DEMONSTRATION OF COUPLING BETWEEN THE PROTONMOTIVE FORCE ACROSS BACTERIORHODOPSIN AND THE FLOW THROUGH ITS PHOTOCHEMICAL CYCLE

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1. Introduction

Any membrane enzyme catalyzing a reaction in which both $[H^+_{in}]$ and $[H^+_{out}]$ are involved, will be influenced by the protonmotive force ($\Delta\tilde{\mu}_{H^+}$) across its membrane, due to the second law of thermodynamics. Examples of electrogenic proton pumps are found among ATPase-complexes and electron-transfer chains [1–3].

The effect of a $\Delta\tilde{\mu}_{H^+}$ on an electrogenic proton pump may be manifested in the inhibition of the flux through the enzyme reaction with increasing, oppositely directed, $\Delta\tilde{\mu}_{H^+}$; this phenomenon is termed coupling. For ATPase-complexes from mitochondria [4], chloroplasts [5] and bacteria [6] this coupling has been shown by measurement of the velocity of ATP-breakdown in vitro. Many electron-transferring enzymes that catalyze concomitant transmembrane proton transport can be reconstituted in artificial membranes and their reaction rates have been shown to be sensitive to the $\Delta\tilde{\mu}_{H^+}$ across the reconstituted membrane [7–9]. This coupling is also well known in intact mitochondria, where it has been termed 'respiratory control' [10].

Several light-driven electrogenic proton pumps exist, such as the electron transfer chain in chloroplasts [11] and bacteriorhodopsin from the purple membranes of *Halobacterium halobium* [12–15].

Abbreviations: S-13, 5-Chloro-3-*tert*-butyl-2′-chloro-4′-nitro-salicylanilide; bR^{LA}_{570}, Light-adapted ground state of bacteriorhodopsin

Although coupling could be demonstrated in parts of the chloroplast electron transfer chain containing cytochromes, no such coupling has ever been demonstrated for a segment of the chloroplast electron transfer chain that does not contain cytochromes or plastoquinone [for review see 11].

In this article we demonstrate the existence of the coupling between the activity of the other light-driven electrogenic proton pump, viz. bacteriorhodopsin, and the $\Delta\tilde{\mu}_{H^+}$ across this enzyme. A system was chosen which consists of bacteriorhodopsin and well-defined lipids only, the so-called bacteriorhodopsin vesicles [16,17]. The reaction rate of the bacteriorhodopsin was determined by measurement of changes in concentration of an intermediate of the photochemical cycle of bacteriorhodopsin after flash-illumination.

2. Materials and methods

Bacteriorhodopsin was isolated as described in ref. 18. Bacteriorhodopsin vesicles which show light-dependent proton uptake were prepared according to the procedure described in [19], with a sonication time of 60 times 15 s. Vesicles in which the majority of the bacteriorhodopsin is incorporated in the in vivo orientation, were prepared according to the procedure of Happe et al. [20]. Cardiolipin (from bovine heart) was obtained from Sigma. 5-Chloro-3-*tert*-butyl-2′-chloro-4′-nitro-salicylanilide (S-13) was a gift of Dr P. Hamm, Monsanto Company, St. Louis. Nigericin and valinomycin were gifts of Dr W. C.

Pettinga, Eli Lilly and Comp., Indianapolis, IN.

Absorption changes were detected with a single beam spectrophotometer built according to a home-made design. The optical path length of the reaction vessel was 1 cm; the intensity of the measuring light was 15 $\mu W/cm^2$ at 415 nm and 50 $\mu W/cm^2$ at 660 nm. Changes in the transmission of the samples were detected with a photomultiplier (R.C.A. 1 P 28), which was shielded from actinic illumination by a suitable combination of a Balzers interference filter and a Wratten Kodak gelatin filter (for measurement at 660 nm a Balzer B-40-660 filter, and at 415 nm a combination of a Balzer B-40-415 filter and a Wratten 47 filter were used). The signal from the photomultiplier was amplified, digitized and stored by a transient recorder (DATALAB DL 922). Usually 64 transients were sampled and added to improve the signal to noise ratio. The averaged data were then plotted on an analogue X–Y recorder (H.P. 7004 B). The reaction cuvette had a volume of 1.6 ml and was thermostatted by means of a thermoelectric module device and a NTC resistor to supply the feedback signal.

