Comparison of the O_{640} photo-intermediate and acid-induced species in membrane patches from *Halobacterium halobium* S_9 and R_1mW strains

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1. INTRODUCTION

Halobacterium halobium, an extreme halophile, has been found to contain bacteriorhodopsin (bR) in a 'purple membrane patch' that is easily isolated in a stable form [1]. It undergoes a photocycle upon illumination and several photo-intermediates have been elucidated by time-resolved resonance Raman, flash-photolysis, and low temperature spectroscopic techniques [2–4]. A commonly accepted scheme of the photocycle is:

$$BR_{568} \xrightarrow[ps]{} K_{590} \xrightarrow[ns]{} L_{550} \xrightarrow[\mu s]{} M_{412} \xrightarrow[ms]{} O_{640} \xrightarrow{ms} BR_{568}$$

where the subscripts denote absorbance maxima of intermediates revealed by difference spectroscopy. The photocycle has been thought to be directly related to proton pumping because the Schiff-base nitrogen between the retinal and the apoprotein undergo deprotonation–reprotonation accompanying formation and decay of the M_{412} intermediate [5]. The protonation states of tyrosine residues have also been postulated to undergo cyclic changes during photocycle activity [6–8].

A white mutant, R_1mW, of *H. halobium* has been discovered which upon retinal addition can reconstitute the functions of bacteriorhodopsin in intact cells [9]. A 'white membrane patch' has been isolated from these cells which, after reconstitution with stoichiometric amounts of retinal, forms chromophore with λ_{max} ~560 nm (dark adapted [9]). Due to the lack of any retinyloxime, formed by light/hydroxylamine treatment of bR, white membrane patches present an ideal system for the study of retinal–protein interactions. Therefore, it was of interest to compare some of the properties of native purple membranes with reconstituted white membrane patches.

Bacteriorhodopsin is known to undergo a transformation in acid pH to a species absorbing with a λ_{max} ~600 nm [10,11]. Difference spectra of the formation of this species show a positive maximum at 640 nm [11]. The formation of this acid species is favored by low ionic strength, low pH and high temperature [11,12]. The O_{640} intermediate of the photocycle has been proposed to be similar, if not identical, to the acid-induced species since it displays the same dependence of pH and temperature [11,12]. Stabilization of the acid-induced species decreases the efficiency of proton pumping in purple membrane-reconstituted liposomes; the acid-induced species might be the photocycle intermediate K [13].

Here we have examined the effects of low pH and temperature on the O_{640} intermediate and absorption characteristics of the retinal-reconstituted white membranes. The results show a good correlation between the acid-induced species and the O_{640} photointermediate. The rate of light-to-dark adaptation of the chromophore in reconstituted white membrane patches and in purple membranes were also compared. The results are interpreted as evidence consistent with:

(i) Stabilization of the 13-*cis* isomeric form of retinal in the reconstituted white membranes;

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(ii) The identification of the acid-induced species as the 640 nm photointermediate.

The observed characteristics of the reconstituted white membrane might arise from differences in the lipid–protein organization of white membrane patches.

2. MATERIALS AND METHODS

Purple membranes from the S_9 strain of *H. halobium* [14] and white membranes from the R_1mW mutant [9] were isolated as described. White membrane patches in 100 mM NaCl and 10 mM Hepes (pH 6.8) at room temperature were fully reconstituted with all-*trans* retinal (dissolved in ethanol). All-*trans* retinal and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) are obtained from Sigma Chemical Co. The reconstituted white membranes were washed 3 times in distilled water before use. The concentration of chromophore in both samples were estimated by the absorbance at 568 nm after light adaptation. The extinction coefficient and M_r used are 63 000 $M^{-1} \cdot cm^{-1}$ and 26 000, respectively [1,15].

Absorbance measurements were obtained with an Aminco DW-2 spectrophotometer. Changes in pH were measured with a Corning Model 130 pH meter and Polymark 1885 electrode from Markson Science Inc. Laser flash-photolysis measurements were obtained as in [14]. Actinic illumination was supplied by a phase-R (model DL-1100) pumped dye laser using Rhodamine 575. Single flash-induced absorbance differences were recorded by a Varian strip-chart recorder via a Biomation 1010 waveform recorder. Unless otherwise mentioned, all experiments were run at 22°C. Illumination of samples for light adaptation was achieved by a 250 W projector lamp.

