The bleaching of purple membranes does not change their surface potential

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A resonance Raman molecular probe was used to measure the surface potential of membrane fragments which contain bacteriorhodopsin. It is shown that the surface potential of the native membranes is identical to that of bleached bacteriorhodopsin. It is therefore concluded that the secondary interactions between the retinal chromophore and the protein, which are known to exist, do not have a long-range effect on the exposure of the bacteriorhodopsin at the membrane's surface.

Purple membrane *Bacteriorhodopsin* *Membrane surface potential*
Chromophore–protein interaction *Molecular dye probe* *Resonance Raman spectroscopy*

1. INTRODUCTION

Bacteriorhodopsin is a photopigment which is found in the cytoplasmic membrane of *Halobacterium halobium*. It is composed of a protein, bacterio-opsin, and a retinal chromophore which is attached to a lysine side chain through a Schiff base linkage [1]. It functions as a light-driven proton pump to establish an electrochemical gradient across the membrane, which is used for ATP synthesis [2,3]. The protein molecule is folded in the membrane into 7 helical segments which span the membrane [4] and are connected to each other at the membranal surface by non-helical segments [5–7]. The retinal is most probably attached to Lys-216 in the helical column near the C-terminus [8].

A question of major importance in understanding the mechanism of the light-activated proton pumping is the nature of the secondary interactions between the retinal and the apoprotein. Resonance Raman studies indicate that such an interaction exists at the protonated Schiff base linkage [9]. Studies of the intrinsic absorption and fluorescence of the aromatic residues in the protein, indicate that the environment of these residues is affected by the retinal, and is modulated by the photochemical cycle [10–13]. CD measurements have shown that removal of the retinal chromophore induces changes in the tertiary structure of the protein [14].

We here address ourselves to the question whether the effect of the retinal on the protein's conformation is localized near the chromophore, or extends to amino acid residues at the surface of the membrane. We have used resonance Raman probes to study the surface potential of the purple membrane in comparison to the bleached membrane, from which the retinal chromophore has been removed, and found them to be virtually identical, indicating that bleaching the bacteriorhodopsin does not affect the exposure of the protein to the aqueous medium at the surface of the membrane.

2. MATERIALS AND METHODS

The purple membrane fragments which contain bacteriorhodopsin were grown and isolated by a standard procedure [15]. Bleaching was achieved

Abbreviation: WW-638, 2-[4-(*p*-*N*,*N*-dimethylanilinyl)-1,3-butadienyl]-3-propylbenzothiazolium iodide

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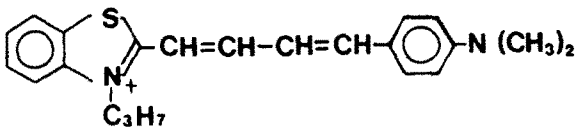
by illuminating a stirred suspension of the membranes in water + 0.25 M NH_2OH (pH 7) with light of $\lambda > 520$ nm (150 W halogen projector lamp + Schott glass filter) for 3–4 h. The apomembrane was pelleted and washed a few times with water in order to remove the hydroxylamine and the retinal-oxime. The concentration of the protein, native or bleached, was 15 μM.

The dye molecule which was used as a resonance Raman probe of the surface potential is 2-[4-(*p*-*N*,*N*-dimethylanilinyl)-1,3-butadienyl]-3-propylbenzothiazolium iodide [16]. It was synthesized by



Professor A. Waggoner of Carnegie-Mellon University (Pittsburgh PA) and coded WW-638. Its concentration in the samples was 50 μM.

Resonance Raman spectra were excited by 50 mW of 457.9 nm light from an Ar^+ laser. The measurement of the scattered light was carried out with a photon counting apparatus interfaced to an Apple II microcomputer. This Raman spectrometer is described in detail in [17].

3. RESULTS AND DISCUSSION

When the surface of a membrane is electrically charged, due to the presence of charged lipid molecules or protein residues, a potential difference exists between the surface and the bulk of the solution in which the membranes are suspended. The relation between the surface potential ψ_o, given in mV, and the surface charge density σ, in electronic charges per $Å^2$, is given by the Gouy–Chapman equation (at 22°C, in aqueous solution):

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

where:
C is the concentration of a 1:1 electrolyte present in the solution [18].

