribute to the overall absorption spectrum of Fig. 2, we have measured the Raman spectra with excitations at different laser wavelengths. When the excitation wavelength falls within an electronic absorption band of a species, the Raman intensity of vibrational modes associated with the absorbing chromophore becomes resonantly enhanced (for review, see Carey, 1978). As seen in Fig. 4, the band at 1,430 cm^{-1} is enhanced, relative to the other bands, when

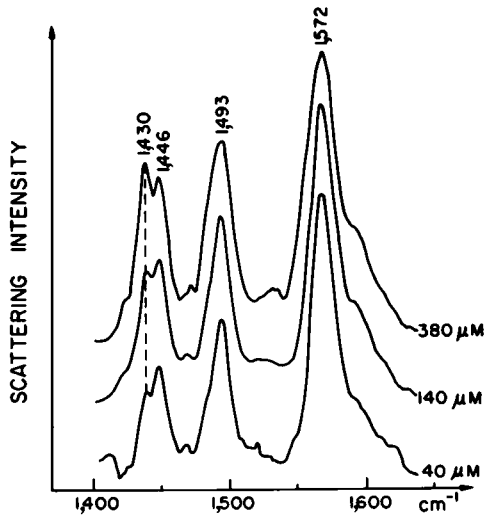

FIGURE 3 Resonance Raman spectra of WW-638 in aqueous solution at various dye concentrations. Excitation was at 457.9 nm with 100 mW, and the spectral resolution was 2 cm^{-1}. pH = 6.0, T = 22°C.

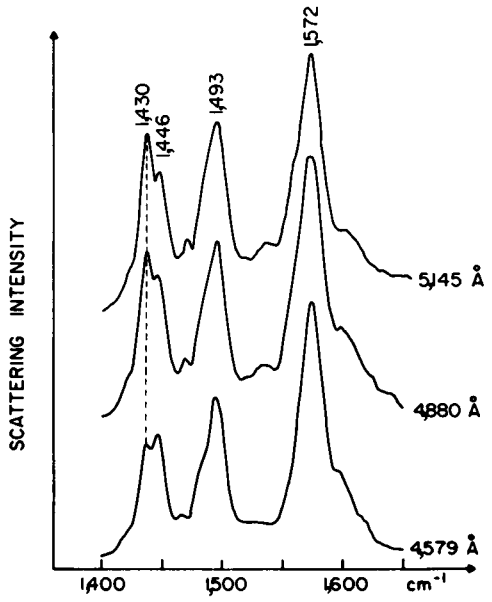

FIGURE 4 Resonance Raman spectra of WW-638 (100 μM), excited at three different wavelengths. Laser power was 100 mW, and spectral resolution 2 cm^{-1}.

increasing the wavelength of excitation. We therefore conclude that when the concentration of the dye was increased, there was a growth in the amount of an aggregated or perturbed form of the dye that absorbs light at a longer wavelength. This behavior is less detectable in the absorption spectrum directly, because the difference between the two absorption profiles is too small. However, the width (FWHM) of the absorption band in water is larger (5,700 cm^{-1}) than in the other solvents of Fig. 1 (3,200–4,000 cm^{-1}), possibly indicating the presence of more than one chromophore in water. Resonance Raman spectroscopy thus provides a sensitive tool for monitoring the distribution of the dye WW-638 between its monomeric and aggregated states.

