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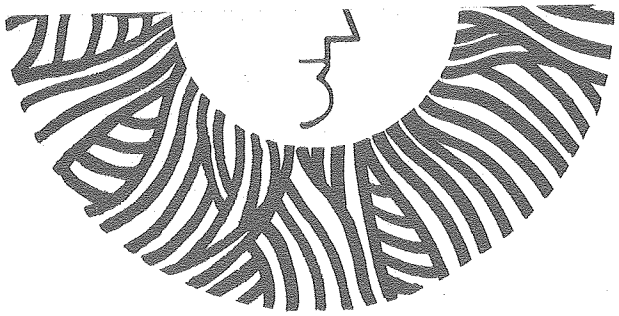

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LIGHT INDUCED SURFACE POTENTIAL CHANGES IN PURPLE MEMBRANES AND BACTERIORHODOPSIN LIPOSOMES

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INTRODUCTION. Bacteriorhodopsin, the retinal containing protein from the purple membrane of Halobacterium halobium is known to function as an electrogenic light activated proton pump (1). On illumination, the chromophore undergoes a photocycle in which its retinal Schiff base is reversibly protonated (2). The detailed molecular mechanism of H^+ translocation is not understood at present, but it may involve the Schiff base alone or other charge separation events, such as tyrosine deprotonation (3,4) or charge displacement in tryptophan (5). These dissociation events could move along a sequence of amino acids thus providing a pathway for the protons across the purple membrane.

Purified purple membranes have been shown to produce pH changes in their suspension medium during steady state illu-

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mination (6), which are strongly pH and temperature dependent (7). Avi-Dor et al (8) have shown that there is a correlation between the accumulation of the phototransient absorbing maximally at 412 nm (M_{412}) and the light induced H^+ release under steady state conditions. Using continuous actinic light of moderate intensity and single turnover laser flashes we have studied the kinetics and stoichiometry of changes in surface charge that arise on purple membranes during proton release and binding.

MATERIALS. Purple membranes were prepared from H. halobium by standard methods (9) and suspended in KCl containing media at different ionic strengths and pH values, at a concentration of 3.2 mg/ml.

Reconstituted bacteriorhodopsin containing liposomes were prepared by sonication (10) with partially purified asolectin at a lipid to protein ratio of 1:20 (mg/mg); the final bacteriorhodopsin concentration was 0.5 mg/ml in a medium containing 100 mM KCl, pH 7.

The positively charged paramagnetic amphiphile 4-(dodecyl dimethyl ammonium)-1-oxyl-2,2,6,6-tetramethyl piperidine bromide (CAT_{12}), which partitions between the membrane and aqueous phases, was synthesized in our laboratory by R.J. Mehlhorn and used as a probe of surface potential (11,12,13) at concentrations that had little effect on the photocycle (Viz. < 7 bound molecules of CAT_{12} per bacteriorhodopsin).

RESULTS. In the presence of purple membranes the spin-probe CAT_{12} partitions between the membrane and aqueous phases showing both membrane (broad) and aqueous (narrow) components to its EPR signal (Fig. 1). The ratio of the free to bound popu-

lations of the probe, designated as the partition P, has been shown to monitor the surface potential of the membrane (12, 13). A change in surface potential will result in a change in the partition of CAT_{12}. Changes in surface potential $\Delta\psi_s$ were calculated from Eqn.(I) which was derived (12) from the Gouy-Chapman theory (see reference 14),

$$\Delta\psi_s = \frac{RT}{zF} \ln \frac{P_1}{P_2} \qquad (I)$$

where P_1 and P_2 stand for the partition of CAT_{12} at two different states; z, F, R, and T are respectively the charge on the spin probe, the Faraday constant, the Universal gas constant and the absolute temperature. The partition P of CAT_{12} was a function of the ionic strength and the pH of the medium, as expected from the Gouy-Chapman theory. The surface potential calculated from Eqn.(I), decreased in the dark by as much as 60 mV as the ionic strength was raised from 2 mM to 300 mM (monovalents) and by 50 mV as the pH was decreased from 11 to 2 (not shown).