The cuvette could be illuminated either through a quartz window in the bottom of the cuvette or with the aid of a flexible lightpipe from above. Samples were illuminated either with short white light flashes, halfwidth 3 μs and a total light energy of 6 mJ/cm^2 per flash or with a combination of the flash light and 'background'-light; continuous white light from a 150 W quartz–halogen tungsten filament lamp, filtered through a Balzer B-40-576 filter (intensity 12 mW/cm^2). Due to the flashes the photomultiplier shows saturation for 1 to 2 ms after the start of the flash. From measurements with samples, without measuring light, it could be inferred that stability of the measurement was regained within 2 ms. pH measurements in the sample cuvette were carried out with an Ingold (Nr. 10 142 3001) glass pH-electrode and an Ag/AgCl reference electrode (Micro Electrodes, MI 401) connected to a Knick pH meter (Type Nr. 640). Measurements of light intensities were carried out with a Photometer/Radiometer (Model 450, E. G. & G.). Before each absorbance measurement the sample was preincubated for 5–10 min to allow the light adaption of the bacteriorhodopsin [21] and the equilibration of the steady state $\Delta\tilde{\mu}_{H^+}$ across the vesicle membrane.

3. Results and discussion

The series of chemical reactions occurring after light absorption of bacteriorhodopsin has been extensively studied [22]. The picture of the photochemical cycle of bacteriorhodopsin that emerges from these studies is the following: after absorption of a photon by bR^{LA}_{570} (the light-adapted ground state), the excited state decays back to the ground state via a series of temperature-dependent dark reactions. The intermediates involved are called K_{635}, L_{550}, M_{412}, N and O_{640}.

After a single turn-over flash at room temperature the return to the dark absorbance level occurs in about 10 ms [22]. The absorbance due to the presence of intermediates of the photochemical cycle of bacteriorhodopsin can be measured at 660 nm [23–25] and this measurement can be used for the determination of the flux through the photochemical cycle. The relevant reactions can be summarized as follows:

$$bR^{LA}_{570} + h\nu \xrightarrow{k_1} bRh^* \xrightarrow{k_2} O_{640} \xrightarrow{k_3} bR^{LA}_{570}$$

Since exact correlation of the occurrence of photointermediates and the release and uptake of protons has not yet been possible [22,26,27] these processes are not made explicit in this reaction scheme; they are reflected by pH dependence of the different reaction constants. The mechanistic basis of a thermodynamic back pressure by a protonmotive force must lie in a pH dependence of some of the reaction constants combined with the location of different steps at different sides of the membrane or in changes in the fraction of bacteriorhodopsin excited at a certain light intensity.

From experiments on the photochemical cycle of bacteriorhodopsin [22,25] it can be inferred that – in our experimental set up – at 660 nm after a short flash a transient increase in absorbance will take place which gives rise to a maximum at $t = \tau$ followed by a decay to the pre-flash level, due to the formation and decay of the O_{640} intermediate. The height (h) of the maximum will depend both on the fraction of bR^{LA}_{570} that is excited by the flash light and on changes in the reaction constants of the reactions of the photochemical cycle, whereas τ will only depend on the reaction constants (and on $\Delta\tilde{\mu}_{H^+}$ via these parameters).

