RAPID LIGHT-INDUCED SURFACE CHARGE CHANGES IN BACTERIORHODOPSIN

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1. Introduction

Purple membranes from *Halobacterium halobium* contain bacteriorhodopsin which is a light-driven electrogenic proton pump [1]. After light excitation the chromophore which is retinal-bound through a Schiff base to a lysyl residue in the opsin, forms several photointermediates (designated K, L, M, O) returning to the ground state at the end of the cycle. The major photochemical and biochemical events involve isomerization of the retinal from all-*trans* to 13-*cis* [2] and dissociation of a proton from the retinal Schiff base [3]. The electrogenic transfer of protons should also involve charge separation within and across the protein. Measurements of photoelectric transient response in oriented purple membranes were assumed to reflect these changes. The experimental systems included either purple membranes or bacteriorhodopsin liposomes attached to lipid-impregnated filters, lipid bilayer, teflon films or multilayers of purple membranes [4–7].

We have used molecular probes to monitor surface potential changes in purple membranes [8,9]. Spin probes were used to measure the photostationary and pulse-induced surface potential charges. However, because of the relatively low sensitivity and the slow response of EPR, the measurements were performed only under experimental conditions which induced a slowdown in the photocycle.

Here, we have developed a highly sensitive optical method having a fast time resolution. The method involves measurements of absorption changes in a pH indicator adsorbed to the purple membranes suspended in a highly buffered solution. It was shown [10] that fixed charges alter the apparent pK_a of adsorbed indicators. Measurements of the kinetics of surface charge changes were based on experiments [11–15] where the rate of protonation and deprotonation of bound pH indicators, as measured by a laser-induced pH jump technique were shown to be dependent on the total surface charge according to Debye's equation [16] for diffusion controlled reactions. A fixed surface charge affects the proton concentration at the diffuse double layer according to Gouy and Chapman [17] at a rate constant demonstrated to be faster at the interface than in the bulk solution [12,13]. Therefore, surface charge changes in purple membranes were calculated from the observed pK_a shift in the adsorbed indicator. Excitation by a laser pulse induced a rapid transient negative charge (formation, app. $t_{1/2} < 0.5$ μs; decay, app. $t_{1/2}$ 0.2 ms) which was followed by a slower transient positive charge change. A stoichiometry of almost 2 charges/photointermediate M was calculated. The relevance of these charge changes to the mechanism of proton pumping is discussed.

Abbreviations: MES, 2-(*N*-morpholino) ethane sulfonic acid

2. Methods

Highly purified purple membranes were prepared from *Halobacterium halobium* [18]. Membranes containing 20 μM bacteriorhodopsin (E_{570} 63 000 $M^{-1} \cdot cm^{-1}$) were suspended in a stirred medium containing 0.2 M NaCl, 0.1 M MES (pH 5) and when indicated, 20 μM bromcresol green, at 25°C. Following light adaptation, absorption changes were measured in a flash photolysis apparatus having time resolution of 50 ns. He,Ne Spectra Physics model 247 laser served as a source for the measuring light (632 nm) which was monitored by an EMI 9659 QB photomultiplier connected to a Biomation model 8100 transient recorder which was interphased to a Nicolet 1170 averager. Alternatively, light from tungsten iodide

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lamp, which was passed through color filters, served as measuring beam. A Molectron model UV 14 N_2 laser (10 ns flash length, 4 mJ/flash at 337 nm) was used for actinic light flashes. The photomultiplier was screened from this light by a monochromator and a Schott interference filter.

3. Results

Surface charge changes were calculated from the light-induced absorption change in the pH indicator bromcresol green adsorbed to purple membranes. This system has 2 chromophores, the pH indicator, which is partitioned between the aqueous and the membranous phases, and bacteriorhodopsin. To resolve the absorption charges in bound indicator from charges in the other components the following criteria should be observed:

(i) The light-induced absorption changes in the bacteriorhodopsin should be subtracted from the combined changes of the chromophore and the indicator;
(ii) There should be minimal absorption of the actinic light by the indicator;
(iii) The absorption changes in the bound and aqueous free indicator should be distinguished;
(iv) The concentration of the bound indicator should be determined.