The fractional conversion (Y) from 568 nm form to the 640 nm form of the chromophore is calculated according to [16]. The equation used is:

$$\frac{R_h - R_n}{R_a - R_n} = Y_h$$

where R is the ratio of absorbance at 640 nm to 568 nm. The subscripts h, a and n denote the ratio R at pH h, at pH 2.0 and at pH 7.0, respectively. As discussed in [16], this equation minimizes the effects of aggregation on calculation of the percentage of the 640 nm species from absorption measurements.

3. RESULTS AND DISCUSSION

The conversion of the 568 nm chromophore to a species which absorbs maximally at ~600 nm as the pH is lowered was found in suspensions of reconstituted white membrane patches. Difference spectra of this process revealed a minimum at 536 nm and a maximum at 630 nm with an isosbestic point at 574 nm (not shown). These parameters are qualitatively similar to that observed with native purple membranes [11,12] and thus suggest that the acid-induced species observed in reconstituted white membranes is similar, if not identical, to that of purple membranes. However, it is found that the pK of the transition is significantly higher in the reconstituted white membranes (fig.1). The acid species is stabilized by low salt [11]. We have also observed this in reconstituted white membrane patches. The pK of the transition for reconstituted white membrane patches was observed to increase from 4.3 in 100 mM NaCl, 10 mM Hepes to ~5.2 in 2 mM Hepes (cf. fig.3). Under identical conditions, the pK of acid transition for native purple membrane patches was increased from ~2.2–3.2 (not shown). The effects of salt concentration are thus comparable between the 2 membrane suspensions.

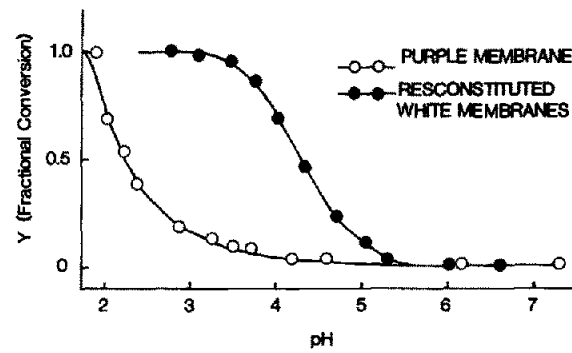

Fig.1. Acid titration of purple membranes and reconstituted white membranes. Membranes for titration were suspended in 100 mM NaCl, 10 mM Hepes at 0.5 mg bR/ml. Fractional conversion to the acid-induced species calculated as in section 2. The pH of the suspensions were adjusted by additions of small aliquots of 1 M HCl or 1 M NaOH.

