SALT REVERSAL OF THE ACID-INDUCED CHANGES IN PURPLE MEMBRANE FROM *HALOBACTERIUM HALOBIUM*

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1. Introduction

The purple membrane of the extreme halophile *Halobacterium halobium* contains only one protein, bacteriorhodopsin, which has a single retinal moiety bound to the protein via a Schiff base linkage to a lysine residue [1,2]. Purple membrane has a broad absorption band centred at 558 nm in the dark and 568 nm in the light [3]. Bacteriorhodopsin acts as a light-driven proton pump thought to drive ATP synthesis in a chemiosmotic manner [3,4]. When illuminated the protein goes through a photochemical cycle that is accompanied by a reversible deprotonation of the Schiff base–protein linkage [5–8]; the phototransients have been labelled *K, L, M, N, O* and *bR* [5], named alphabetically in the order in which they are believed to appear after absorption of light.

In [9] we showed that a new spectral species of bacteriorhodopsin is formed at acid pH values having an $A_{\max}$ ~610 nm, and we suggested that acid conditions may stabilise phototransient *O*. We discuss here the effects of salts on the acid species of purple membrane and provide evidence that the acid species is formed in response to changes in membrane structure.

2. Materials and methods

Growth of *H. halobium* strain R1 and isolation of the purple membrane were performed as in [9,10]. SDS–gel electrophoresis and amino acid analysis were carried out as before to check purity and concentration. For all static spectral experiments, purple membrane was used at ~2 μM protein in glass distilled water to minimise buffering effects. Acid titrations were carried out as in [9]. pH 3.08 was chosen for the investigation of the effects of salts on the acid spectrum as, at 22°C, the acid spectrum is about 75% developed.

Salts were added to the acidified membrane in 0.5–100 μl aliquots from stock salt solutions whose concentrations were checked by a chloride assay using silver nitrate and Mohr's indicator. Lithium chloride was 'laboratory reagent' grade, lanthanum chloride was 'spectroscopic reagent' grade and aluminium chloride was 'general purpose' grade; all other chemicals were 'analytical reagent' grade.

Flash photolysis experiments were conducted with the instrumentation and methods in [11].

3. Results

3.1. *Addition of salts to acidified purple membrane at 22°C*

Addition of salts to purple membrane acidified to pH 3.08 resulted in a loss of the A_{610} peak together with the appearance of an A_{560} peak (see fig.1), the process having a single isosbestic point at 585 nm. The extent of the spectral change produced can be described by defining a pseudo-equilibrium constant:

$$K_{\mathrm{eq}} = \frac{A_x}{(1 - A_x)X}$$

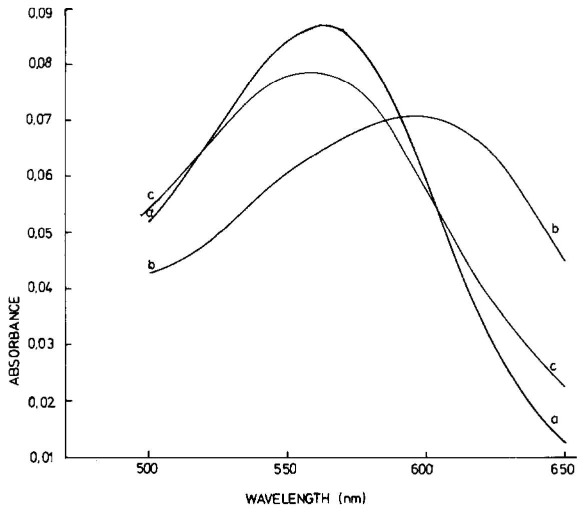

Fig.1. Absorbance of purple membrane in distilled water, acid, and acid and salt. Purple membrane was 1.82 μM at 22°C in: (a) distilled water; (b) in HCl at pH 3.08; (c) in HCl (pH 3.08) and 66 μM $CaCl_2$.

where A_x is the fraction of the total spectral change at 610 nm due to the salt species and X is the concentration of cation present (assuming complete ionization). In carrying out these experiments, it was found that this defined K_{eq} varied with purple membrane preparations used; however, the overall spectral changes were constant. Ranges for these K_{eq} are given in table 1. Experiments with monovalent, divalent and trivalent chloride salts gave the effect described above; however, a pattern developed in the progression to trivalent ions. K_{eq} increased by almost 2 orders of magnitude each time the positive charge was increased by one when using the group I cations Li^+, Na^+, K^+, Rb^+ and Cs^+, and the larger monovalent cations ammonium (NH_4^+) and choline ($N(CH_3)_3H^+$); the group IIA cations Ba^{2+}, Ca^{2+} and Mg^{2+}; the group IIIA cation Al^{3+}; and the trivalent cation La^{3+} from the lanthanide series. Although the cations in each particular charge class vary quite extensively in physical and chemical properties, no significant variation was found among their K_{eq} within the class.