A typical recording of the transient absorption change in bacteriorhodopsin at 632 nm induced by a 10 ns laser pulse is shown in fig.1a. A larger change is seen when bromcresol green is added to the same purple membrane suspension (fig.1B). Subtraction of the changes obtained with bacteriorhodopsin alone from the change in the combined system yields the absorption change of the indicator. This absorption change is not due to direct absorption of light by the indicator since no change was observed when light pulses were shone on bromcresol green solution devoid of the purple membranes. The change is likely to be due to interaction of protons with the membrane-bound indicator since the presence of 100 mM MES buffer decreases beyond detectability pH changes in the aqueous phase. If not buffered adequately the indicator in the aqueous phase is also expected to undergo absorption changes due to the transient proton release induced by light pulses [19]. Indeed larger absorption changes were observed when these measurements were done in a weakly buffered solution (not shown).

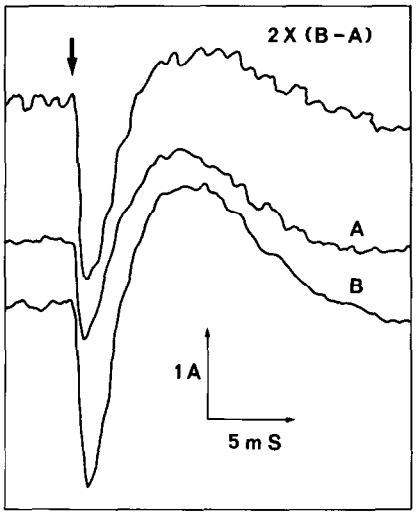

Fig.1. Light-induced absorption changes in bromcresol green adsorbed to purple membranes. Following 10 ns laser flash indicated by an arrow, the absorption changes in purple membrane suspension were monitored at 632 nm: (A) time course of the absorption changes in bacteriorhodopsin; (B) bacteriorhodopsin and bromcresol green; 2 × (B − A), two-fold expansion of the net changes in bromcresol green obtained from subtraction of (A) from (B). The recordings are results of accumulation of 4096 signals at 10 Hz intervals, recorded in a suspension containing 20 μM bacteriorhodopsin, 20 μM bromcresol green, 200 mM NaCl and 100 mM MES (pH 5) at 25°C.

The light-induced absorption changes could be resolved into 4 major kinetically distinguishable components.

(i) A decrease in absorption which followed a first-order kinetics having a $t_{1/2} < 0.5$ μs (fig.2). The recording of this change was done with a time resolution which was only slightly faster than the observed change. It is possible therefore, that the decrease in absorption was faster than what was measured;
(ii) A decay of this change with a $t_{1/2}$ 0.2 ms;
(iii) An increase in absorption ($t_{1/2}$ = 1 ms);
(iv) A decay to the ground state with $t_{1/2}$ = 2.5 ms.

At 632 nm, a decrease in absorbance of bromcresol green is caused by increase in the proton concentration while an increase in absorbance is caused by alkalization. Such changes in the bound indicator were assumed to be caused by a light-induced charge changes in the purple membrane which cause an app. pK_a shift in the indicator. Based on this assumption, ΔpK_a was calculated by:

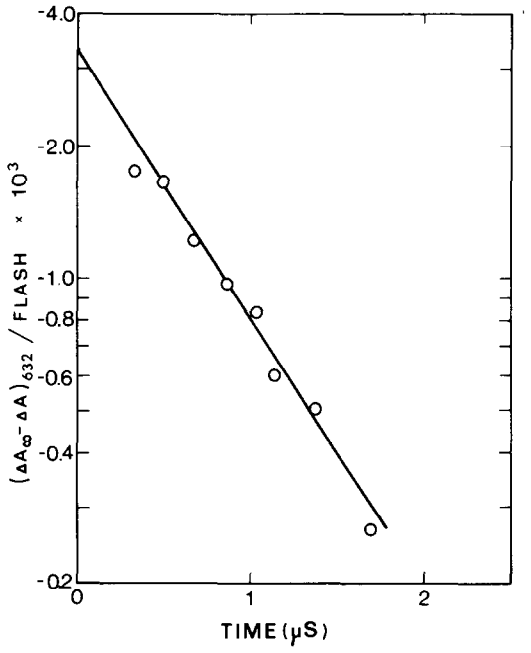

Fig.2. A semilogarithmic plot of the light-induced decrease in absorption of bromcresol green adsorbed to purple membranes. Experimental conditions were as in fig.1, except for the transient recorder which monitored at 0.1 μs/address.

