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Bacteriorhodopsin in ice

Accelerated proton transfer from the purple membrane surface

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The photocycle and the proton pumping kinetics of bacteriorhodopsin, as well as the transfer rate of protons from the membrane surface into the aqueous bulk phase were examined for purple membranes in water and in ice. In water, the optical pH indicator pyranine residing in the aqueous bulk phase monitors the H^+-release later than the pH indicator fluorescein covalently linked to the extracellular surface of BR. In the frozen state, however, pyranine responds to the ejected H^+ as fast as fluorescein attached to BR, demonstrating that the surface/bulk transfer is in ice no longer rate limiting. The pumped H^+ appears at the extracellular surface during the transition of the photocycle intermediate L_{550} to the intermediate M_{412}. The Arrhenius plot of the M formation rate suggests that the proton is translocated through the protein via an ice-like structure.

Bacteriorhodopsin; Purple membrane; Optical pH indicator; Proton transfer; Membrane surface; Flash spectroscopy

1. INTRODUCTION

The electrochemical gradient for protons across a biological membrane is not only determined by the transport activity of the proton pump, but also affected by the interaction of protons with the membrane surface. Because of the strong buffering power of the lipid headgroups [1], the membrane/bulk interface might represent a transient reservoir for at least part of the established proton gradient. Furthermore, an efficient proton-conducting pathway along the surface of membranes has been postulated [2]. Protons vectorially translocated across the light-driven proton pump bacteriorhodopsin (BR) residing in the purple membrane (PM) or in reconstituted lipid vesicles, are retained at the membrane surface for hundreds of microseconds before released into the aqueous bulk phase [3,4]. Equilibration with the bulk phase is accelerated only to some extent by the presence of mobile buffer molecules in the aqueous medium [5,6]. Therefore, optical pH indicators such as pyranine or fluorescein residing in the aqueous bulk phase distant to the membrane, monitor proton release significantly later than pH indicator dyes covalently linked to the extracellular surface of BR or to the lipid headgroups [3,4].

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Upon absorption of a photon, BR undergoes a photochemical cycle passing various intermediates: J_{610}, K_{590}, L_{550}, M_{412}, N_{530}, O_{640} (the subscripts correspond to the absorbance maxima of the respective intermediates [7]). For the accurate description of the photocycle, backreactions from M and N have been introduced [8]. With BR as a light-energized H^+-pump, a pH-jump experiment can be performed in the presence of a natural membrane. The rate of diffusion controlled transfer of protons from the surface into the bulk is a function of the surface properties [9]. To gain insight into the molecular mechanism of the surface/bulk transfer as well as to correlate proton release and photocycle kinetics, BR and pH indicators quickly frozen in ice were studied by time-resolved flash spectroscopy. Under these conditions, pyranine in the bulk phase responds to the ejected protons as fast as fluorescein covalently attached to BR demonstrating that the surface/bulk transfer is in ice no longer rate limiting. The pumped H^+ appears at the extracellular surface during the L-M transition. Our data suggest that protons in BR are translocated through the protein via an ice-like structure. These results are of relevance for the understanding of energy-transducing processes across biological membranes.

2. MATERIALS AND METHODS

2.1. *Sample preparation*

Purple membranes were isolated from *Halobacterium halobium*, strain ET1001 [10,11]. The time-course of pH changes in the aqueous

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bulk phase was monitored with the pH indicator dye pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate; laser grade, Kodak) as described elsewhere in detail [6,12]. Due to the 3-4 negative charges, pyranine is highly water soluble and does not bind to the negatively charged PM. In addition, it has suitable optical properties and a pK of 7.2 [13]. The pH indicator fluorescein (5(6)-carboxyfluorescein succinimidyl ester, Molecular Probes, Eugene, USA) was covalently conjugated via a carboxamide linkage to the extracellular surface of BR. Under the labeling conditions applied (i.e. pH 8.1, molar fluorescein/BR ratio of 100, 5.5 h incubation at 22°C), 1.0 fluorescein was bound per BR, which did not affect the photo- or the pumping cycle. Fluorescein conjugates with the α-amino group of the N-terminal amino acid in all of the 5–20% precursor BR molecules in the sample (carrying up to 13 additional amino acids at the N-terminus [14]) as well as with the ϵ-amino group of lysine 129 in BR. Both conjugation sites are located at the extracellular side of the PM (Heberle and Dencher, paper submitted).