At room temperature, light of moderate intensity converts only a very small fraction of bacteriorhodopsin into its M_{412} intermediate, making it difficult to measure light induced phenomena. Hence it is convenient, when studying the correlation between the photocycle and surface charge changes, to increase the steady state concentration of M_{412}; this may be done by using high light intensities or by slowing down the rate of its decay. Continuous illumination at high light intensities may damage the system. We have used the antibiotics Valinomycin and Beauvericin (8) at a 1:1 molar ratio with the protein, to slow down the decay of M_{412}. Under these conditions the spectral changes in the high field aqueous EPR line

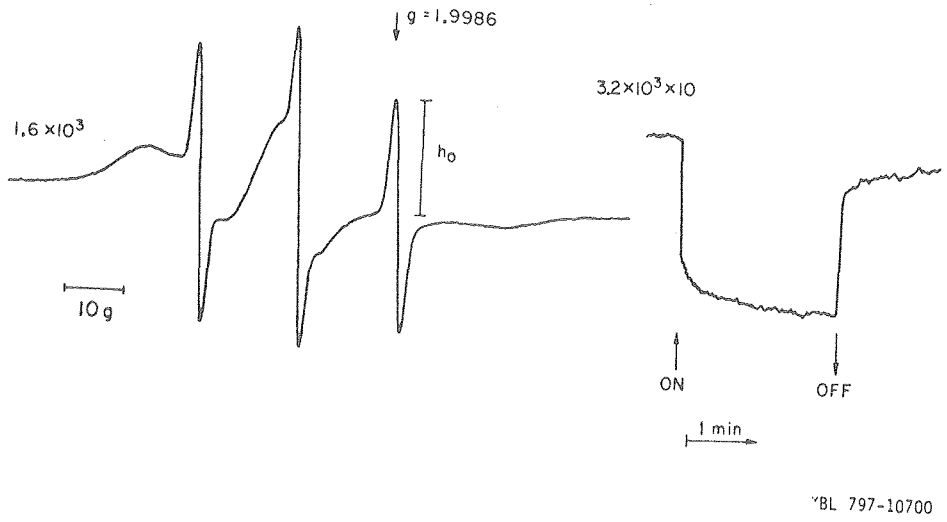

Fig. 1. EPR spectrum and light induced amplitude changes of the high field aqueous CAT_{12} signal in a purple membrane suspension. 30 μℓ sample containing 0.128 mM bacteriorhodopsin, 0.128 mM Valinomycin, 0.128 mM Beauvericin, 90 mM KCl, 1 mM CAT_{12}, pH 6.1 at room temperature. The light (intensity 12.4 mW/cm², quartz iodide lamp) induced changes in h_o were measured in a Varian E-109E spectrometer.

of CAT_{12} during continuous illumination, are shown in Fig. 1. A reversible decrease in the free aqueous population of CAT_{12} during illumination could mean one of two things: (a) the surface potential of the purple membrane becomes more negative when H^+ are released or (b) the purple membrane becomes more hydrophobic during that process. Control studies with the uncharged spin probe 2N11(2,2-dimethyl-5,5-methyl nonyl-N-oxazolidinyloxyl) suggested that there was very little change in the hyrophobicity of the purple membrane during illumination.

The light induced change in surface potential is independent of pH between 5.5 and 8 decreasing at lower pH's and increasing at higher pH's with pK values at about pH 4 and pH 9. The change in surface potential seems to be independent of ionic strength up to about 100 mM (monovalents) decreasing only at higher ionic strengths. It is clear from Eqn.(II) that the

same change in ψ_s at two different ionic strengths (pH's) will correspond to a higher change in surface charge density at the higher ionic strength (lower pH).

It was found (8) that in the presence of Valinomycin and Beauvericin, the increment in H^+ concentration in the assay medium of the purple membrane suspension, during continuous illumination, increased with ionic strength.

The equation relating the surface charge density σ to the surface potential ψ_s and the ionic concentration c, is

$$\frac{e\,\psi_s}{2kT} = \sinh^{-1}\ \frac{500\,\pi}{DRT}\,.\frac{\sigma}{\sqrt{c}} \qquad (II)$$

where k and D are the Boltzmann and the aqueous dielectric constants, e the unit electronic charge and the other constants as for Eqn.(I).