The Surface Potential on Purple Membrane Fragments

The resonance Raman spectrum of the dye WW-638 in a suspension of purple membrane fragments containing bacteriorhodopsin is shown in Fig. 5 (the lowest curve). There are two striking differences between this spectrum and that of the free dye in water, shown in Fig. 3: the intensity of the band at 1,430 cm^{-1} is extremely strong relative to the 1,446 cm^{-1} band and the splitting of bands between 1,540 and 1,610 cm^{-1}. Yet, the absorption spectrum of the dye, as shown in Fig. 2, is practically unchanged compared with the aqueous solution, indicating that the dye does not dissolve in the membrane fragments, but remains within the polar water medium. However, the presence of negatively charged protein groups from the bacteriorhodopsin molecules, at the surface of the membrane fragments, establishes a negative surface potential that attracts the positively charged dye molecules. This increase in the local concentration of the dye molecules near the surface of the membrane fragments also causes a splitting in the vibrational mode of the conjugated polyenic mode at 1,573 cm^{-1} (see Fig. 3) into several bands at 1,530, 1,547, 1,571, 1,594, and 1,600 cm^{-1}. The intrinsic resonance Raman bands of bacteriorhodopsin's chromophore, retinal, in the BR_{570} and M_{412} states that appear at 1,530 and 1,565 cm^{-1}, respectively (Lewis et al., 1974; Mendelsohn, 1973), contribute very little to the measured Raman spectra in Fig. 5, because the dye WW-638 has a much stronger Raman scattering efficiency than bacteriorhodopsin. The existence of the multitude of bands may indicate a stronger perturbation of the dye than that observed at the increased concentration in Fig. 3. Indeed, the ratio of intensities at 1,430 and at 1,446 cm^{-1} in the lower plot of Fig. 5 is about five times higher than in aqueous solution (Fig. 3). Thus, in the presence of bacteriorhodopsin, the dye is strongly perturbed by aggregation, and possibly also by binding to the membranal protein. We shall therefore use the resonance Raman spectrum of the dye as a sensitive probe to monitor the surface potential on the purple membrane fragments.

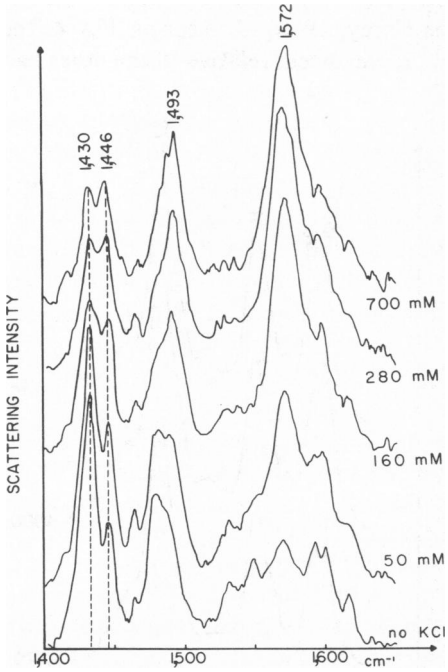

FIGURE 5 Resonance Raman spectra of WW-638 (60 μM) in aqueous suspensions containing 20 μM bacteriorhodopsin as purple membrane fragments, and varying concentrations of KCl. Excitation was with 100 mW of 457.9 nm light, and spectral resolution was 1 cm^{-1}.

Surface potential is a general property at interfaces between different phases when one of them is electrically charged. According to the theory proposed by Gouy and Chapman, the magnitude of the potential at the surface of the charged membrane, ψ, is given by Eq. 1 (McLaughlin, 1977):

$$\sinh\left(\frac{ze\psi}{2kT}\right) = A \cdot \sigma \cdot C^{-1/2} \qquad (1)$$

where σ is the density of the electronic charges per square ångström of surface, C is the electrolyte concentration, and z is its ionic charge. In the presence of a 1:1 electrolyte and at 22°C, one obtains

$$\sinh\left(\frac{\psi}{50.86}\right) = 136.4 \cdot \sigma \cdot C^{-1/2}. \qquad (2)$$

ψ is in milivolts. The electrolyte concentration profile near the surface follows Boltzmann's distribution. When a charged dye molecule is present in the solution, it will also form a concentration gradient near the surface. When the membrane surface potential is negative and the dye bears a positive charge, as does WW-638, its concentration at the surface will be higher than in the bulk solution, which should reflect itself in an enhanced Raman scattering intensity at 1,430 cm^{-1}. Increasing the electrolyte (KCl) concentration in the sample will decrease ψ, and indeed as can be seen in Fig. 5, the intensity ratio I_{1446}/I_{1430} decreases.

To evaluate the absolute surface potential from the

above-mentioned intensity ratio, which is proportional to the ratio of monomeric and aggregated and/or perturbed dye molecules, we measured the resonance Raman spectra at increasing KCl concentrations until no further change was observed in the relative intensities of the spectral bands. At this point, where the surface potential is negligibly small, we designate $P_o = (I_{1446}/I_{1430})_{\psi=0}$. At any other, lower KCl concentration, when the intensity ratio is P_i,