For flash photolysis of PM in ice, only a fast freezing procedure is applicable. The sample was frozen at −70°C and thereafter equilibrated at the desired temperature for at least 30 min in the flash photometer. By slow-freezing, water crystallizes into large ice crystals and the PM fragments will sink to the bottom of the cuvette. In order to extract the response of a pH indicator from the optical contributions of the photocycle, a measurement of a sample with buffer (10 mM imidazole) was numerically subtracted from a measurement without buffer. This can be reliably done only with homogeneous samples. For the measurement in supercooled water, freezing was prevented due to the presence of PM and 150 mM KCl, and by stirring.

2.2. *Flash photolysis*

BR was excited by a XeCl-excimer pumped dye laser (Rhodamine 6G; $\lambda = 580$ nm ± 20 nm; pulse duration: 10 ns; flash energy: 1 mJ; repetition rate: ≥0.25 Hz). For a light-adapted sample of $OD_{BR} = 0.8$–1.0, a 10–15% photocycle turnover was obtained. The monitoring beam was polarized under 54.7°. Digitization of the photomultiplier voltage was performed by a fast two-channel A/D converter (20 MHz maximal sampling rate, 64 kbyte memory and 8-bit resolution per channel). 50–100 measurements were averaged and the linear data were reduced to about 800 logarithmic equidistant points. The optimal fit to the data was achieved with the program MINIFIT. The algorithm is based on a non-linear regression method (Marquadt, [15]) and up to 9 exponentials can be applied.

3. RESULTS AND DISCUSSION

Upon flash excitation, BR actively transports protons in microseconds from the interior of the protein into the extracellular medium. The released protons are subsequently taken up in milliseconds at the intracellular side of BR. The time-course of vectorial H^+-translocation can be accurately determined only with a pH indicator that is covalently bound to the extracellular side of BR [3,4]. The response of the fluorescein carboxamide conjugate of BR in water is depicted in Fig. 1(F). When BR is photoactivated by a short laser flash, the absorbance of fluorescein transiently decreases reflecting the protonation of the optical pH indicator. This corresponds to the transfer of H^+ from the interior of the protein to the extracellular surface. The protonation occurs concurrently with the rise of the photocycle intermediate M (second phase in Fig. 1,M). The subsequent deprotonation of fluorescein proceeds within two steps well separated in time. At first, the H^+-excess at the surface dissipates into the bulk. This process is also reflected by