For the calculation of surface charge changes using Eqn.(II), two assumptions were made: (i) that the surface charge distributions are always homogeneous on the two sides of the purple membrane, and (ii) that the spin probe CAT_{12} only monitors what happens in the lipid phase, where it is probably located. The first assumption has not been tested; as regards the second, we know that the lipid occupies ∿37% (1) of the total area of the purple membrane and that the hyperfine splitting of the EPR signal of the bound component of the probe is ∿60 gauss at room temperature (Fig. 1); this suggests indeed that the probe may be in the constrained lipid environment of the purple membrane.

The charge density changes under steady state illumination were related to the level of M_{412} photointermediate under similar conditions. Table I shows the steady state levels of M_{412} at different pH values and for different light intensities

TABLE I

STOICHIOMETRY OF LIGHT INDUCED STEADY STATE CHARGE CHANGES AND M_{412} INTERMEDIATE LEVEL IN PURPLE MEMBRANE

pH	Light Intensity (mW/cm^2)	M_{412} (Abs.)	$\frac{M_{412}}{BR}$	$\Delta\psi_s$ (mV)	$\frac{Charge}{BR}$*	$\frac{Charge}{M_{412}}$
7.1	3.8	0.110	0.022	-0.082	0.022	1.04
7.1	2.9	0.058	0.011	-0.056	0.015	1.06
8.1	3.8	0.178	0.035	-0.121	0.033	0.94
8.1	2.9	0.103	0.020	-0.074	0.020	1.01

*The stoichiometry was calculated from measurement of changes in surface potential and the level of M_{412} at continuous illumination and at room temperature; the sample was assayed in a flat cell as described in Fig.2.

as well as the number of charge changes per M_{412} obtained when purple membranes were suspended in 90 mM KCl and 5 mM Tris buffer. Approximately one negative charge per M_{412} was induced by light under these conditions.

That the antibiotics (Valinomycin + Beauvericin) are not required to observe surface potential changes can be shown by assaying at lower temperatures and higher pH values, both of which slow down the decay of the M_{412}. A temperature analysis of surface potential changes will be published elsewhere.

In all cases of light induced surface potential changes, a fast and a slow phase could be detected after turning off the light. Since the generation of the M_{412} is much faster at room temperature than the limit of sensitivity of our EPR instrumentation (0.2 ms), we studied the laser-flash induced decay of M_{412} and the corresponding decay of the surface potential change monitored with CAT_{12}. A typical decay curve for the change in the high field aqueous line of CAT_{12} is shown