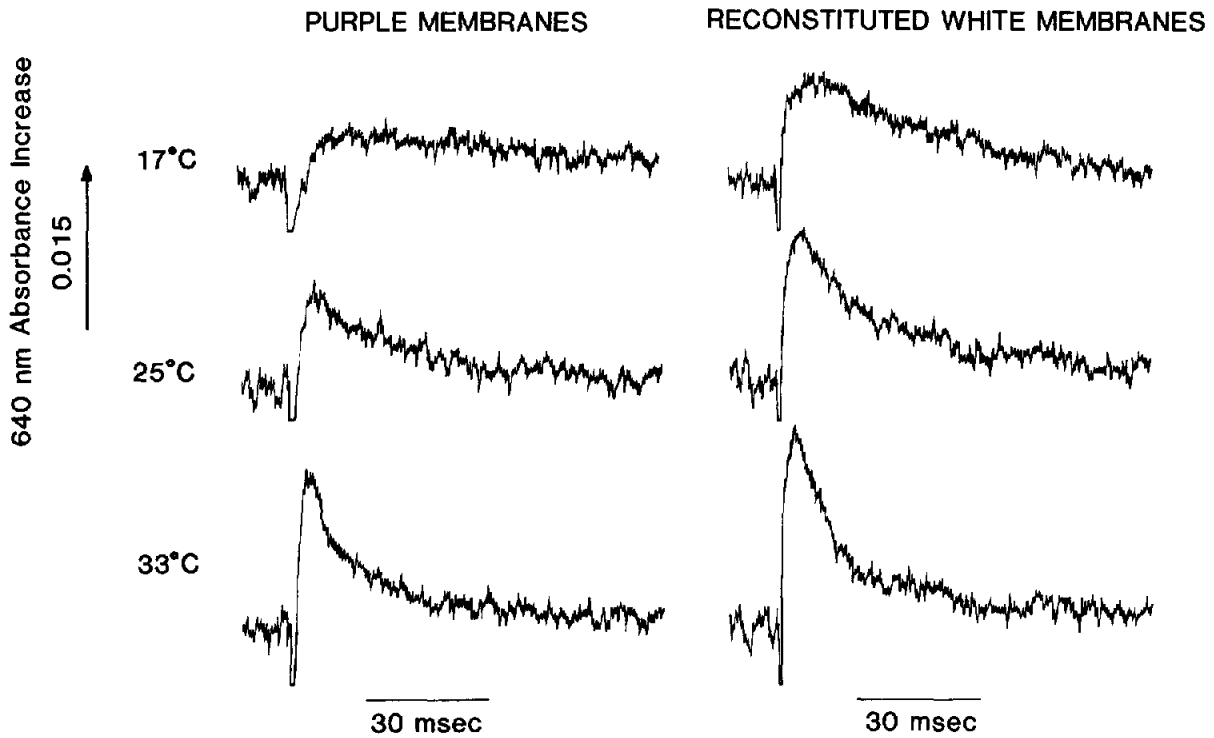

Fig.2. Effects of temperature on the laser flash-induced absorbance changes at 640 nm. Purple membranes and reconstituted white membranes were suspended in 2 mM Hepes (pH 6.1) at 0.15 mg bR/ml. The pH of the suspension did not change by > 0.1 units in the temperature range studied.

The O_{640} photointermediate has been correlated with the acid-induced species in purple membranes [11,12]. The flash-induced absorbance increase at 640 nm in purple membranes and reconstituted white membranes is compared at different temperatures (fig.2). Both preparations show stabilization of the O intermediate by higher temperature, thus confirming that the O intermediate of the reconstituted white membranes is similar to that of purple membranes. It is of interest to note that the 640 nm absorbance increase is larger in the reconstituted white membranes under the various temperatures studied, suggesting that the O intermediate is stabilized in the reconstituted white membranes. In 2 mM Hepes, pH titration of the 640 nm absorbance change shows an acid inhibition with an app. pK ~5.2 (fig.3). This pK is observed to shift to 4.2 when the reconstituted white membranes are suspended in 100 mM NaCl (not

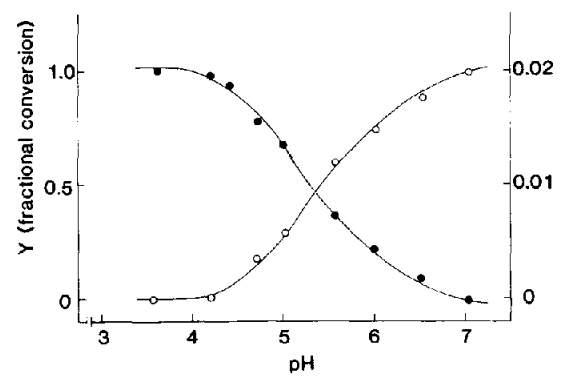

Fig.3. Effects of pH on the 640 nm absorption transient and absorption characteristics in reconstituted white membranes. The fractional conversion (-•-•-) of the acid-induced species is calculated from absorption spectra of the samples at indicated pH. Other conditions were as in fig.2. Titration was done at 22°C.

shown). Although this observation is consistent with the suggestion that the acid-induced species is the O intermediate, it does not preclude the possibility that the acid species can be another form of bacteriorhodopsin. Thus, as the acid-induced species is formed, a different photocycle with no intermediate absorbing maximally at 640 nm might be followed by this population of chromophores [10]. To correlate the acid-induced species with the O intermediate in the reconstituted white membranes, we have studied the temperature stability of the acid species after almost full conversion of the 568 nm chromophores by low pH to the 600 nm form. Lowering the temperature from 22–6°C induced a blue-shift of the absorption maximum from 596–582 nm with an apparent isosbestic point at ~575 nm while increasing the temperature to 35°C only slightly increased the absorbance without any changes in absorption maximum (fig.4). Reconstituted white membranes at pH 7.0, and purple membranes at pH 7.0 and pH 3.8 all showed no significant changes in absorption maxima under identical conditions (not shown). Thus, we conclude from this observation that the acid-induced species, similar to the O intermediate, is also favored at higher temperatures and destabilized at lower temperatures in reconstituted white membrane patches. These observations