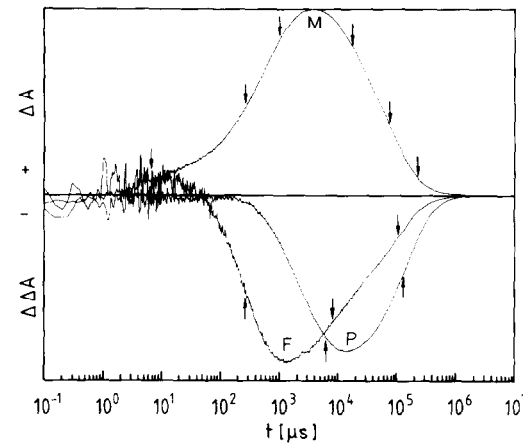

Fig. 1. Kinetic comparison of the M intermediate (M) with the response of the pH indicators fluorescein (F, covalently attached to BR) and pyranine (P, located in the aqueous bulk phase) in water; $T = 3$°C, 150 mM KCl, $c_{BR} = 13$ μM, $\lambda_M = 412$ nm, $c_F = 13$ μM, $\lambda_F = 489$ nm, $c_P = 40$ μM, $\lambda_P = 457$ nm. The time constants for the kinetic processes are indicated by arrows: $\tau(M_1) = 6.8$ μs, $\tau(M_2) = 311$ μs, $\tau(M_3) = 1.2$ ms, $\tau(M_4) = 20$ ms, $\tau(M_5) = 82$ ms; $\tau(M_6) = 247$ ms; $\tau(F_1) = 287$ μs, $\tau(F_2) = 9.2$ ms, $\tau(F_3) = 111$ ms; $\tau(P_1) = 6.5$ ms; $\tau(P_2) = 135$ ms.

the protonation of the pH indicator pyranine which resides in the aqueous bulk phase (Fig. 1,P). Pyranine is protonated within the same time as the membrane surface is partly deprotonated which is measured by fluorescein. The complete deprotonation of both indicators reflects the H^+-reuptake by BR. (A detailed description of these processes will be presented elsewhere.)

The difference in the H^+-detection time for the two indicators suggests that the protons are retained at the membrane surface for several hundred microseconds. In order to gain further insight into the molecular mechanism of the H^+-transfer from the Schiff base via the membrane/water interface into the aqueous bulk phase, we performed the same experiment as in Fig. 1, but in fast frozen ice (Fig. 2). Under this condition, the protonation of pyranine which was delayed by one order of magnitude in water as compared to fluorescein (Fig. 1), occurs in ice with almost the same time constant. The small difference may arisen from the distance of the negatively charged pyranine to the PM. Also, the characteristic three exponential response of surface bound fluorescein (appearance of H^+ at the surface, surface/bulk transfer, H^+-reuptake by BR) has vanished. Moreover, pyranine now monitors H^+-release by BR concomitantly with the rise of M. The most simple way of explaining this fast response of pyranine is to relate it to an enhanced mobility of H^+ in ice. However, the rate of H^+-diffusion in ice is still in controversy. One of the first determinations yielded a 20-fold higher H^+-mobility in ice as compared to liquid water [16]. During the past decades smaller values for H^+-diffusion in ice were reported [17]. The most recent

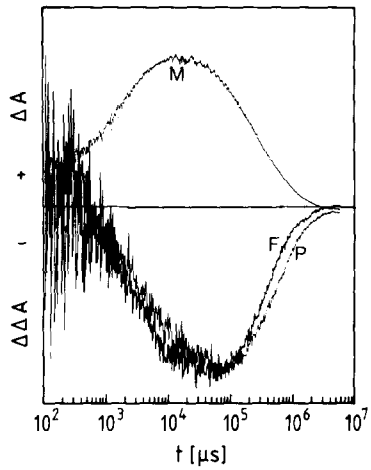

Fig. 2. The photocycle (M) and the proton pumping cycle (F, fluorescein; P, pyranine) in ice. $T = -15°C$; other conditions as in Fig. 1.

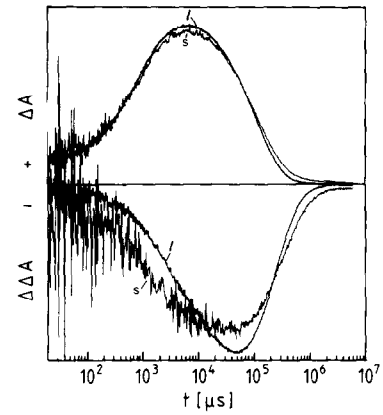

Fig. 3. Effect of the water/ice transition on the kinetics of the pH indicator pyranine and the M intermediate. $T = -6°C$, other conditions as in Fig. 1; smooth trace (l): in water; noise trace (s): in ice.

measurement [18] gave an even higher value of proton mobility in water by a factor of two at least as compared with ice. But the situation in a biological system is more complex. The surface of the membrane is composed of various acids and bases (amino acid sidechains, lipid headgroups) which are involved in H^+-migration [9]. A direct effect of ice on the pK of these protonable groups cannot be ruled out. Furthermore, the membrane/water interface consists of highly structured water [19]. For ice having a similar structure, the probability for a H^+ at the surface to enter the bulk ice phase is equal or even greater than to react with surface groups. For H^+-conductance along the membrane surface, the protonable surface groups must be able to rotate [2]. If the rotational motion is suppressed in ice, this 'proton-wire' will be interrupted. Consequently, the protons have a greater chance to diffuse into the surrounding medium.

