Studies of an Acid-Induced Species of Purple Membrane from *Halobacterium Halobium*

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A new spectral species of the purple membrane of *Halobacterium halobium* has been observed below pH 3.2. The formation of this new species is temperature-dependent and is favoured by increasing temperatures up to the physiological range of the organism. The rate of formation at pH 3.0 and 22°C is $7.9 \times 10^{-3} \text{ s}^{-1}$. The spectral distribution and temperature-dependence of the new species suggest that it may be phototransient *O*, stabilized by low pH. Flash-photolytic experiments in the pH range 7.2–2.7 show a pH-dependence corresponding to the static events and are consistent with a single protonation of bacteriorhodopsin below pH 3.22. These results can also be interpreted in terms of the stabilization of phototransient *O* at low pH. The temperature-dependence of the formation of the acid-induced species may reflect a relationship with the phase transition of the membrane.

The purple membrane of the extreme halophile *Halobacterium halobium* acts as a light-driven proton pump (Oesterhelt & Stoeckenius, 1973; Racker & Stoeckenius, 1974), which, when illuminated, brings about a vectorial movement of protons across the cell membrane, resulting in a pH gradient across the membrane (Oesterhelt & Stoeckenius, 1973). Purple membrane contains a single chemical species of protein, bacteriorhodopsin, which constitutes 75% of the dry weight of the membrane and has a single retinal moiety per protein molecule bound via a Schiff-base linkage to a lysine residue (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976). The absorption maximum of the retinylidene-protein is shifted from 370 nm, characteristic of a retinylidene-Schiff base, to a broad absorption band centred at 558 nm in the dark and 568 nm in the light (Oesterhelt *et al.*, 1973), which is indicative of further protein-retinal interactions.

When illuminated, the protein goes through a photochemical cycle that is accompanied by a reversible deprotonation of the Schiff-base linkage (Lozier *et al.*, 1975; Kaufman *et al.*, 1976; Sherman *et al.*, 1976b; Lozier *et al.*, 1976). The phototransients have been labelled *K*, *L*, *M*, *N*, *O* and *bR* by Lozier *et al.* (1975), named alphabetically in the order in which they are believed to appear after absorption of light by bacteriorhodopsin. The spectral characteristics have been discussed by Lozier *et al.* (1975) and Chu Kung *et al.* (1975).

By suspending purple membrane in concentrated

salt solutions saturated with diethyl ether, Oesterhelt & Hess (1973) have shown that the phototransient *M* formed after bleaching with light can be stabilized, and a similar result has been obtained by Yoshida *et al.* (1977) with guanidine hydrochloride. The present paper describes the effect of acid pH on purple membrane and shows that the properties of the spectral species produced resemble those of phototransient *O*.

Materials and Methods

Halobacterium halobium, strain R1, was grown and the purple membrane extracted and purified as described by Oesterhelt & Stoeckenius (1974). The bacteria were grown in 10-litre batches and illuminated by eight 150 W photoflood bulbs. The concentration and purity of the bacteriorhodopsin were measured by amino acid analysis and the purity was further checked by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The purple membrane used for experiments was in the light-adapted state.

Amino acid analysis was carried out on membrane samples that were hydrolysed in sealed evacuated tubes at 105°C in 5.7 M-HCl and 4.73 mM-2-mercaptoethanol for 24 h. The hydrolysate was separated and analysed with a Rank Hilger J180 mark 1 Chromaspek amino acid analyser and a Digico Micro 16V computer with teletype. We used a linear gradient method of elution and a cycle time of 70 min, essentially according to the manufacturers' instructions.

The protein concentrations of samples were calculated by using the molecular composition values, of lysine (7), arginine (7), aspartic acid (15), glutamic acid (16), valine (20) and phenylalanine (13), as published by Bridgen & Walker (1976) for a mol.wt. of 25000.

For the acid titrations, purple membrane was suspended in glass-distilled water at a protein concentration of $2\text{--}3\text{ }\mu\text{M}$ and the initial pH measured with a type GK 2312C Radiometer (Copenhagen, Denmark) concentric electrode and a type 26 Radiometer pH-meter. The protein concentration was

chosen deliberately to minimize buffering effects. Depending on the size of pH change required, small samples of 0.1M- or 2.0M-HCl were added to the purple-membrane preparation with a Hamilton syringe, the final pH values being calculated in every case.

All spectra and single-wavelength observations were recorded by using a Cary 118c spectrophotometer at the required temperature in a cell of 1 cm path length.