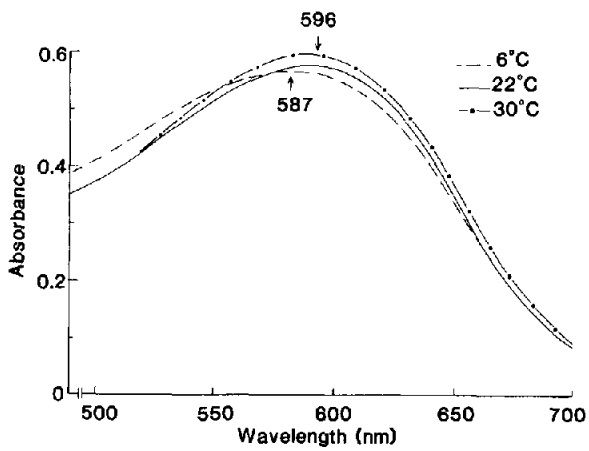

Fig.4. Effect of temperature on the acid-induced species in reconstituted white membranes. Reconstituted white membranes were suspended in 2 mM Hepes at 0.26 mg bR/ml at pH 3.8. The pH of the suspension did not change by > 0.1 units in the temperature range studied.

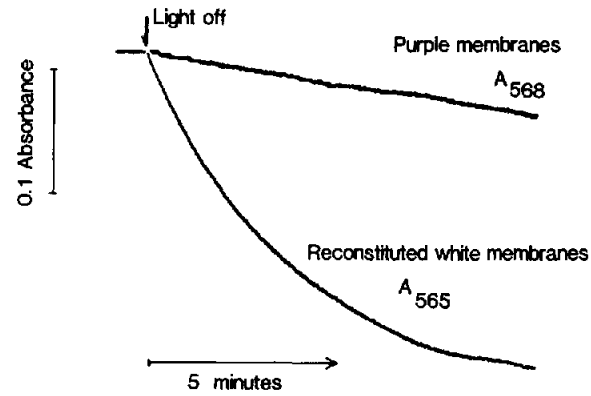

Fig.5. Light-to-dark adaptation rate of native purple membranes and reconstituted white membranes. Conditions were as in fig.2, except at pH 6.2 and 22°C.

tions suggest a strong similarity in characteristics between the acid species and the O intermediate in reconstituted white membrane patches.

It is known that the acid stabilized species contains a higher percentage of 13-*cis* retinal [10] and that low pH facilitates the light–dark adaptation rate of bacteriorhodopsin in native purple membranes [17]. The dark-adapted form contains a much greater percentage of 13-*cis* retinal as compared to the light-adapted form [10]. If the effects of low pH are due to the stabilization of the 13-*cis* conformation of retinal, it would be predicted that reconstituted white membrane patches should show a faster rate of light-to-dark adaptation. The rate of dark adaptation is considerably enhanced in reconstituted white membranes to a $t_{1/2}$ of ~2 min (fig.5) as compared to 60 min for native purple membranes [7]. The light-minus-dark difference spectrum of reconstituted white membranes shows a minimum at 505 nm, a maximum at 588 nm and an isosbestic point at 541 nm (not shown). These are all characteristics of the light–dark difference spectrum of purple membranes [17], indicating that the dark species in reconstituted white membranes is probably the same as that in native purple membranes.

Certain properties of reconstituted white membrane patches differ from that of native purple membranes. These include changes in the apparent p*K* for the formation of the acid-induced species and the inhibition of the 640 nm photocycle

intermediate; which are increased in the retinal-reconstituted white membrane patches; and an increased rate of light-to-dark adaptation in reconstituted white membrane patches. These differences might be caused by stabilization of the 13-*cis* conformation of retinal in the reconstituted white membranes. In any case, it seems unlikely that they arise from amino acid composition differences of bacterio-opsin in the mutant since the R1mW strain of *H. halobium* is a spontaneous mutation that involves synthesis of retinal [9]. An alteration in the primary sequence of the bacterio-opsin protein is unlikely. In fact, preliminary studies (in collaboration with F. Goñi and D. Chapman, University of London) indicate that lipid composition of the white membrane patches is different from that of purple membranes. Thus, stabilization of the 13-*cis* isomeric form of retinal might be due to the alteration of the membrane environment of the chromophore. Nevertheless, the increased p*K* for the formation of the 600 nm species in reconstituted white membranes will certainly facilitate its comparative study with the 568 nm species since aggregation at low pH, necessary for observation of the 600 nm species in bR, can be avoided.

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