BACTERIORHODOPSIN: BIPHASIC KINETICS OF PHOTOTRANSIENTS AND OF LIGHT-INDUCED PROTON TRANSFER BY SUB-BACTERIAL *HALOBACTERIUM HALOBIUM* PARTICLES AND BY RECONSTITUTED LIPOSOMES

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1. Introduction

It is well established that the bacteriorhodopsin of *Halobacterium halobium* acts as a proton-pump in intact bacteria [1–3] as well as in vesicles [4,5] and reconstituted liposomes [6–9]. Isolated purple membrane fragments release protons upon illumination [10]. Reversible de-protonation of the Schiff-base linkage between retinal and lysine [11] occurs on forming the 412 nm-intermediate of the photochemical reaction cycle [12–17]. Proton pumping by bacteriorhodopsin is probably a direct consequence of protonation and deprotonation during the cycle.

In this communication it is shown that light-induced proton release and proton uptake by sub-bacterial particles or reconstituted proteoliposomes (which have a reversed membrane orientation [16]) show biphasic kinetics. Furthermore, the decay of the 412 nm-intermediate of bacteriorhodopsin also shows biphasic kinetics. This similarity in the kinetics of the macroscopic and microscopic processes may, however, be coincidental. It is suggested that the biphasic proton movement results from a combination of two processes: a faster process of association or dissociation, and a slower process reflecting transport across the membrane.

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2. Materials and methods

Halobacterium halobium was grown as described by Danon and Stoeckenius [18]. The sub-bacterial vesicles were prepared by sonication according to MacDonald and Lanyi [4] with minor modifications. The integrity of the vesicles was examined by the NADH–menadione reductase test [4,19] and verified by fragiligraph [20]. The vesicles were inside-in and intact (90 ± 5%).

Proteoliposomes were prepared according to the method of Racker [6,9] from purple membranes (about 20 nmol of bacteriorhodopsin) and a lipid mixture of 25 mg egg phosphatidylcholine plus 0.25 mg dicetylphosphate. 5 ml amounts of aqueous suspensions of this mixture were sonicated for 20 min under argon in a Laboratories Supplies sonicator. After 10 min of sonication, the pH of the suspension was brought to its desired final value (5.2). Bacteriorhodopsin concentrations were determined using a molar extinction coefficient of 63 000 M^{-1} cm^{-1} at 570 nm [10].

Light-induced pH changes in suspensions of equilibrated and pre-illuminated preparations of vesicles or proteoliposomes were measured at constant temperature with a Radiometer GK 2321C combined glass electrode connected to a Radiometer 64 pH meter with output to a high-speed Varian A-25 recorder; response time 0.5 s. The recorder scale was calibrated by additions of μl quantities of approximately 1 mM HCl to the reaction vessel. Suspensions were illuminated by a slide projector (24 V, 150 W). Light intensities were measured with a Y.S.I Kettering 65 A radiometer.

Purple membrane was prepared as described by Oesterhelt and Stoeckenius [21]. A conventional flash photometry system was used to study the decay of the 412 nm-intermediate. The air-filled flash tubes were operated at 20 kV and gave a total light energy of about 400 J.

3. Results

Figure 1A shows a typical measurement of the light-induced proton transfer by sub-bacterial vesicles (4.4 mg protein), suspended in 2.5 ml of 4 M NaCl– 1 mM HEPES* (pH 7.0) solution. The appearance of protons in the suspending solution in the 'on' reaction was kinetically very similar to their disappearance in the 'off' reaction, and the same extents were observed. Figure 1B shows the kinetic analysis of the 'off' process. The proton concentrations were plotted on a semi-logarithmic scale versus time, showing clearly that the process exhibited biphasic kinetics (closed circles). The second phase followed first-order kinetics ($k_2 =$ 0.068 s^{-1}; 25°C), while the first phase combined this process and a faster process. By linearly extrapolating the slow phase as shown, and plotting the differences

between the observed and extrapolated concentration values semi-logarithmically as before (open circles), we obtained the rate of the rapid process ($k_1 = 0.29$ s^{-1}; 25°C). The fraction of protons disappearing in the fast process (calculated at $t = 0$) was estimated as 49%. A similar kinetic analysis of the 'on' process in the same sample showed the same features, with rate constants of 0.29 s^{-1} and 0.11 s^{-1} for the fast and slow processes, respectively. Here 51% of the protons ejected were related to the rapid process. Repeated experiments of this kind in various batches of sub-bacterial vesicles yielded slightly different values of k_1 and k_2, but the ratio between them remained practically constant. Furthermore, the fraction contributed by each process was in all cases 50 $\pm$ 20%.

