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# The surface potential on the purple membrane measured using a modified bacteriorhodopsin chromophore as the spectroscopic probe

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The surface potential of the purple membrane was measured by a novel method by using an artificial bacteriorhodopsin whose chromophore was 13-CF<sub>3</sub> retinal instead of retinal. When attached to the apoprotein by a Schiff base, the intrinsic pK of the 13-CF<sub>3</sub> chromophore is around 7.3. The apparent pK of this pigment depends on the surface potential and thus on the electrolyte concentration. This allowed us to determine the surface charge density using the Gouy-Chapman equation. The surface charge density was found to be  $-1.65 \pm 0.15 \times 10^{-3}$  electronic charges per Å<sup>2</sup> or about 2 negative charges/bacteriorhodopsin. This large value for the surface potential probably explains both part of the strong apparent association of divalent cations with the membrane and the effect of low salt concentrations on light-induced proton release from the purple membrane.

Bacteriorhodopsin; Purple membrane; Surface potential; Surface charge; Retinal; Absorption spectroscopy

## 1. INTRODUCTION

Bacteriorhodopsin is the only protein in the purple membrane patches of *Halobacterium halobium*. This light-driven proton pump acts as a proton-motive source for the synthesis of ATP (for reviews see [1–3]). The membrane-spanning and folded bacteriorhodopsin has some segments of its primary structure exposed at the two surfaces. These segments contain many charged amino acid residues. Thus, along with important contributions from the charged lipids of the membrane, the surface charge density of the purple membrane (see [4–6] for general reviews) will reflect the mode of bacteriorhodopsin's folding in the membrane.

Several different techniques have been used to measure the surface charge density, and thus the surface potential, of the purple membrane [7–14].

Unfortunately, the results span a range of almost two orders of magnitude. The novel method employed here is more direct than most previous methods and thus is likely to be more reliable.

The chromophore of bacteriorhodopsin is retinal attached to the apoprotein via a protonated Schiff base linkage. When the Schiff base is deprotonated, the absorption spectrum shifts dramatically to the shorter wavelengths. We have employed an artificial bacteriorhodopsin pigment possessing a Schiff-base pK around 7.3 which was derived from the 13-CF<sub>3</sub> retinal analog. This rather low pK compares with a pK of >12 for native bacteriorhodopsin [15]. Since a negative surface potential causes accumulation of protons near the membrane's surface, its magnitude can be inferred from a spectroscopic comparison of the actual pK with the apparent pK under different conditions. Bacteriorhodopsin in the purple membrane can thus serve as a spectroscopic probe of its own surface charge density.

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## 2. MATERIALS AND METHODS

*Halobacterium halobium* cells were grown and the purple membrane fragments purified by standard procedures [16]. Bacterio-opsin was prepared by hydroxylamine extraction of the retinal, and it was reconstituted with 13-trifluoromethyl retinal [15].

The pH titration of the bacteriorhodopsin fluoro-analog was performed by adding NaOH into a solution containing 1 M NaCl or 0.2 M MgSO<sub>4</sub>. Under these conditions the surface potential is quite small and the true pK can be obtained. For the calculation of the surface charge density, the suspension of the membrane fragments in double distilled water was titrated with aliquots from a concentrated NaCl solution. Absorption spectra were measured with a Perkin-Elmer Lambda-9 spectrophotometer.

## 3. RESULTS

The method which we employ in this study to measure the surface charge density on the purple membrane is based on the following reasoning. A negative surface charge density generates a negative surface potential, which attracts protons to the vicinity of the membrane's surface, lowering the local pH. Addition of an electrolyte tends to reduce the surface potential, according to the Gouy-Chapman equation, until eventually the local pH equals that in the bulk of the solution. If the local pH is close to the pK of the pigment's chromophore, changing the local pH will affect the absorption spectrum of the pigment. Thus, the 'titration' of the pH near the surface by changing the salt concentration, as followed by the pigment's absorption spectrum, depends on the surface charge density.

Since the pK of the Schiff base in bacteriorhodopsin is ca.13 [17], it is impossible to use the native chromophore as its own probe to measure the surface charge density, because the bulk pH would have to be set in this range. Therefore, we have used an artificial pigment containing a 13-CF<sub>3</sub> retinal analog, where the pK is drastically lowered due to the presence of the electron-withdrawing CF<sub>3</sub> group. This artificial pigment maintains normal photoactivity [15], as well as efficient proton pumping capacity [18].