Flash-photolysis experiments were conducted with the instrumentation and methods described by Gib-

Fig. 1. Acid titrations of purple membrane in water at different temperatures

Small samples of 0.1 M-HCl were added to purple-membrane suspensions ($2.73\text{ }\mu\text{M}$ -protein) at the appropriate temperature, and the spectrum was recorded after equilibrium had been reached. Only a few of the spectra are shown. Path length of spectrophotometer cell was 1 cm. (a) Temperature = 5°C ; curve (i) pH 6.80; (ii) pH 3.88; (iii), pH 3.70; (iv), pH 3.58; (v), pH 3.48; (vi), pH 3.02. (b) Temperature = 15°C ; curve (i), pH 6.80; (ii), pH 4.18; (iii), pH 3.88; (iv), pH 3.70; (v), pH 3.58; (vi), pH 3.48; (vii), pH 3.40; (viii), pH 3.30; (ix), pH 3.00. (c) Temperature = 25°C ; curve (i), pH 6.80; (ii), pH 4.18; (iii), pH 3.88; (iv), pH 3.70; (v), pH 3.58; (vi), pH 3.48; (vii), pH 3.00. (d) Temperature = 35°C ; curve (i), pH 6.80; (ii), pH 4.48; (iii), pH 4.18; (iv), pH 3.88; (v), pH 3.70; (vi), pH 3.48.

son & Greenwood (1965). A Bausch and Lomb monochromator (500mm grating, 1200 grooves/min, $f=4.4$) was used in conjunction with the photolysis apparatus, which delivered a flash of white light of 200J having a duration of 300 μ s. The glass cell holding the sample had a 4cm light-path and was fitted with quartz windows and surrounded by a water jacket. Purple membrane was used at a protein concentration of 7–8 μ M. All experiments were carried out at a temperature of 15°C.

Volumetric HCl solutions were bought from British Drug Houses, Poole, Dorset, U.K.; all other chemicals were A.R. grade. Glass-distilled water was used throughout.

Results

Acid titration produced a distinct spectral species of purple membrane, the spectrum of which varies with temperature (see Fig. 1). During the course of an acid titration, the appearance of the species absorbing maximally at 600–610nm was preceded by a decrease in the absorption at 560nm and showed a single isosbestic point, which, however, varied between 592 and 608nm depending on the purple-membrane preparation used. The apparent pK of the transition was wavelength- and temperature-dependent, and the entire process was found to be reversible by observing a regeneration of the original spectrum when the pH was restored to 6.80 with NaOH.

Table 1 shows the change measured in the spectrophotometer at various wavelengths after the addition

of acid to purple-membrane preparations. Exposure to bright sunlight after removal from the spectrophotometer did not change the final spectrum. It is obvious that the process observed in the spectrophotometer is very slow; however, this does not represent the total absorption change on going from pH 6.80 to 3.00. The absorption change caused by the slow reaction represents approximately one-third of the expected change over the spectral region explored, the remaining change being attributable to one or more fast phases. The rate of the slow process appeared to be independent of wavelength, with a value of $7.93 \times 10^{-3} \pm 1.62 \times 10^{-3} \text{ s}^{-1}$ (at P 0.05) or $7.93 \times 10^{-3} \pm 0.75 \times 10^{-3} \text{ s}^{-1}$ if only those regions of the spectrum are considered where the change in absorption was large (i.e. 610–660nm inclusive). Fig. 2 shows the overall difference spectrum after a transition from pH 6.80 to 3.00 and compares it with the contributions made by the fast and slow processes. Fig. 3 shows that the difference spectra recorded over the pH range 6.80–3.60 show a unique isosbestic point at 593nm that corresponds to that observed for the rapid phase in Fig. 2. The difference spectrum (v) in Fig. 3 corresponds to the overall difference spectrum seen in Fig. 2, but it is evident from the lack of an isosbestic point for spectra (iv) and (v) in Fig. 3 that the final stage in the pH titration cannot be described by a simple two-state transition. It seems reasonable to equate this second process with the slow change seen in Fig. 2, although the fast and slow processes both contribute to the increase in A_{640} .

Flash-photolysis experiments at 640nm showed

Table 1. *Kinetics of the slow absorbance change after adding acid to purple membrane*

To 3ml of purple membrane (initial concentration of protein, 2.43 μ M) in water at 22°C, 30 μ l of 0.1 M-HCl was added to give a final pH of 3.00. Spectra were recorded in a 1 cm-path-length cell. Change in absorbance from that at pH 6.80 is positive (+), negative (–) or no change (0).