$$\psi_i = \frac{kT}{ze} \ln \frac{P_i}{P_o}. \quad (3)$$

We have measured the resonance Raman spectra of WW-638 in suspensions of bacteriorhodopsin fragments, at KCl concentrations from 0 to 0.7 M, above which no additional changes in the spectrum were observed. The limiting value of the ratio I_{1446}/I_{1430} at high salt concentration was thus used to calculate ψ_i at any salt concentration, in each set of measurements, through Eq. 3. We found that at pH 6.0 the surface potential is -34.5 ± 2.0 mV. This value is somewhat lower than that obtained by Carmeli et al. (1980), who have used an ESR probe that partitions into the lipid layer as a function of the surface potential. Plotting sinh $(\psi_i/50.86)$ vs. $C^{-1/2}$ as shown in Fig. 6, and using a least-squares fit, we obtain a line whose slope yields a surface charge density σ of $-(5.3 \pm 0.6) \cdot 10^{-4}$ electronic charges per square ångström. As can be seen in Fig. 5, the splitting of the Raman bands above 1,500 cm^{-1}, caused by aggregation or even binding of WW-638, also disappears upon increasing the electrolyte concentration, until the spectrum of the free dye in aqueous solution (Fig. 3) is reproduced. Indeed, practically identical results on the surface potential could be obtained by using the differential changes in intensities at 1,570 cm^{-1} and 1,594 cm^{-1}. We preferred, however, to use the bands at 1,430 cm^{-1} and 1,446 cm^{-1} as monitors, because of better resolution.

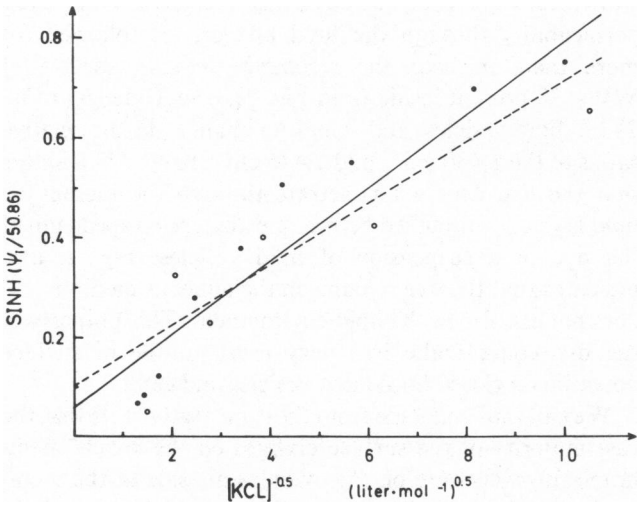

FIGURE 6 The functional dependence of the purple membranes' surface potential, ψ_i, on the electrolyte concentration C according to Eq. 2. The surface potentials in the photostationary state (● and full line) and in the bR_{570} state (○ and broken line), were calculated from the intensity ratio I_{1446}/I_{1430} in the steady state and fast-flow resonance Raman spectra, respectively. The slopes of the linear least-squares fits yield the surface charge densities.

These values of surface potential and charge density correspond to the purple membrane fragments in their photostationary state. This state is reached in the laser beam, which activates the bacteriorhodopsin photocycle and probes the Raman spectrum of WW-638, simultaneously. The photostationary mixture that is reached within the illuminated volume in the sample contains mainly bacteriorhodopsin in the M_{412} state, because of the saturating laser-light intensity. The exact composition of this mixture is hard to evaluate due to the diffusion into and out of the illuminated spot. Because of this dual role of the laser beam, we could not perform a measurement of the surface potential when the bacteriorhodopsin was unilluminated, i.e., when it was in the BR_{570} state. To obtain such measurements, we have performed kinetic resonance Raman studies (Mathies et al., 1976; Callender et al., 1976; Marcus and Lewis, 1977) with the sample solution flowing through a capillary tube, with an average dwell time in the laser beam of 8 μs. Under these conditions the probed sample volume contains ~90% BR_{570}, and most of the rest is K_{610}. At these conditions, the surface potential at zero electrolyte concentration was found to be -29.5 ± 2 mV. A plot of sinh $(\psi_i/50.86)$ vs. $C^{-1/2}$ is shown in Fig. 6, and from it we calculated the charge density σ as $-(4.4 \pm 0.6) \cdot 10^{-4}$ electronic charges per square ångström. When the flow rate was decreased so that the average dwell time within the illuminated spot was 170 μs, and so was composed mainly of M_{412} (Marcus and Lewis, 1978), the surface potential and charge density were like those in the steady state measurement shown in Fig. 5, and described above. Such time-resolved resonance Raman measurements could be used to monitor the kinetics of evolution of the surface potential, and to correlate these with the steps in the photochemical cycle of bacteriorhodopsin. We have found recently (Ehrenberg and Meiri, 1983) that bleaching bacteriorhodopsin by removal of the retinal chromophore does not alter the surface potential. Thus, the exposure of the protein to the membrane's surface is not affected by the protein-chromophore interactions that exist at the active site.