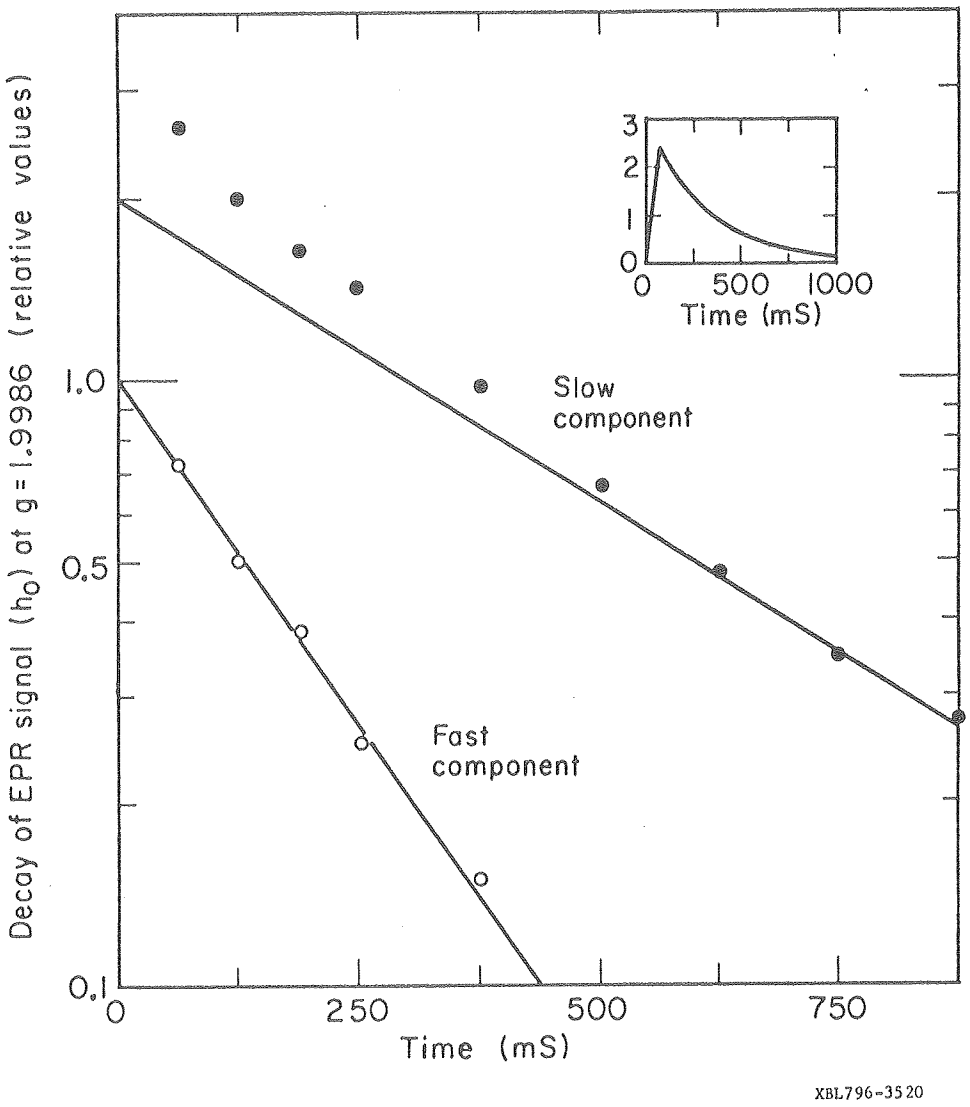

Fig. 2. Kinetics of the flash induced decay of CAT_{12} binding to purple membranes. The flash induced changes in the amplitude of the high field aqueous EPR signal of the probe were monitored on a CAT 400 signal averager. The inset is a trace-average of 12 flashes (150 ns rise-time, 0.2 joules/flash from Phase-R 1100 dye laser with Rhodamine 575). The sample (in a flat cell, 0.245 mm light path) contained 0.256 mM bacteriorhodopsin, 0.256 mM Valinomycin, 0.256 mM Beauvericin, 90 mM KCl, pH 6.1. The EPR time constant was 8 ms.

in Fig. 2. The decay kinetics could be decomposed into a fast and a slow component; Table II shows the half-times for the

decay kinetics of M_{412} measured in the presence of CAT_{12} in the flash photolysis apparatus, and for the change in surface potential $\Delta\psi_s$ at two different pH values. As can be seen from Table II the kinetics of both the fast and the slow components of surface potential changes are slower than the kinetics of M_{412} decay. This cannot be due to a limitation of the response time of the spin-probe, since the change in the EPR signal can be generated in a few ms, indicating that the response time of the probe is at least in the ms range.

TABLE II

KINETIC CONSTANTS FOR THE DECAY OF THE FLASH INDUCED CHANGE IN THE CAT_{12} EPR SIGNAL AND THE M_{412} PHOTOINTERMEDIATE IN PURPLE MEMBRANES.

The decay of the light induced CAT_{12} signal change was assayed under the conditions of Fig. 2. The decay of the M_{412} was assayed in a flash photolysis apparatus, using the same ionic concentration, the same ratio of bound CAT_{12} per bacteriorhodopsin and the same stoichiometry of antibiotics but with 4.1 μM bacteriorhodopsin.

pH	Fast component $\tau_{1/2}$ (ms)		Slow component $\tau_{1/2}$ (ms)	
	CAT_{12}	M_{412}	CAT_{12}	M_{412}
6	120	58	344	220
8	138	80	710	530