Bacteriorhodopsin reconstituted with 13-trifluoromethyl retinal has an absorption band peaking at 625 nm when the Schiff base linkage between the retinal and a lysine's amino group of the protein is protonated. Upon deprotonation at high pH, the major absorption band shifts to

440 nm [15]. To obtain the true pK of the Schiff base, independent of surface charge effects, we titrated the pigment in 1 M NaCl. With this electrolyte concentration, only a small residual surface potential still exists, and usually more than 95% of the surface potential is abolished [13,19]. The mathematically fitted titration curve, shown in fig.1, exhibits a pK at  $7.3 \pm 0.1$ . This value can be contrasted with the previously measured pK of 8.0 [15], obtained at low electrolyte concentration, where the surface potential caused the surface pH to be lower than the bulk pH.

Based on this pK of 7.3 and the absorption spectrum of the pigment, we were able to calculate the surface pH (pH<sub>s</sub>) and thus the surface potential ( $\psi_s$ ) of the purple membrane, at different NaCl concentrations with a constant bulk pH (pH<sub>∞</sub>), using eqns 1 and 2 [13]:

$$\text{pH}_s = \text{pK} - \log \frac{[\text{BRH}]}{[\text{BR}]} \quad (1)$$

$$\text{pH}_s = \text{pH}_\infty + \frac{e\psi_s}{2.3 kT} \quad (2)$$

[BRH], the concentration of the pigment with a protonated Schiff base, is proportional to its absorbance at 625 nm; [BR], the concentration of the unprotonated pigment, is proportional to the decrease in absorbance at 625 nm upon addition to

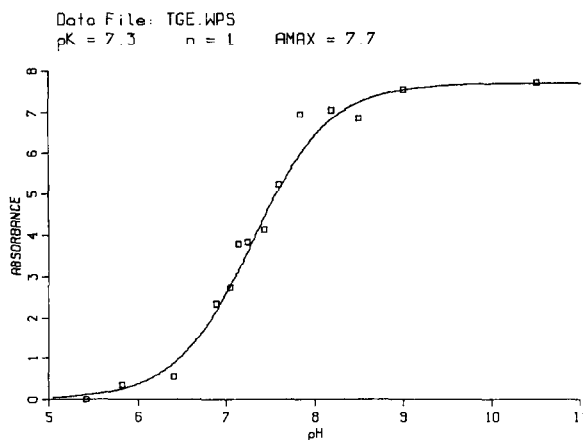


Fig.1. Titration data and a fitted curve of bacteriorhodopsin with 13-trifluoromethyl retinal, in a solution of 1 M NaCl. The titration is followed by the decrease of absorbance of the protonated Schiff base form of the pigment, at 625 nm.

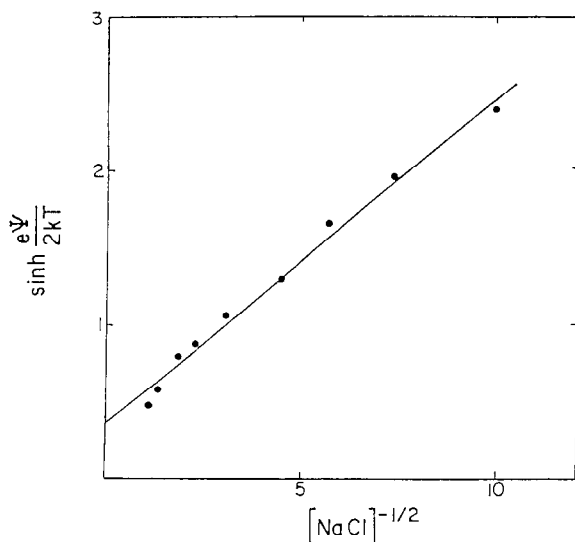


Fig.2. A Gouy-Chapman plot (see eqn 3 in the text) of the surface potential as calculated from eqn 2, vs the concentration of NaCl. The bulk pH of the solution was 7.4. The straight line is a linear least-squares fit of the measured points.