Repeating the same experiment with sub-bacterial particles loaded with and suspended in 4 M KCl instead of NaCl revealed the same kinetics, with comparable rate constants [Eisenbach, Garty, Rotterberg and Caplan, manuscript in preparation]. The only difference between vesicles loaded with KCl and NaCl was the extent of the pH changes. In NaCl-loaded sub-bacterial particles at high light intensity an influx of protons follows the acidification as a results of Na$^+$/H$^+$ exchange [22]. Thus, in order to avoid complications in the kinetic analysis, we used low light intensity in fig.1. In KCl-loaded vesicles, on the other hand, the light intensity could be raised with a resultant big increase in the extent of the pH change, but the rate

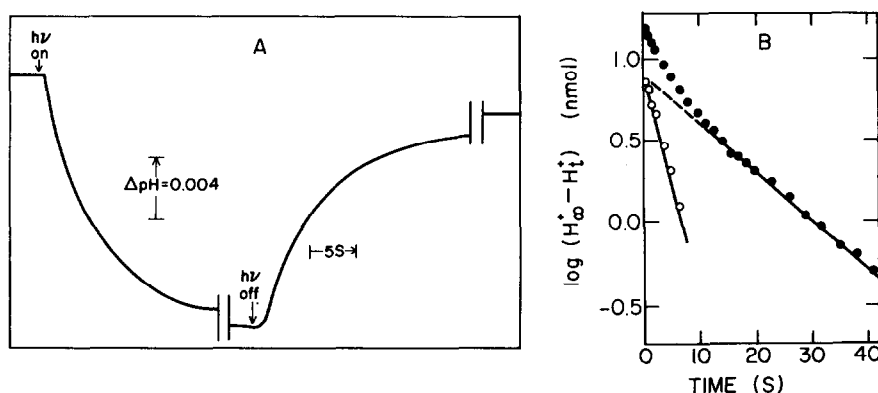*Abbreviations*: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

Fig.1. Light-induced pH changes in sub-bacterial vesicles from *H. halobium*. A. Vesicles (4.4 mg protein) were suspended in 2.5 ml of 4 M NaCl–1 mM HEPES (pH 7.0) at 25°C, equilibrated until a constant pH was reached, and pre-illuminated and re-illuminated at 580 ± 20 nm (interference filter). I = 25 W/m². B. The kinetic analysis of the 'off' reaction shown in A. (●) Experimental values of the H$^+$ concentration. (○) The rapid process, calculated by subtraction of the extrapolated (dashed) line from the experimental points, as described in the text.

Table 1

Salt dependence of kinetic parameters of light-induced proton uptake by bacteriorhodopsin (bR) proteoliposomes

Light	[NaCl]	Total extent (eq. H$^+$/mol bR)	Fast process		Slow process	
			k_1 (s^{-1})	Extent (eq. H$^+$/mol bR)	$k_2 \times 10^2$ (s^{-1})	Extent (eq. H$^+$/mol bR)
On	25 mM[a]	2.0 ± 0.2	0.24 ± 0.06	0.8 ± 0.1 (40%)	4.4 ± 0.5	1.2 ± 0.2 (60%)
	150 mM	3.6 ± 0.1	0.32 ± 0.01	1.3 ± 0.1 (35%)	7.0 ± 0.1	2.3 ± 0.1 (65%)
	500 mM	4.5 ± 0.1	0.30 ± 0.02	1.4 ± 0.1 (30%)	9.5 ± 0.2	3.1 ± 0.1 (70%)
	2 M	4.4 ± 0.1	0.20 ± 0.01	b	b	b
Off	25 mM[a]	2.1 ± 0.3	0.23 ± 0.01	0.7 ± 0.1 (33%)	3.2 ± 0.2	1.4 ± 0.2 (67%)
	150 mM	3.7 ± 0.1	0.15 ± 0.02	1.6 ± 0.1 (40%)	2.9 ± 0.3	2.1 ± 0.1 (60%)
	500 mM	4.5 ± 0.1	0.12 ± 0.01	2.3 ± 0.1 (50%)	2.1 ± 0.3	2.2 ± 0.2 (50%)
	2 M	4.4 ± 0.1	0.16 ± 0.02	2.1 ± 0.2 (50%)	2.5 ± 0.3	2.3 ± 0.2 (50%)

[a] At 25 mM NaCl the pH electrode was more unstable than at other NaCl concentrations
[b] At 2 M NaCl k_2 (on) became indistinguishable from k_1 (on).

3.0 ml suspension of sonicated proteoliposomes prepared from 99% egg lecithin plus 1% dicetylphosphate (w/w) in the NaCl concentration given in the table, were tested at 30°C for their ability to take up protons upon illumination and to release protons upon switching the light off. Illumination was carried out by passing light through a 3–69 Corning cut-off filter; light intensity 1–2 × 10^3 W/m^2. The curves of proton transfer obtained were found to represent a sum of two apparently first-order processes (see fig.1B), the extents and kinetic constants of which are given in the table. The values given are averages of duplicate experiments. The deviations are those between the extreme values and the average. N.B. At different salt concentrations different liposomal preparations were used. The differences between measured data are much bigger between preparations (20–30%) than between duplicate experiments with one preparation (5–10%).

constants were practically unchanged [Eisenbach et al., loc. cit.].