The photochemical conversion of bacteriorhodopsin is thus seen to be involved with a change of ~5 mV in the surface potential of the purple membrane fragments, which is caused by an increase of $0.9 \cdot 10^{-4}$ negative electronic charges per square ångström of the membrane surface. This light-induced change in potential is larger than the change of -0.25 mV reported by Carmeli et al. (1980) and reflects the larger extent of photochemical conversion due to the higher light intensity used by us. The lipids comprising the membrane are uncharged, therefore the surface charges arise from the bacteriorhodopsin molecules. The change in the surface charge could arise from

deprotonation of a protein group on one side of the membrane and/or protonation of a different group at the other membrane surface, in the process of the unidirectional transport of protons across the membrane, as suggested by Carmeli et al. (1980). It seems unlikely to us that a significant change could occur in the pK of a side chain of an amino acid localized at the aqueous interface. It is more likely that the increased surface charge density reflects an increased exposure of amino acid side chains, which are buried inside the intra-membranal protein volume and become exposed to the surface and deprotonate. Another possibility could be that the bacteriorhodopsin molecule as a whole, or segments of the molecule, undergo a vertical displacement in the membrane, caused by a change in the protein-lipid interaction. Such a vertical displacement which was observed before (Borochov and Shinitzky, 1976) would change the charge density at the surface.

The Sidedness of the Membrane Potential

As will be shown later, the dye WW-638 does not permeate through a lipid bilayer. Therefore it can be used to monitor the potential of each surface separately. We have reconstituted the bacteriorhodopsin-containing membrane fragments into sonicated lecithin vesicles, and, having the dye either incorporated into the intravesicular trapped volume, or present only in the outside medium. When bacteriorhodopsin is reconstituted into lecithin vesicles, its sidedness is inverted relative to the intact cell, so that the cytoplasmic side of the membrane fragments now faces the outer side of the vesicles (Happe et al., 1977). We have checked each sample to ensure that it was properly reconstituted by observing the alkalinization of the suspension when it was illuminated by a slide projector lamp. This indicated uptake of protons from the outside due to inward pumping. We found that when the dye was trapped inside the liposomes at a concentration of 200 μM, its resonance Raman spectrum (trace *a* in Fig. 7), was identical to that in water solution or with membrane fragments at high salt concentration. Adding KCl to the suspension of vesicles during the sonication did not affect the spectrum of the trapped dye. These results indicate that the potential on the inner side of the vesicle membrane is zero or positive. On the other hand, when the dye was present only in the extravesicular volume, a surface potential of -6 mV was calculated from the resonance Raman spectra of these vesicles with and without KCl in the solution (traces *b* and *c* of Fig. 7), using Eq. 3. The potential was also decreased when the sample with the dye was re-sonicated, and so the dye also penetrated into the inner vesicle volume where, as discussed above, there is no negative surface potential. The observation that on vesicles containing the purple membrane fragments the surface potential is ~5 times smaller than on the isolated fragments can be explained by the fact that the surface charges originating from the protein are "diluted" in the vesicular phosphatidylcholine surface