Reconstituted bacteriorhodopsin containing liposomes pump protons from the external to the internal space during steady state illumination (10). It is not known whether all the bacteriorhodopsin molecules are incorporated into the liposomal membrane with one orientation only, but it is assumed that most of them are pumping protons into their interior (1). Using a spin labeled amine, Tempamine, which monitors ΔpH (15),

and a spin-labeled phosphonium probe, which responds to transmembrane potentials (16)(a gift of W.L. Hubbell), we have been able to show that during steady state illumination and in the absence of antibiotics, the pH inside the liposomes decreases and it also becomes more positive relative to the outside. Although the transmembrane potential is negative in the external space of the liposomes, the outer surface potential, as assayed with the spin probe CAT_{12}, becomes more positive. The change in surface potential per mg of bacteriorhodopsin in the preparation is about equal but opposite in sign to that measured in the purple membranes. Addition of Nigericin (0.6 μM) completely abolishes the pH gradient, does not affect the transmembrane potential, but doubles the change in surface potential (Table III). Valinomycin (in the presence of KCl) is required at very high concentrations (> 80 μM) to completely abolish the transmembrane potential and we are not sure whether or not this is due to a specific interaction with the protein (which is known to slow down the photocycle). At lower Valinomycin concentrations (< 2 μM) the rate of the pH gradient generation increases, as expected, with only a slight

TABLE III

THE EFFECT OF ANTIBIOTICS ON THE LIGHT INDUCED ΔpH, $\Delta\psi_s$ AND TRANSMEMBRANE POTENTIAL $\Delta\psi$ IN BACTERIORHODOPSIN LIPOSOMES.

The reaction mixture contained 100 mM KCl, liposomes containing 0.5 mg/ml of bacteriorhodopsin, pH 6.5 at room temperature. The light induced ΔpH was measured by Tempamine uptake, $\Delta\psi$ by the change in distribution of the Phosphonium spin probe, and the $\Delta\psi_s$ by changes in the distribution of CAT_{12}.

Antibiotics	% of control		
	ΔpH	$\Delta\psi_s$	$\Delta\psi$
0.6 μM Nigericin	5	205	95
3 μM Valinomycin	150	20	70

increase in the total pH gradient established, while the surface potential changes decrease.(In table III the number given for ΔpH in the presence of Valinomycin actually corresponds to the rate of generation of the gradient).

DISCUSSION. The data presented here indicate that the changes in surface charge of purple membranes during steady-state illumination can be described as the appearance (disappearance) of approximately one negative (positive) charge on the surface of the protein per M_{412} photointermediate. Previous studies (8) have shown that in the presence of Valinomycin and Beauvericin, and under steady-state illumination, the number of protons released per M_{412} formed approaches 0.9 in the presence of salts, and that this number is a factor of 2 - 3 greater than what is observed in the absence of salts. Under conditions in which Eqn. (II) is valid (ie. $c > 0$) we have found that the number of negative charges per M_{412} appearing at the surface of purple membranes increases from 0.4 at 5 mM KCl to 1.08 at 90 mM KCl, assuming that M_{412} levels are unchanged under these conditions, as shown in (8).

The decay of the surface charge changes closely follows the decay of the M_{412} intermediate dependence on pH and response to antibiotics, but are slightly slower. Biphasic decay kinetics of M_{412} have been previously reported (1, 17). The slightly slower kinetics of the surface potential changes, could indicate that CAT_{12} measures the dissociation of an amino acid residue in the protein which occurs after the reprotonation of the retinal Schiff base and constitutes a step in the movement of protons across the protein.

The liposome data indicate that the side of the bacteriorhodopsin that takes up protons from the medium becomes more

positively charged during steady-state illumination. It could be that during steady-state illumination an accumulation of protons takes place at this surface. This observation is in contrast with results obtained with thylakoid membranes (18), which also take up protons, but drive electrons to their outer surface during steady-state illumination. Whatever the origin of the increase in positive charge density on this surface of the bacteriorhodopsin molecule, our results suggest that the number of negative charges appearing from the bacteriorhodopsin side which releases protons has to be greater than 1 per M_{412}.

Our Nigericin and Valinomycin data in the liposome studies show that the changes in the outer surface potential are inversely correlated with the pH gradients that are established during steady-state illumination. Lower pH gradients may permit a greater accumulation of protons on the outer surface of reconstituted bacteriorhodopsin molecules during steady-state illumination whereas faster rates of proton uptake may prevent their accumulation.

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