That the response of pyranine depends on the H_2O-structure was further corroborated by measurements in ice and in water at the same temperature (Fig. 3). Although the rise and decay kinetics of M are almost the same (cf. below), the protonation of pyranine is accelerated in ice. However, the protonation is not a single exponential function. Two exponentials are required to fit the data traces, both in ice and water. The ratio of the amplitudes of the fast and slow protonation reaction is 2:1 in ice, whereas it is 1:2 in water. This is an indication for a certain ordering of the supercooled water. The H^+-reuptake is slightly slower in ice than in water. This is explained by the fact that the slow decay component of M exhibits a discontinuity in the Arrhenius plot (Fig. 4) indicating that the N decay is delayed in ice (N participates in the M decay via the M ← N backreaction [8]). Because the N decay determines the H^+-reuptake [20,21], the pyranine deprotonation in ice is also decelerated as compared to water.

In Fig. 4, the temperature dependence of the processes involved in rise and decay of the M intermediate is shown. Although six exponentials are required for the description of the measurements in water, only four exponentials are depicted. The higher turbidity of the frozen samples results in a lower signal-to-noise ratio which permits to fit only four exponentials with sufficient accuracy. The lines in the Arrhenius plot represent a least-squares fit of the data points for the temperature range from 10 to 50°C. The filled symbols correspond to measurements in ice. The rates of fast and slow rise of M (attributed to the L–M equilibrium [8]) as well as the fast M decay (attributed to the M–N step) are not influenced by the transition from water to ice (Fig. 4). Rise and decay of M are determined by the deprotonation of the retinal Schiff base and its reprotonation, respectively. If the H_2O-molecules around the Schiff base and in the H^+-pathway [22,23] behave as bulk water, a discontinuity in the transition to ice is to be ex-

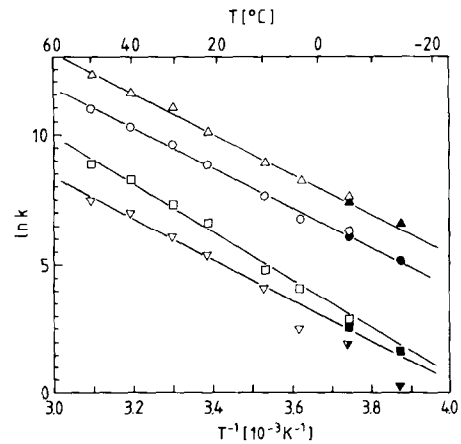

Fig. 4. Arrhenius plot for the rate constants [s^{-1}] of rise and decay of the M intermediate. (△) M_2 (fast rise); (○) M_3 (slow rise); (□) M_4 (fast decay); (▽) M_5 (slow decay); filled symbols represent measurements in ice.

pected. Since this is not observed (Fig. 4), we conclude that the water molecules of the H^+-conducting pathway across BR are highly structured. The discontinuity in the rate of the slow M decay component may be due to an increase in pH caused by the transition from water to ice, e.g. the molar concentration of H^+ in ice is by three orders of magnitude lower than in water [24]. A higher pH will decelerate the decay of N [21].

4. CONCLUSIONS

(i) The diffusion of protons from the membrane surface to the pH indicator pyranine in the bulk phase is accelerated in ice as compared to water.

(ii) The proton release time matches the fast rise time of the M intermediate.

(iii) The proton is translocated through the protein via an ice-like structure, both in ice and under physiological conditions.

(iv) In water only pH indicators covalently bound to the extracellular side of BR are able to accurately measure the rate of proton release.

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