The positively charged WW-638 molecules are distributed in the bulk and in the Debye layer of a varying potential near the membrane surface, and when the latter is negatively charged, the dye's concentration near the membrane will be higher than in the bulk. Like many other dye molecules which tend to form dimers at high concentrations [19], WW-638 will also form increased concentrations of an aggregated state in the layer of solution close to the membrane's surface. The resonance Raman spectrum of the dye, which is sensitive to the molecular conformation, can be used to distinguish between the aggregates and the monomeric dye molecules [20].

As can be seen in fig.1, the Raman spectrum of the dye is strongly affected by the concentration of KCl, reflecting the effect of the electrolyte on ψ_o.

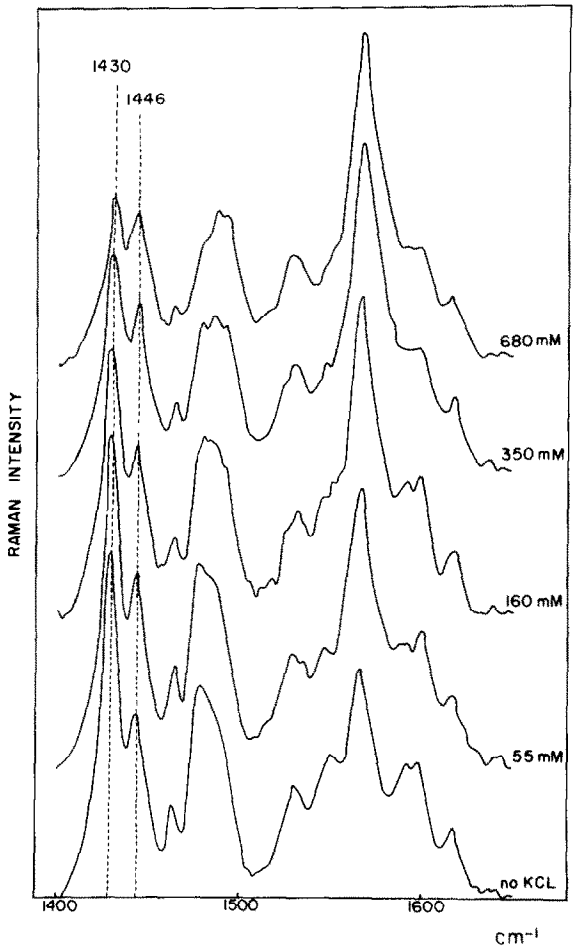


Fig.1. Resonance Raman spectra of WW-638 (50 μM) in solutions containing purple membrane fragments, and KCl at the marked concentrations. Excitation of the spectra was with 50 mW of 457.9 nm light, spectral resolution was 1 cm^{-1}.

Bacteriorhodopsin contributes only slightly to the resonance Raman spectrum, at 1530 cm^{-1} and at 1565 cm^{-1} [21]. At KCl concentration approaching 1 M, ψ_o is practically dissipated and the spectrum of the dye approaches that in aqueous solution. The ratio of the Raman bands at 1430 cm^{-1} and at 1446 cm^{-1} is proportional to the ratio of aggregated to monomeric dye and, therefore, to the surface potential [20]. The value of the potential can thus be calculated by the equation:

$$\psi_o = 25.43 \cdot \ln \frac{P_i}{P_o}$$

where:

P_i is the ratio of the above-mentioned Raman bands under the measured conditions;

P_o is the ratio at high KCl concentration; i.e., zero potential.

The membrane surface potential, when no KCl was added, was found to be 22 ± 2 mV, and from the slope of

$$\sinh\left(\frac{\psi_o}{50.86}\right)$$

vs $C^{-1/2}$ fitted by a least squares analysis and shown in fig.3, we calculated the surface charge density, $\sigma = 7.1 \cdot 10^{-4}$ charges$\cdot$Å^{-2}. We have found some variations in ψ_o and σ between different batches of purple membranes, and our results are also slightly different from those in [22], who used an ESR probe to study the surface potential. However, the differences may be attributed, at least partially, to a possible removal of part of the C-terminus end of the protein, which may occur spontaneously upon storage of the membranes [23].