Table 1

Variation of pseudo K_{eq} with salt

Salt	K_{eq} (mM^{-1})
Monovalent salts	
LiCl, KCl, KNO_3, NaCl, CsCl, RbCl, NH_4Cl, $NH(CH_3)Cl$	0.056 – 0.189
Divalent salts	
$CaCl_2$, $MgCl_2$	11.1 – 13.1
Trivalent salts	
$LaCl_3$, $AlCl_3$	400 – 555

Bacteriorhodopsin was 1.68 μM/at 22°C, pH 3.08, and K_{eq} is defined in section 3.1

For comparison, potassium nitrate was also used, and although not completely identical to potassium chloride in strength, it also fell into the range expected for monovalent salts. The concentration of the nitrate solution was not known as exactly as those of the chloride salts, as they were determined by a chloride assay.

Although the decrease in A_{610} is linear up to 70% of the change seen, there is no clear indication of binding to specific sites on the protein as double reciprocal plots of the data are not linear. Extrapolation of the linear part of the curve in fig.2 would suggest ~90 divalent ions binding/protein molecule, which is an improbable situation.

3.2. *Titrations of purple membrane with acid in the presence of calcium chloride*

In order to more clearly understand the cause of

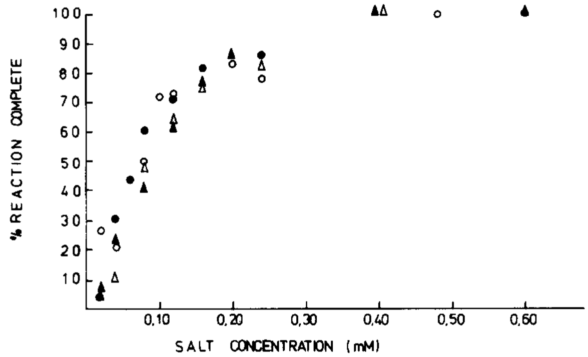

Fig.2. Binding curve for magnesium chloride and calcium chloride to purple membrane at acid pH. Purple membrane was 1.82 μM (pH 3.08) at 22°C. Measurements were made at 610 nm, with the open circles (○), closed circles (●) and closed triangles (▲) representing results from separate experiments using $CaCl_2$, and the open triangles (△) representing results using $MgCl_2$.

the salt effect, acid titrations were carried out on 1.68 μM suspensions of purple membrane in 66 μM $CaCl_2$ at 22°C. In view of the problems encountered with 'salting out' of purple membrane at high salt concentrations and contamination at the very low salt concentrations, 66 μM $CaCl_2$ was chosen as the most feasible for the work.

The acid titration followed the same pattern as that in the absence of salt, having at least two clear phases of spectral change, the first resulting in a decrease and broadening of the peak at 565 nm with a single isosbestic point at 597 nm, and a second, at lower pH values, giving the final peak at 610 nm seen for the acid form of purple membrane. However, in the presence of $CaCl_2$, considerably more acid was required to achieve the same spectral change, and the pK of the second phase was shifted from 3.65 to 2.80.

3.3. *Effect of temperature on purple membrane/acid/salt mixtures*

At 8.5°C, acidified purple membrane suspensions (pH 3.08) in 66 μM $CaCl_2$ showed a single A_{560} peak. On raising the temperature, two phases of spectral change were seen, the first change resulting in a lowering and broadening of the 560 nm peak with a single isosbestic point at 648 nm and the second change resulting in a further decrease of A_{560} and the formation of a new peak at ~610 nm with an isosbestic point at 573 nm. This biphasic pattern is similar to that seen in the absence of salt, excepting a shift of the initial isosbestic point from 597 to 648 nm. However, the temperature required to achieve 50% of the absorption change seen during the second phase has increased from 23°C in the absence of salt for the acidified purple membrane to 39°C when in a 66 μM $CaCl_2$ solution. These temperature changes are reversible, and the thermodynamical quantities associated with the second phase reveal no effect of salt. Table 2 summarises these observations.

Table 2
Summary of parameters measured from the variation of acidic purple membrane with temperature

	Presence of salt	Absence of salt
1. Temperature at which phase II is 50% complete	39°C	23°C
2. $\Delta H°$ (kJ. mol^{-1})	180.0 ± 4.6	188.2 ± 67
3. $\Delta S°$ (kJ. mol^{-1} . K^{-1})	0.577 ± 0.015	0.636 ± 0.022
4. Isosbestic point		
(a) phase 1	648 nm	610 nm
(b) phase 2	573 nm	573 nm

Both experiments were carried out at pH 3.08 in a 1 cm path-length cell at 1.68 μM bacteriorhodopsin. The experiment in the presence of salt had $CaCl_2$ added to final 66 μM conc. ΔA_{610} was measured because it is large during phase 2 and has negligible contribution from phase 1