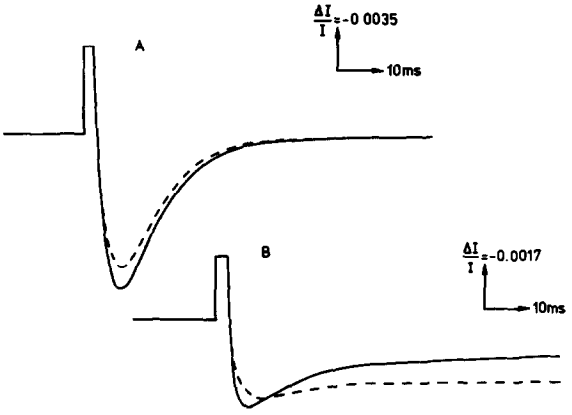

Fig.1. Flash-induced 660-nm absorbance changes in purple membranes and in bacteriorhodopsin vesicles, with and without background illumination. (A) Purple membranes (0.4 mg protein/ml) in 150 mM KCl pH 6.0 and 28°C. Full line: – ; dashed line: + 570-nm illumination. (B) Bacteriorhodopsin vesicles. Bacteriorhodopsin: 1 mg/ml; soy-bean phospholipids 10 mg/ml; medium 150 mM KCl pH 6.0, temperature 27°C. Full-line: – ; dashed line: + 570-nm illumination.

In fig.1A the time change of the absorbance at 660 nm of a suspension of purple membranes in 150 mM KCl at pH 6.0 and 28°C is shown. For a short period after the flash the photomultiplier shows saturation which prohibits the resolution of absorbance changes taking place in less than 2 ms. After this period the absorbance changes reported here, exactly agree with other reports in the literature [25]. The dashed line in this figure confirms the predicted independence of τ and dependence of h on the fraction of the bacteriorhodopsin molecules, excited in one flash; here the fraction of molecules, present in the bR^{LA}_{570}-form has been diminished by continuous illumination with low intensity 570 nm light.

A completely different picture emerges if this experiment is repeated with a preparation of bacteriorhodopsin vesicles (cf. fig.1B). The flashes give rise to proton transport and absorbance changes due to the presence of photointermediates of bacteriorhodopsin. In the dark periods between the flashes (± 2.5 s) the photointermediates can decay to the bR^{LA}_{570}-form and also a large part of the transported protons can leak back. After some 5–10 min the net proton uptake ceases (at an extent of less than 20 ngeq. H^+/mg bacteriorhodopsin); the steady state in which the back leakage matches the light-driven proton transport, is attained. From the proton uptake the proton motive force can be estimated (see ref. 28): about 15 mV. Such a small $\Delta\tilde{\mu}_{H^+}$ is not expected to have a large effect on the kinetics of the intermediates of the photochemical cycle. Figure 1B shows the results of an experiment, in which the steady-state $\Delta\tilde{\mu}_{H^+}$ was increased by additional continuous illumination with 570-nm light.

A complication arises: The transient 660 nm absorbance change already observed in fig.1A is superimposed on a second, apparent absorbance change that decays much more slowly. The small wavelength dependence of the latter does not correlate with the absorbance spectrum of any of the intermediates in the photochemical cycle (not shown). On the other hand, the former change was shown to have the wavelength dependence characteristic for the O_{640} intermediate [23,24]. We conclude that the slowly decaying absorbance change is not due to light absorbance by intermediates of the photochemical cycle of bacteriorhodopsin (it may be caused by changes in the light-scattering characteristics of the vesicles). The slowly-decaying, apparent, absorbance change reaches its maximum more rapidly than can be detected with the present instrumentation. From this point onwards it decays to its pre-flash level in an approximately linear way. (This is inferred from measurements with 702 nm light, which none of the intermediates of the photochemical cycle absorbs [23,24].) In the following only the rapidly-decaying absorbance change will be discussed.

Reconsidering fig.1B, we can now conclude that additional 570-nm illumination does indeed change the kinetics of the photochemical cycle: With increasing background illumination h decreases and τ increases due to a decrease in k_2 with a constant k_3.