$$\Delta pK_a = pK_a - pK_a'$$

$$pK_a' = pH - \log\left(\frac{HA + \Delta HA}{^-A - \Delta HA}\right)$$

where pK_a and pK_a' are the pK_a of the bound indicator in the dark and in the light-induced states, respectively; HA and ^-A are the concentrations of the protonated and of the dissociated forms of the pH indicator; and ΔHA is the light-induced change in the concentration of the protonated indicator. The concentration of the bound indicator was calculated from the dissociation constant of the binding of the indicator to purple membranes. Binding was measured by mixing a known concentration of the indicator with purple membranes. Following incubation, the membranes were precipitated by centrifugation. The concentration of bound indicator was calculated from the difference in the concentration of the indicator before and after the removal of purple membranes. Under the experimental conditions used in the experiments, an app. K_a = 30 μM was determined.

Surface potential changes ($\Delta\psi_s$) were calculated according to a known relation [20]:

$$\Delta\psi_s = \Delta pK_a \times 60\ mV$$

From the calculated light-induced $\Delta\psi_s$ and the ψ_s measured in [9] for a given ionic strength the surface charge density σ (in electronic charges/Å^2) were calculated according to the Gouy–Chapman equation [17]:

$$\sinh\frac{ZF\psi_s}{2RT} = 136\frac{\sigma}{\sqrt{c}}$$

where, ψ_s (in mV) is related to the ionic concentration (c molar) and to σ. The change in charge was related to the concentration of the photointermediate, M, measured from its absorbance change at 632 nm [21]. In separate experiments, the concentration of M measured at 632 nm was found to be in agreement with measurements done at 412 nm under identical experimental conditions. A formation of ~2 negative charges/M was followed by the formation of ~1 positive charge/M (table 1).

Table 1
Calculation of light-induced surface charge changes in purple membranes

Charge polarity	Bacteriorhodopsin		Bromcresol green			
	ΔA_{632}	M_{412}	ΔA_{632}	$\Delta\psi_s$	Charge	Charge
	Flash	BR*	Flash	Flash	BR	M_{412}
Negative	5.02×10^{-3}	25.1×10^{-3}	2.55×10^{-3}	−0.315	44.3×10^{-3}	1.77
Positive	5.02×10^{-3}	25.1×10^{-3}	1.14×10^{-3}	+0.14	19.6×10^{-3}	0.78

* *Abbreviations:* BR, bacteriorhodopsin; ψ_s, surface charge

Data were calculated from the experiments in fig.1

Absorption changes could also be a result of a change in the hydrophobic interaction between the membrane and the bound indicator. However, although hydrophobic interaction is probably the major force which induced the adsorption of this reagent to the purple membranes, no change in the spectrum or the extinction coefficient of the bound indicator was observed. Therefore, a light-induced change in hydrophobicity that might alter the environment of the adsorbed reagent and its partition between the membrane and the aqueous phases does not seem likely to cause absorption changes.

There is no change in the quantum yield for activation of the photocycle throughout the absorption spectrum of the bacteriorhodopsin [2]. Therefore, it was advantageous to activate the pump by laser pulse at 337 nm where the output was maximal while the ratio between the extinction coefficients of the bacteriorhodopsin and bromcresol green at pH 5 was the same as the ratio at the peak of the visible absorption band of the bacteriorhodopsin at 570 nm (fig.3). Indeed, the light-induced absorption changes in bromcresol green increased as a function of the increase in the concentrations of both the indicator between the range of 0–20 μM (fig.4) and of the bacteriorhodopsin.

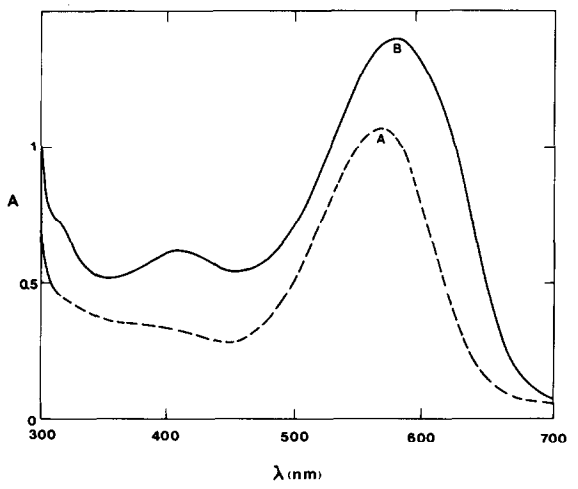

Fig.3. Spectra of bromcresol green and bacteriorhodopsin. Absorption spectra of (A) 17 μM bacteriorhodopsin and (B) bacteriorhodopsin + 20 μM bromcresol green were measured in a medium containing 100 mM MES (pH 5) and 200 mM NaCl.