λ (nm)	Percentage of spectral change caused by slow phase of reaction	Direction of change		$10^3 \times$ Pseudo-first-order rate constant (s^{-1})
		(a) Slow reaction	(b) Fast reaction(s)	
520	35.3	–	–	7.83
530	33.3	–	–	6.89
540	34.3	–	–	7.05
550	34.6	–	–	9.49
560	34.6	–	–	9.49
570	2	–	–	
580	0	0	–	
590	3	+	–	
600	59.3	+	+	8.17
610	46.0	+	+	7.21
620	42.5	+	+	7.27
630	35.0	+	+	7.84
640	33.0	+	+	7.84
650	33.0	+	+	7.75
660	29.1	+	+	8.16
670	24.6	+	+	8.07
690	0	0	+

Fig. 2. Difference spectrum between purple-membrane suspensions at pH 6.80 and 3.00

—, Full measured difference spectrum; ○, measured contribution of the slow phase of reaction; Δ, calculated contribution of the fast phase(s) of reaction. Concentration of protein in purple-membrane suspensions was $2.43 \mu\text{M}$; temperature was 22°C ; path length of spectrophotometer cell was 1 cm.

Fig. 3. Difference spectra between purple-membrane suspensions at pH 6.80 and other pH values

Curve (i), pH 4.08; (ii), pH 3.78; (iii), pH 3.60; (iv), pH 3.23; (v), pH 3.04. Concentration of protein in purple-membrane suspension was $2.56 \mu\text{M}$; temperature was 22°C ; path length of spectrophotometer cell was 1 cm.

biphasic kinetics of opposite sign in the pH range 5.25–2.90, and of the same sign below pH 2.90 (see Fig. 4, which shows a family of typical reaction profiles observed as a function of pH). Analysis of the fast and slow components of these changes reveals that both phases appear to be pH-dependent in rate and extent, although it is more difficult to characterize the slow phase because of its absence at the upper and lower bounds of the pH range (see Fig. 5).

Fig. 4. Flash-photolysis records

A family of typical reaction curves is shown, representing changes in A_{640} for purple membrane in water and HCl at (i) pH 5.25, (ii) pH 3.22, (iii) pH 2.92, (iv) pH 2.70, after a brief but intense photolytic flash. All experiments were carried out at 15°C .

At pH 6.80 the absorption spectrum of purple membrane was found to be temperature-dependent in a reversible manner with an isosbestic point at 529 nm, and, although the changes in A_{570} are small, it is clear that the data in Fig. 6(a) can be used to evaluate certain thermodynamic quantities. Fig. 6(b) shows an Arrhenius plot of these data, which yields a single standard-enthalpy change of $103 \text{ kJ} \cdot \text{mol}^{-1}$ and a standard-entropy change of $0.34 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. A similar experiment performed at pH 3.18 and 630 nm is presented in Fig. 7(a) together with an Arrhenius plot that yields a single standard-enthalpy change of $137 \text{ kJ} \cdot \text{mol}^{-1}$ and a standard-entropy change of $0.46 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. The thermodynamic constants for the data presented in Figs. 6 and 7 are summarized in Table 2. The point of maximal rate of change of absorbance with temperature has shifted from 32°C in Fig. 6(a) (570 nm; pH 6.80) to 23°C in Fig. 7(a) (630 nm; pH 3.18).

Discussion

Various transient species formed during the photochemical cycle in the purple membrane of *H. halobium* have been described in several laboratories. To study the properties of such intermediates in detail it is necessary physically to isolate these photoproducts or else generate them by some other means. One of the phototransients that has been observed by a number of workers (Lozier *et al.*, 1975; Slifkin & Caplan, 1975; Sherman *et al.*, 1976a,b) has an absorbance maximum at 640 nm and has been designated as phototransient O. By treating purple membrane with acid as described in the Results section of the present paper, we have induced changes in the system that result in the formation of

Fig. 5. Analysis of the flash-photolysis kinetics

The rates (k) and extents (ΔA) of the fast and slow phases of the flash-photolytic experiments recorded at 640 nm and 15°C, some reaction traces of which are shown in Fig. 4, are plotted against pH. The curves here represent (a) $\log k_{\text{fast}}$ against pH, (b) ΔA_{fast} against pH, (c) $\log k_{\text{slow}}$ against pH and (d) ΔA_{slow} against pH.

an apparently distinct spectral intermediate (see Figs. 1 and 3) whose properties resemble those of phototransient *O*.

Fig. 1 shows that the formation of the acid-induced intermediate is very dependent on temperature and further that this dependence is complex, as evidenced by the absence of a single isosbestic point at temperatures above 5°C. It is clear from Fig. 1 that raising the temperature appears to facilitate the production of the 640 nm-absorbing species, and this behaviour is consistent with the temperature-dependence of the phototransient *O* as described by Sherman *et al.* (1976a,b) and Lozier *et al.* (1975).