We have repeated this type of measurement with bleached membranes at the same concentration of protein and dye. The resonance Raman spectra of the dye at various concentrations of the electrolyte are shown in fig.2, and the dependence of

$$\sinh\left(\frac{\psi_o}{50.86}\right)$$

on $C^{-1/2}$ appears in fig.3. The surface potential, in the absence of KCl, is 18 ± 2 mV and the surface charge density is $6.3 \cdot 10^{-4}$ charges$\cdot$Å^{-2}. Thus, the surface potential and the charge density of native purple membranes is about 10–15% higher than that of bleached membrane. Nevertheless, this difference is probably due solely to the fact that the measured surface potential of the native membranes mainly reflects the situation when the bacteriorhodopsin pigment is converted to the intermediate M_{412}, which is the main constituent in the volume of the sample which is illuminated by the laser beam. We have indeed found [20] that the surface potential and charge density of the purple membrane are increased by 18% when the bacteriorhodopsin molecules are converted from the initial bR_{570} state to the M_{412} intermediate, in the illuminating laser light. Thus, when the photochemical effect on the surface potential is considered, there is no difference in surface electrical properties between native and bleached purple membranes.

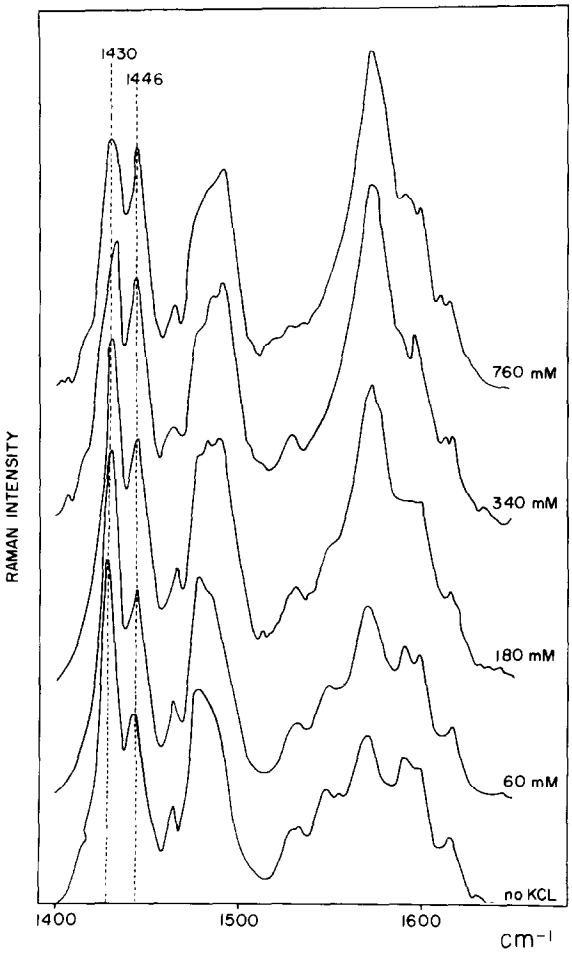


Fig.2. Resonance Raman spectra of WW-638 in solutions containing bleached purple membrane fragments and varying KCl concentrations. All experimental conditions were as described in fig.1.

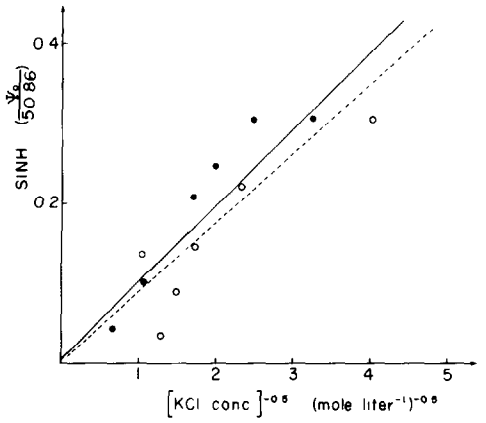


Fig.3. The variation of the surface potential, ψ_0, on native (●——●) and bleached (○---○) membrane fragments, with the electrolyte concentration, according to the Gouy–Chapman equation.

Our results indicate that removal of the retinal chromophore from the apoprotein bacterio-opsin does not change the way the protein is exposed to the surface of the membrane in which it is imbedded. On the other hand, the intrinsic protein absorption, fluorescence and CD spectra, show great dependence on the presence of the retinal chromophore [10–14], and a major part of the effect was attributed to the increased interaction of tyrosines and tryptophanes with water upon bleaching [11]. Combining both results shows that whatever secondary interactions exist between the retinal and the protein, they are localized and affect those regions in the protein which are buried inside the membrane, but these interactions do not influence the folding of the protein at the surface of the membrane. In other words, the hydrophilic interactions at the membrane's surface are not influenced by chromophore–protein interactions in the bulk lipid environment.

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