NaCl;  $e$ ,  $k$  and  $T$  have their usual meaning. The surface charge density,  $\sigma$ , is obtained through eqn 3 [4,13]:

$$\sinh \frac{e\psi_s}{2kT} = A\sigma C^{-1/2} \quad (3)$$

$C$  is the bulk concentration of a symmetric, monovalent electrolyte and  $A$  equals  $136.2 \text{ M}^{1/2}$  at  $20^\circ\text{C}$ , if  $\sigma$  is expressed in electronic charges per  $\text{\AA}^2$ . The results of an NaCl titration at a bulk pH of 7.4 are linearized in fig.2, and a fitted least-squares line is shown. The satisfactory adherence of the data to the Gouy-Chapman equation is evident. The slope of the fitted line yields  $\sigma = -1.65 \pm 0.15 \times 10^{-3} \text{ charges/\AA}^2$  or, with an area of  $1150 \text{ \AA}^2/\text{bacteriorhodopsin}$ , about  $-2 \text{ charges/bacteriorhodopsin}$ .

#### 4. DISCUSSION

The surface charge density on the purple membrane has been measured by a variety of direct and indirect methods, yielding quite different results [7–14]. Each of these methods carries with it a set of implicit or explicit assumptions. For example, some methods use extrinsic probes or involve

manipulations which alter the divalent cations normally tightly bound to the purple membrane [20]. Moreover, some techniques measure just one side or just regions of purple membrane while others determine an average value, including both sides of the membrane.

Our approach of using bacteriorhodopsin itself as an intrinsic probe is less prone to problems which haunt techniques relying on the use of extrinsic probes. It is unlikely that the fluorinated retinal has any major effect on the folding of the bacteriorhodopsin at the surface, since even extracting the retinal altogether, to produce bleached bacterio-opsin, changes the surface charge density by less than 15% [21], showing that the protein is anchored at the membrane's surface and that it is not greatly disturbed even by large changes at the chromophore binding site. Our method is also noteworthy in that it provides information on the surface charge density from the perspective of a specific place on the purple membrane, the chromophore binding site. Within these limitations, we therefore feel confident that the surface charge density of the purple membrane we obtained,  $-1.65 \pm 0.15 \times 10^{-3} \text{ charges/\AA}^2$ , is a reasonable estimate.

The purple membrane is a particularly interesting subject for the determination of its surface charge density for two reasons. First, the simple structure of the membrane and the wealth of structural information available should allow one to begin to understand the precise physical basis for its surface potential – what charged groups are involved and how they are spatially arranged. Secondly, the physiological activity of the purple membrane, light-driven proton pumping, might be expected to be influenced by the surface potential.

The proposed folding pattern of bacteriorhodopsin in the purple membrane [22], together with the proposed distribution [23] of the charged lipids [24,25] in the purple membrane, allow us to initially estimate that the side of the purple membrane which contains the N-terminal end of bacteriorhodopsin would have a charge density of about  $-4 \times 10^{-3} \text{ charges/\AA}^2$ , while the C-terminal side of the membrane would have a charge density, due just to these charges, of about  $-12 \times 10^{-3} \text{ charges/\AA}^2$ . The five or so divalent cations tightly associated with the purple membrane [20,26], prob-

ably on its C-terminal side, would be expected to modify this later value to also about  $-4 \times 10^{-3}$  charges/Å<sup>2</sup>. These rough values for the average surface charge density are remarkably close to the value we determined at the particular location on the purple membrane, the Schiff base of bacteriorhodopsin.

An important side benefit of our measurement is that it strongly suggests that the Schiff base itself can be directly titrated by external protons and is thus accessible to them; this has already been suggested by Druckmann et al. [17], but our results are obtained under much less restrictive conditions than their experiments.

Finally, we should comment on some possible consequences of this high surface charge. As already noted by Marinetti [27], at a value of surface charge density very close to that which we have determined, purple membrane would be expected to 'condense' counter ions to the surface, so that they are always associated with the purple membrane, irrespective of the ionic strength [28]. Thus, at the charge density which we have determined, the cations may undergo condensation to the surface. This mechanism may underlie the close association of divalent cations with the purple membrane, whose removal normally leads to the purple to blue membrane color transition [20]. A second consequence of the rather high surface potential of the purple membrane is its influence on light-driven proton transport across the membrane. We recently have obtained evidence that varying the surface potential can greatly influence this transport process [29].

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