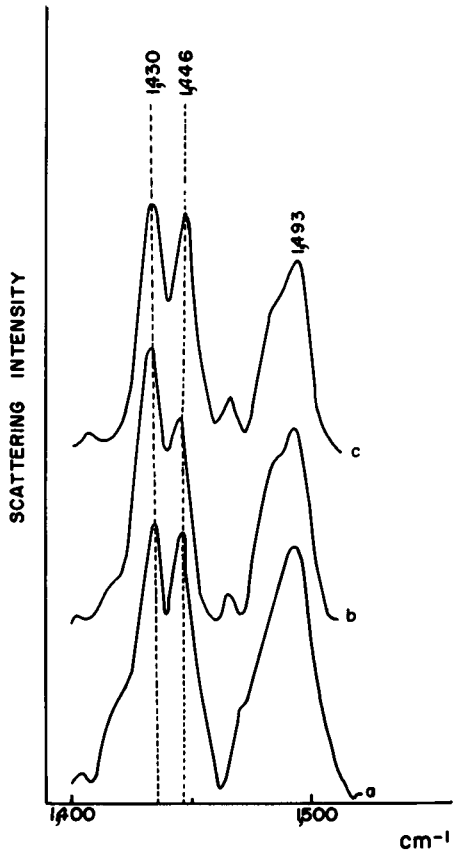

FIGURE 7 Resonance Raman spectra of WW-638 in a suspension of sonicated lecithin vesicles (10 mg/ml) reconstituted with bacteriorhodopsin (20 μM) · pH = 6.0. (*a*) The dye is trapped inside the vesicles. (*b*) The dye is outside the vesicles. (*c*) The vesicles were sonicated in 0.7 M KCl, and the dye was added outside the vesicles only.

which does not carry charges. As a check on the dye's permeability through the lipid bilayer, we followed for more than an hour the resonance Raman spectra of WW-638 present inside (*a* in Fig. 7), or outside (*b* in Fig. 7) the lipid vesicles, and found no changes in the relative ratios of the 1,430 cm^{-1} and 1,446 cm^{-1} bands. This shows that the dye does not penetrate through the membrane lipid layer. As discussed before, the absorption spectrum of the dye in a suspension of lipid vesicles, Fig. 1, also indicates that the dye remains in the aqueous medium and does not dissolve in the lipid environment. This property of the dye could make it a very useful probe of surface potential on closed liposomes, vesicles and cells.

We can thus conclude from these measurements that the vast majority of the surface charges on the purple membrane fragments are on the cytoplasmic side of the membrane which, when reconstituted into lipid vesicles, faces the outer side. This fits nicely with the accepted models of bacteriorhodopsin's folding (Khorana et al., 1979; Ovchinnikov et al., 1979) that place 2–4 amino acids with negatively charged side chains in excess of those with positive charges close to the cytoplasmic membrane surface, in the NH_2-terminus segment, which extends into the

cytoplasm, and in the protein segments that protrude into the cytoplasm and connect neighboring helices. These models indicate a zero net charge on the external surface of the membrane. A calculation using the size of the two-dimensional unit cell of the purple membrane (Unwin and Henderson, 1975) and the surface charge density of $-(4.4 \pm 0.6) \cdot 10^{-4}$ electronic chargers per square ångstöm, measured by us, and assuming that it is all on one side of the membrane, as discussed above, yields 1.8 net negative charges per bacteriorhodopsin molecule. Such an asymmetric orientation of a membrane protein, which involves a segregation of surface charges is a phenomenon that was encountered in a few cases of membrane-bound proteins (Weinstein et al., 1982). This electrostatic effect was shown to be a factor that could thermodynamically stabilize a preferred membranal orientation of such asymmetrically placed proteins. It should be noted that studies of purple membranes' adsorption to polylysine surfaces (Fisher et al., 1978) and ferritin binding to purple membranes (Neugebauer et al., 1978) have also indicated an asymmetric charge distribution on the membranes with the cytoplasmic side being more negative.

We wish to mention a certain limitation of the application of the Gouy-Chapman theory, which may interfere with the exact evaluation of the surface-charge density. A basic assumption in the theory on surface potential is that the charges are uniformly dispersed on the surface. However, when considering charged groups on membrane-bound proteins, the discrete charge effect may cause deviations from the Gouy-Chapman theory (Haynes, 1974), therefore the charge density calculated from such measurements may have some inherent error. We have tried to minimize this problem by sonicating the purple membrane fragments before reconstitution to avoid formation of large and isolated purple patches on the liposomes.

Our results show, therefore, that the steady state charges are on the cytoplasmic side of the membrane. Our results also indicate that the cytoplasmic side of the membrane is where the change in potential occurs during the photocycle. Yet this last conclusion cannot be reached unambiguously, because the light-induced change in the surface potential, when using liposomes to determine the sidedness, is expected to be even smaller than the 5 mV that we have obtained with the membrane fragments in water because of the dilution of bacteriorhodopsin in uncharged lipid. But if our results, which indicate no light-induced change in charges on the external surface, could be unambiguously corroborated, they would mean that the change in surface potential reflects light-activated configurational alterations of protein segments near the membrane's surface rather than deprotonation of an amino acid side chain at the surface, as part of the process of proton pumping, because the latter effect should lead to an increased negative charge density on the external side of the membrane (the internal side in inside-out reconstituted liposomes) from which protons are extruded during the photocycle. Thus, acidic groups at the surface of the membrane probably only shuttle a proton which is transferred to them from inner environments in the membrane (Nagle and Morowitz, 1978), and so their state of protonation at the surface does not change in the process of proton pumping.