Decrease of the steady state $\Delta\tilde{\mu}_{H^+}$ by means of ionophores gives rise again to an increase in h and a decrease in τ (k_2 returns to its original, or even higher value). The change in $\Delta\tilde{\mu}_{H^+}$ can be elicited either by an uncoupler (S-13, see fig.2A) or by the combined addition of nigericin and valinomycin (fig.2B). Changes similar to the ones reported here at pH 6.0 can be observed at pH 7 or pH 5.

We interpret the changes in kinetics of the photochemical cycle of bacteriorhodopsin upon changes of

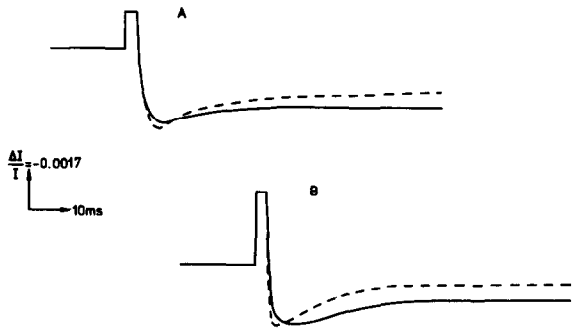

Fig.2. $\Delta\tilde{\mu}_{H^+}$-Depedence of the kinetics of the photochemical cycle of bacteriorhodopsin, measured at 660 nm. (A) Bacteriorhodopsin 1.5 mg/ml; soy-bean phospholipids 10 mg/ml; medium 150 mM KCl pH 6.0 temperature 27°C. The vesicle suspension was illuminated with 570 nm light. Full line: no additions; dashed line: 10 μM S-13. (B) Conditions as in legend to fig.2A, except for (i) the bacteriorhodopsin concentration: 1 mg/ml and (ii) the addition: 1 μM valinomycin plus 1 μM nigericin instead of S-13.

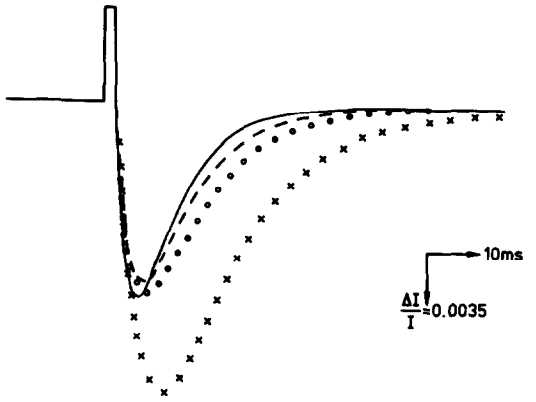

Fig.3. pH-Dependence of the kinetics of the photochemical cycle of bacteriorhodopsin. Purple membranes: 0.4 mg protein/ml, 150 mM KCl, temperature 28°C. (——): pH 6.7; (- - -): pH 5.8; (○): pH 4.5; (×): pH 3.85. Absorbance measurements were carried out at 660 nm.

the intensity of illumination and upon addition of ionophores as a manifestation of the regulatory effect of the $\Delta\tilde{\mu}_{H^+}$ on the kinetics of the photochemical cycle of bacteriorhodopsin.

Since the kinetics of the photochemical cycle of bacteriorhodopsin are pH dependent [see 25] – as would be expected for a regulated electrogenic proton pump – one might argue that the observed changes in the kinetics of the photochemical cycle of bacteriorhodopsin are due to changes in average – or intravesicular-pH rather than in $\Delta\tilde{\mu}_{H^+}$. With the data from fig.3 and fig.4, this explanation can be excluded. In fig.3 the pH-dependence of the photochemical cycle of bacteriorhodopsin is shown. Between pH 7 and pH 5 no large changes in the kinetics of O_{640} occur; it is not until pH 4 that large changes do occur. Under our conditions a $\Delta\tilde{\mu}_{H^+}$ of maximally one pH-unit can exist (estimated by use of ref. 28), which makes a pH – rather than a $\Delta\tilde{\mu}_{H^+}$ – regulation of the kinetics of the photochemical cycle of bacteriorhodopsin unlikely. In fig.4, the experiment of fig.1B is repeated, but now vesicles are used, that have an inverse direction of net proton movement. Therefore, in this preparation, the net pH change upon illumination is opposite to that in the experiment described in fig.1B. These liposomes too, show an increase in τ with increasing $\Delta\tilde{\mu}_{H^+}$ (due to a decrease in k_2; fig.4A) and a return to the original value of τ, induced either by the addition of S-13 or by the addition of valinomycin plus nigericin (fig.4B). This (fig.1B and fig.4) proves the regulatory role of $\Delta\tilde{\mu}_{H^+}$ rather than of pH on the kinetics of the photochemical cycle of bacteriorhodopsin.