3.4. *Flash photolysis experiments in acid/salts*

Because the low pH values have such a marked effect on phototransient O [9], flash photolysis experiments were carried out on purple membrane at low pH values in the presence of certain monovalent salts. These previous results showed that phototransient O is favoured at first, as the pH is lowered, concurrent with the first spectral phase seen statically. Over this pH range, biphasic kinetics of opposite sign are observed at 640 nm, the wavelength at which phototransient O absorbs maximally, and it is the initial increase in absorbance which is associated with formation of O while the slow decreasing phase is its decay. The amount of phototransient O seen with the flash reaches a peak as the first spectral phase is completed. The rate of formation of O reaches a minimum and the rate of decay is slowed. As the pH is further lowered and the static spectral titration reaches its second phase, the amount of phototransient O begins to decrease. Its rate of formation now increases, the data indicating single protonation, and the rate of decay continues to decrease. These phenomena are associated with the static equilibration of O.

In the present experiments, flash kinetics were observed at 640 nm in the presence of acid (pH 3.50) and a range of monovalent salts. The results, given in table 3 show that in the presence of salt, the rates of formation and decay are increased; also the amount of phototransient O is greater.

4. Conclusion

We have shown [9] that a new spectral species of purple membrane is produced at low pH values.

Table 3
Flash kinetics data

Salt	ΔA	k_1 (ms^{-1})	k_2 (ms^{-1})
Control (no salt)	0.184	0.145	0.041
LiCl	0.335	0.155	0.063
KCl	0.329	0.150	0.060
NaCl	0.221	0.143	0.053
CsCl	0.278	0.195	0.053

Experiments were carried out at pH 3.50 and 22°C with purple membrane at ~2.6 μM. Salt was 80 mM. ΔA represents the amount of phototransient *O* observed; k_1 is the rate of formation and k_2 is the rate of decay

It was noted that acid conditions favoured the formation of phototransient *O* in flash photolysis, and we suggested that the final acid species obtained statically was phototransient *O*. Its accumulation was then assumed to be due to acid-induced changes in the purple membrane making it possible for the bacteriorhodopsin to cycle through *O* as an alternative pathway for reprotonation in returning to its resting state, *bR* [5].

The present results show a loss of the acid peak in the presence of salts. Because the effect differed quite dramatically with the charge of the cation present at constant chloride concentration, the phenomenon was assumed to be mainly, if not exclusively, due to the cation.

In view of our earlier spectrokinetic observations which revealed that single protonation of bacteriorhodopsin occurs at the pH used in the present experiments, competitive binding by cations to the protein was considered. However, a binding curve for $CaCl_2$ gave a result indicating ~90 binding sites/bacteriorhodopsin molecule which seems very unreasonable. The observed non-linearity in double reciprocal plots indicates that there was no independent reversible binding of cations. Our data excludes the possibility of binding to a large number of sites as the uniformity of spectral effect of ions within the same charge class is not consistent with the expected steric hindrance to the larger ions. This is particularly true of the comparison between Al^{3+} and La^{3+}, where Al^{3+} is known to form large complexes with water involving 13 Al^{3+} ions [12].

It is more likely that these salts adhere to the surface of the membrane very tightly, perhaps in the form of a Stern double layer [14] and cause the spectral changes from which a binding curve can be plotted showing many apparent binding sites. The discovery of negative phospholipids in purple membrane [13] would suggest that the salts are modifying the membrane structure by forming positive layers at the surface.

Flash photolysis results also indicate a reversal of the acid state of the membrane in which high equilibrium levels of phototransient *O* are achieved. It appears from the data that the purple membrane at pH 3.50 and 22°C is into the second spectral phase seen statically. Therefore, the increase in the amount of phototransient *O* and the faster rates of formation and decay in the presence of salt indicate that the state of the membrane is shifted towards that which would be observed at a slightly higher pH in the absence of salt.

Our findings that cations affect the temperature dependence of the acid spectrum can be compared with reports that cation layers alter phase transition phenomena in membranes. Studies of drugs binding to membrane surfaces show that when a positively-charged drug binds, the phase transition temperature of the membrane is lowered. However, if other types of positively-charged ions are added they may displace the bound drug and reverse this transition temperature depression [14,15]. Our present data follows this pattern with hydrogen ions lowering the apparent phase transition temperature from 32 to 23°C [9] and the cations used here reversing that change. In light of the recent controversy over the existence of a true phase transition at 32°C in purple membrane [16], we might add that there are spectral changes seen in purple membrane with temperature [9], indicating that there is a change in the state of the membrane and the midpoint of this change is at 32°C. If not a true phase transition, then this temperature-induced change seems to be affected by charge in the same manner as a phase transition. Discontinuities in lipid microviscosity profiles of purple membrane at about 30°C have been suggested [17].

We conclude that acid causes changes in the purple membrane which alter the light cycle of bacteriorhodopsin, and cation layers at the membrane surface displace protons and reverse that effect. Because of the high ionic strength of its natural environment, this study has relevance to the reaction cycle of bacteriorhodopsin in vivo.

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