Biphasic phenomena are also observed in light-induced proton uptake by proteoliposomes (table 1), which have reversed membrane orientation [16]. In such systems salt concentrations are more easily varied than those of more intact halobacterium systems that require high salt concentrations. We observed that the extent of light-induced proton uptake by the liposomes increased with increasing concentration of NaCl (table 1, cf. ref. [6]) or KCl (not shown). This effect might be due either to a direct effect on the proton pump or to an increase in the rate constant of the slow phase of the 'on' process, due to an increased cotransport of Cl$^-$, leading to a larger ΔpH across the membrane. However, if the latter explanation were correct, a similar increase in the rate constant with increasing NaCl concentration should be observed in the 'off' process. This is not seen.

The biphasic kinetics of proton movement observed may reflect a combination of processes. The possibility of its occurrence due to different populations in sub-

bacterial particles and proteoliposomes could be ruled out, since we observed that with a given preparation the relative extents of the two phases could be varied by 20–40% of the total by changing the temperature, the pH, or the light intensity [Eisenbach et al., loc. cit.; Bakker and Caplan, manuscript in preparation].

The decay of the 412 nm-species (or the reappearance of the 570 nm-species) represents the protonation of the bacteriorhodopsin [11]. Thus, we may expect this intermediate to reflect the kinetics of the microscopic proton transfer. The photo-transients of bacteriorhodopsin are advantageously studied on purple membrane fragments, since both faces of the membrane are exposed to the same solution and no potential differences can exist across it. Both the formation of the 570 nm-species and the decay of the 412 nm-species were kinetically completely identical. When the purple membrane was suspended in water, a monophasic first-order kinetics of the 412 nm-species decay was observed ($k = 150$ s^{-1}; 20°C, pH 6.8). However, increasing the concentration of NaCl (to above 0.3 M) changed the mode of the kinetics from monophasic
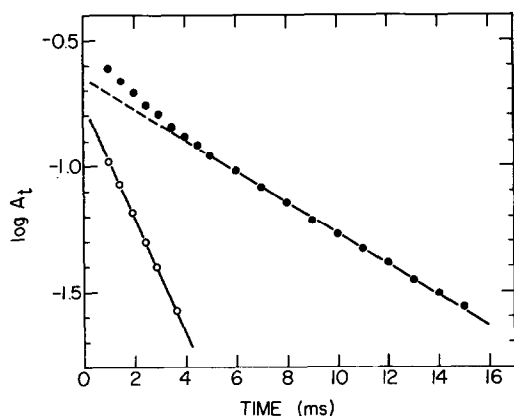
Fig.2. The kinetics of the decay of the 412 nm-species of bacteriorhodopsin. Purple membrane fragments (5 μM) were suspended in 1.6 M NaCl solution at pH 6.8 (20°C). The kinetic analysis was performed as described in the text in relation to fig.1. (Zero time was taken at the absorbance maximum preceding the decay). (●) Experimental values. (○) The rapid phase, calculated as described in fig.1. $k_1 = 660$ s^{-1}; $k_2 = 120$ s^{-1}.

to biphasic. Figure 2 shows that this biphasic behaviour represents the sum of two first order processes and is thus reminiscent of our previous observations in the case of the proton transfer (fig.1B).

4. Discussion

The above experiments show the existence of biphasic kinetics in different preparations from *H. halobium*. In all three cases described the kinetics may be represented as a sum of two exponentials, i.e. two first-order (or pseudo first-order) processes. However, the biphasic decay of the 412 nm-intermediate, which is 3 orders of magnitude faster than proton transfer, can hardly be attributed to the same origin as the pH changes caused by sub-bacterial particles or proteoliposomes. This conclusion is supported by the different dependence of the pH changes and the decay on the nature of the salt and its concentration in the solution.

Possible reasons for the occurrence of a biphasic kinetics of proton transfer include coupling to transport of other ions; two proton pumps (e.g. two configurations of bacteriorhodopsin, each pumping at a different rate); more than one type of leak; and inter-

action with the proton—ATPase pathway. If the cause of the biphasic kinetics is the same for both sub-bacterial particles and proteoliposomes, the last alternative can be ruled out since no ATPase was incorporated into the proteoliposomes. Since the rapid process of the proton transfer operates only briefly, it is unlikely to represent a transport process. Morever, any model which postulates the existence of 2 proton pumps is unlikely, since Henderson and Unwin's electron microscopic analysis of the purple membrane [23] does not show any evidence of two protein structures. It seems reasonable, therefore, that only the slow phase of the pH change represents a net transport phenomenon. This suggestion is further supported by the findings that external effects such as pH, temperature, and presence of permeant ions increased the extent of the slow process several-fold while that of the rapid process was hardly affected [Eisenbach et al., loc. cit.]. Further studies which have been carried out in our laboratory [Eisenbach et al., Bakker and Caplan, loc. cit.] suggest that the rapid process of the proton transfer represents dissociation of protons from the vesicular membrane (or association of protons to the liposomal membrane) resulting from pK shifts of the protein during its light-induced conformational change. The significance of the biphasic kinetics of the 412 nm-intermediate decay is still unknown.

Acknowledgements

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