The solid-line spectrum (no symbols) in Fig. 2 recorded at room temperature represents the spectral difference between purple membrane at pH 3.00 minus that at pH 6.80 and may be compared with the difference spectrum for phototransient *O* minus phototransient *bR* published (Fig. 3, spectrum III) by Dencher & Wilms (1975). The difference in peak amplitude between the two difference spectra, that in the present paper and that of Dencher & Wilms (1975), can be explained on the basis of the temperature difference between the experiments, formation of phototransient *O* being favoured at high temperature. Inspection of Fig. 3 shows that there are several

Fig. 6. Effect of temperature on the spectrum of purple membrane at pH 6.80

Fig. 6(a) shows the variation of A_{570} with temperature, and Fig. 6(b) shows an Arrhenius plot of this experiment. The equilibrium constant was calculated from the initial and final values for the A_{570} (A_i and A_f) and that at the temperature under consideration (A_T), according to the equation $K_{eq} = (A_i - A_T)/(A_T - A_f)$. A_f was calculated by doubling the absorbance difference between A_i and A_T at the point where $d(A_T)/dT$ was maximal (T is temperature, K). Path length of spectrophotometer cell was 1 cm; concentration of protein in the purple-membrane suspension was $2.73 \mu M$.

Fig. 7. Effect of temperature on the spectrum of purple membrane at pH 3.18

Fig. 7(a) shows the variation of A_{630} with temperature, and Fig. 7(b) shows an Arrhenius plot of this experiment. K_{eq} and A_f were calculated in the same way as those in Fig. 6. Path length of the spectrophotometer cell was 1 cm; concentration of the protein in the purple-membrane suspensions was $2.73 \mu M$.

Table 2. *Thermodynamic quantities calculated from Figs. 6(b) and 7(b)*

Purple-membrane concentrations are $2.73 \mu\text{M}$ with respect to protein concn. Errors are calculated from the standard errors of the slope and intercept of a regression of x on y with the data in Figs. 6(b) and 7(b).

pH	Standard-enthalpy change ($\text{kJ} \cdot \text{mol}^{-1}$)	Standard-entropy change ($\text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)	Standard-free-energy change at 25°C ($\text{kJ} \cdot \text{mol}^{-1}$)
6.80	108.7 ± 4.1	0.35 ± 0.01	4.40
3.18	136.9 ± 3.1	0.46 ± 0.01	-0.48

processes involved in the acid-induced conversion. Studies of the kinetics of these events indicated that there is an initial fast change that may itself be complex and that we have not resolved: stopped-flow experiments could possibly elucidate the nature of this phase. The subsequent slow reaction has been analysed and the data are presented in Table 1, the rate of $7.9 \times 10^{-3} \text{s}^{-1}$ being independent of wavelength. This slow process is associated with the final shift of equilibrium to the new spectral species, whose properties suggest that it may be phototransient *O*. The contribution made by each phase to the final difference spectrum at pH 3.00 is shown in Fig. 2.

Since the cycle of intermediates involved in the possible mechanism of protonation and deprotonation has been largely developed on the basis of flash-photolytic experiments (Lozier *et al.*, 1975; Kaufman *et al.*, 1976; Sherman *et al.*, 1976b; Lozier *et al.*, 1976), it was decided to investigate the influence of pH on the photochemistry of phototransient *O*. Fig. 4, which represents a family of reaction curves recorded at 640 nm after a brief but intense photolytic flash, clearly shows that the flash kinetics are dependent on pH. Fig. 5 presents an analysis of the rate and amplitude of the traces observed in Fig. 4. As the pH is lowered to 3.22 the size of the observed overshoot at 640 nm increases to a maximum, and this could be explained as a favouring of the regeneration of phototransient *bR* via phototransient *O*. It is also noteworthy in Fig. 3 that, as indicated by the isosbestic point over this pH region, a simple transition is occurring. In the pH range below pH 3.22, the size of the overshoot decreases and the logarithm of the rate of the fastest process observed increases linearly with a slope of 1 when plotted against $\log[\text{H}^+]$. This indicates that a protonation process appears to be involved in the formation of phototransient *O*. If the species absorbing in the region of 640 nm that we see in the static pH titrations is phototransient *O*, the decrease in the size of overshoot below pH 3.22 could be explained by a stabilization of phototransient *O* in this pH range.

The evidence presented here is consistent with the formation of phototransient *O* as a result of protonation of bacteriorhodopsin, although not necessarily at the Schiff-base linkage of retinal to the protein. The nature of the pH-induced phenomena that are described in the present paper requires further investigation and it will be interesting to see to what extent these phenomena are related to the observed favouring of pathways involving phototransient *O* at the higher temperatures in the normal physiological range of *H. halobium*. The results presented in Figs. 6 and 7, showing the influence of temperature on the absorbance of the purple-membrane preparation at pH 6.80 and 3.18, suggest that the lower pH may facilitate the temperature-induced process. The point of maximum slope in Fig. 6(a) (pH 6.80) occurs at 32°C , whereas that of Fig. 7(a) (pH 3.18) occurs at 23°C . The nature of the process is not clear, but it is known (Sherman *et al.*, 1976b) that the membrane undergoes a phase transition at about 32°C .

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