We are at present checking additional dye molecules which could be used in a similar way, as resonance Raman probes of either positive or negative surface potentials, with even higher sensitivity than the dye which was employed in this study.

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REFERENCES

Bellamy, L. J. 1975. The Infrared Spectra of Complex Molecules. Chapman and Hall Ltd., London. 1–433.

Borochov, H., and M. Shinitzky. 1976. Vertical displacement of membrane proteins mediated by changes in microviscosity. *Proc. Natl. Acad. Sci. USA* 73:4526–4530.

Braiman, M., and R. Mathies. 1982. Resonance Raman spectra of bacteriorhodopsins primary photoproduct: evidence for a distorted 13-*cis* retinal chromophore. *Proc. Natl. Acad. Sci. USA* 79:403–407.

Callender, R. H., A. Doukas, R. Crouch, and K. Nakanishi. 1976. Molecular flow resonance Raman effect from retinal and rhodopsin. *Biochemistry.* 15:1621–1629.

Carey, P. R. 1978. Resonance Raman spectroscopy in biochemistry and biology. *Q. Rev. Biophys.* 11:309–370.

Carmeli, C., A. T. Quintanilha, and L. Packer. 1980. Surface charge changes in purple membranes and the photoreaction cycle of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 77:4707–4711.

Ehrenberg, B., and Z. Meiri. 1983. The bleaching of purple membranes does not change their surface potential. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 164:63–66.

Ehrenberg, B., Z. Meiri, and L. M. Loew. 1984. A microsecond kinetic study of the photogenerated membrane potential of bacteriorhodopsin with a fast responding dye. *Photochem. Photobiol.* In press.

Fisher, K. A., K. Yanagimoto, and W. Stoeckenius. 1978. Oriented absorption of purple membrane to cationic surfaces. *J. Cell. Biol.* 77:611–621.

Garty, H., G. Klemperer, M. Eisenbach, and S. R. Caplan. 1977. The direction of light induced pH changes in purple membrane suspensions. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 81:238–242.

Happe, M., R. M. Teather, P. Overath, A. Knobling, and D. Oesterhelt. 1977. Direction of proton translocation in proteoliposomes formed from purple membrane and acidic lipids depends on the pH during reconstitution. *Biochim. Biophys. Acta.* 465:415–420.

Haynes, D. H. 1974. 1-anilino-8-naphthalenesulfonate: fluorescent indicator of ion binding and electrostatic potential on the membrane surface. *J. Membr. Biol.* 17:341–366.

Johnson, J. H., A. Lewis, and G. Gogel. 1981. Kinetic resonance Raman spectroscopy of carotenoids: a sensitive kinetic monitor of bacteriorhodopsin mediated membrane potential changes. *Biochem. Biophys. Res. Commun.* 103:182–188.

Kasahara, M., and P. C. Hinkle. 1977. Reconstitution and purification of the D-glucose transporter from human erythrocytes. *J. Biol. Chem.* 252:7384–7390.

Kasha, M., 1963. Energy transfer mechanisms and the molecular exciton model for molecular aggregates. *Radiat. Res.* 20:55–71.

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

Koyama, Y., P. R. Carey, R. A. Long, W. G. Martin and H. Schneider. 1979. A resonance Raman and electronic absorption probe of membrane energization. Quinaldine red in cells of *streptococcus faecalis. J. Biol. Chem.* 254:10276–10285.

Lewis, A., M. A. Marcus, B. Ehrenberg, and H. Crespi. 1978. Experimental evidence for secondary protein-chromophore interactions at the Schiff base linkage in bacteriorhodopsin: molecular mechanism for proton pumping. *Proc. Natl. Acad. Sci. USA.* 75:4642–4646.

Lewis, A., J. Spoonhower, R. A. Bogomolni, R. H. Lozier, and W. Stoeckenius. 1974. Tunable laser resonance Raman spectroscopy of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 71:4462–4466.