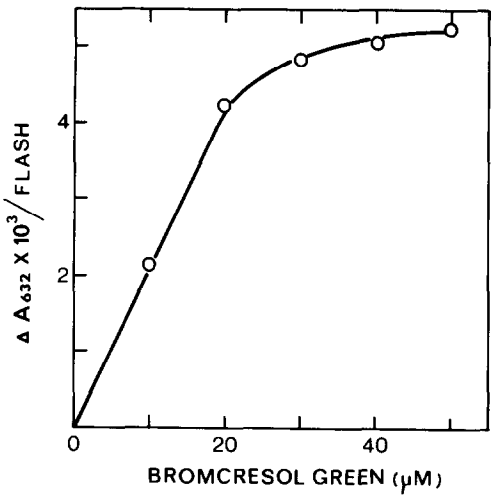

Fig.4. Concentration dependence of the light-induced absorption changes in bound bromcresol green. The light-induced ΔA_{632} in the bound indicator were plotted as function of total concentration of bromcresol green. Other experimental conditions were as in fig.1.

4. Discussion

A negative surface charge should be formed in an electrogenic proton pump where the release of protons is faster than its reassocation [19]. However, the light-induced formation of a negative charge in bacteriorhodopsin was >1000-fold faster than the release of protons. What was measured here could have been charge separation which preceded proton release. A formation of negative charge could result from breakage of a salt bridge between the protonated retinal Schiff base and a fixed counter anionic residue in the protein [22]. The discplacement of the protonated Schiff base could be due to photoisomerization of the retinal form all-*trans* to 13-*cis* configuration. The photoisomerization of the retinal may occur at the ps time scale resulting in the formation of the K photointermediate [22]. However, the full displacement of the Schiff base in the protein is less rapid. Therefore, the negative charge measured here at $t_{1/2} < 0.5$ μs may represent the full displacement of the Schiff base. Since the formation of the negative charge is slightly faster than the K → L transition ($t_{1/2} = 1$ μs [1]) this negative charge may be one of the reactions giving rise to the L photointermediate. In [9] the light-induced formation of the negative potential could not be resolved because of limitation of measurements with spin probes.

The major kinetic component in the decay of the negative charge ($t_{1/2}$ = 0.2 ms) is slightly slower than the rise of M photointermediate [1]. It is possible that the neutralization of the negative charge follows the dissociation of proton from the retinal Schiff base. These results agree with the kinetic results in [9] where the decay of the light-induced negative surface potential measured with spin probe was also found to be slower than the decay of M. The positive surface charge seen in these experiments was not detected in the earlier experiments probably because the changes were not large enough to be detected by the spin probe technique. The kinetics of the formation and the decay of the positive charge changes and that of the last intermediate in the photocycle are similar [1]. They possibly measure the kinetics of rebinding of protons to the protein which is followed by neutralization of charge as the pump returns to ground state.

The surface charge changes measured with the molecular probe are qualitatively similar to measurements of capacitative photoresponse observed with purple membranes and with bacteriorhodopsin liposomes attached to planar membranes. There, both negative and positive transient photocurrents were recorded. In systems where light activation was induced by a short laser pulse, the 2 opposite transient photocurrents could be resolved into 4 components [4–7] having kinetic constants in the range observed in the experiments conducted here with the pH indicator. From studies where the orientation of the purple membranes was determined it was suggested [6] that the fast transient photocurrent is due to a migration of a positive charge towards the cytoplasmic side of the bacteriorhodopsin.

These experiments were done with a purple membrane suspension. Therefore the side at which the charge appeared could not be determined. However, the same probe is being utilized presently with cell envelopes and with bacteriorhodopsin incorporated into liposomes. It is possible that the polarity of the charge could be determined since the pump is oriented in opposite directions in these two systems.

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