Changes in extinction coefficient of bR^{LA}_{570} or one

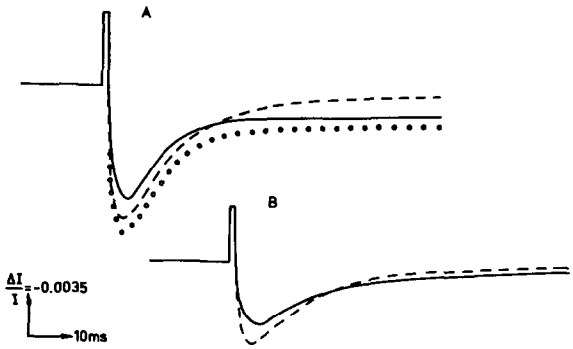

Fig.4. Effect of $\Delta\tilde{\mu}_{H^+}$ on the 660 nm kinetics of the photochemical cycle of bacteriorhodopsin in in vivo orientation. (A) Bacteriorhodopsin:1.5 mg/ml; cardiolipin: 0.6 mg/ml; 150 mM KCl pH 6.6; temperature 28°C. The vesicle suspension was illuminated with 570 nm light. Full line: no additions; dashed line: 10 μM S-13. Open circles: no background illumination, no additions. (B) Conditions as in the legend to fig.4A, except for (i) the pH: here pH 7.0 and (ii) the addition: 1 μM valinomycin plus 1 μM nigericin instead of S-13.

of its photointermediates due to electrochroism have not been reported until now. From (1) the consideration that under our experimental conditions low electrical potentials exist across the vesicle membranes [28]; (2) the finding that the observed effects of $\Delta\tilde{\mu}_{H^+}$ on the kinetics of the photochemical cycle were more sensitive to nigericin than to valinomycin; and (3) the very broad absorbance maximum of O_{640}, while the measurements were carried out near the maximum of O_{640} [24], we think that electrochroism will not have contributed to a significant extent to the effects of $\Delta\tilde{\mu}_{H^+}$ on the absorbance changes.

The possibility that bacteriorhodopsin can undergo more than one photochemical cycle [24,29–31] does not affect the relevance of our conclusions: All authors who discuss the possibility of bypass photochemical pathways [23,29,30,32 but contrast 24] seem to agree with others that the proton transferring photochemical cycle involves the O_{640}-intermediate [22,25,33]. Thus it is justified to take the O_{640}-intermediate as an indicator of the flow through that photochemical cycle that is involved in light-driven proton transfer across the liposomal membrane. Conversely our finding that the kinetics of the appearance of the O_{640}-intermediate are sensitive to $\Delta\tilde{\mu}_{H^+}$ in the predicted [28,34] manner, may further contribute to the appreciation of O_{640} as an intermediate in the proton transporting photochemical cycle.

A question to be elucidated in future experiments is the exact reaction between BRh* and O_{640} of which the kinetics are changed by the $\Delta\tilde{\mu}_{H^+}$. From the consideration that k_3 is independent of the $\Delta\tilde{\mu}_{H^+}$, it is tempting to conclude that both proton release and proton uptake in the photochemical cycle, occur before the decay of O_{640} to bR^{LA}_{570}.

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