Loew, L. M., L. Simpson, A. Hassner, and V. Alexanian. 1979. An unexpected blue shift caused by differential solvation of a chromophore in a lipid bilayer. *J. Am. Chem. Soc.* 101:5439–5440.

Marcus, M. A., and A. Lewis. 1977. Kinetic resonance Raman spectroscopy: dynamics of deprotonation of the Schiff base of bacteriorhodopsin. *Science (Wash. DC).* 195:1328–1330.

Marcus, M. A., and A. Lewis. 1978. Resonance Raman spectroscopy of the retinylidene chromophore in bacteriorhodopsin (bR_{570}), bR_{560}, M_{412}, and other intermediates: structural conclusions based on kinetics, analogues, models, and isotopically labeled membranes. *Biochemistry.* 17:4722–4735.

Mathies, R., A. R. Oseroff, and L. Stryer. 1976. Rapid flow resonance Raman spectroscopy of photolabile molecules: rhodopsin and isorhodopsin. *Proc. Natl. Acad. Sci. USA.* 73:1–5.

McLaughlin, S. 1977. Electrostatic potentials at membrane-solution interfaces. *Curr. Top. Membr. Transp.* 9:71–144.

Meiri, Z., Y. Berezin, A. Shemesh, and B. Ehrenberg. 1983. Microcomputer controlled flash photometry with submicrosecond resolution and Raman spectroscopy. *Appl. Spectrosc.* 37:203–207.

Mendelsohn, R. 1973. Resonance Raman spectroscopy of the photoreceptor-like pigment of *Halobacterium halobium. Nature (Lond.).* 243:22–24.

Nagle, J. F., and H. J. Morowitz. 1978. Molecular mechanisms for proton transport in membranes. *Proc. Natl. Acad. Sci. USA.* 75:298–302.

Neugebauer, D. C., D. Oesterhelt, and H. P. Zingsheim. 1978. The two faces of the purple membrane. II. Differences in surface charge properties revealed by ferritin binding. *J. Mol. Biol.* 125:123–135.

Oesterhelt, D., and B. Hess. 1973. Reversible photolysis of the purple complex in the purple membrane of *Halobacterium halobium. Eur. J. Biochem.* 37:316–326.

Oesterhelt, D., and W. Stoeckenius. 1973. Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. USA.* 70:2853–2857.

Oesterhelt, D., and W. Stoeckenius. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* 31:667–678.

Ovchinnikov, Y. A., N. G. Abdulaev, M. Y. Feigina, A. V. Kiselev, and N. A. Lobanov. 1979. The structural basis of the functioning of bacteriorhodopsin: an overview. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100:219–224.

Pande, J., Callender, R. H., and Ebrey, T. G. 1981. Resonance Raman study of the primary photochemistry of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 78:7379–7382.

Panizzi, J. C., G. Davidovics, R. Guglielmetti, G. Mille, J. Metzger, and J. Chouteau. 1971. Etude des spectres de vibration du benzothiazole et des oscillations des liaison CH de quelques uns de ses derives. *Can. J. Chem.* 49:956–964.

Pick, U. 1981. Lipòsomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. *Arch. Biochem. Biophys.* 212:186–194.

Ruckdeschel, F. R. 1981. Basic Scientific Subroutines. Byte McGraw Hill Inc., Peterborough, NH 2:7–88.

Stockburger, M., W. Klusmann, H. Gattermann, G. Massig, and R. Peters. 1979. Photochemical cycle of bacteriorhodopsin studied by resonance Raman spectroscopy. *Biochemistry.* 18:4886–4900.

Terner, J., C.-L. Hsieh, and M. A. El-Sayed. 1979. Time-resolved resonance Raman characterization of the bL_{550} intermediate and the two dark adapted bR_{560}^{DA} forms of bacteriorhodopsin. *Biophys. J.* 26:527–541.

Tokutomi, S., T. Iwasa, T. Yoshizawa, and S. Ohnishi. 1980. Flash induced fast change on purple membrane surface detected by spin label method. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 114:145–148.

Unwin, P. N. T., and R. Henderson. 1975. Molecular structure determination by electron microscopy of unstained crystalline specimens. *J. Mol. Biol.* 94:425–440.

Weinstein, J. N., R. Blumenthal, J. van Renswoude, C. Kempf, and R. D. Klausner. 1982. Charge clusters and the orientation of membrane proteins. *J. Membr. Biol.* 66:203–212.