BACTERIORHODOPSIN MONOMERS PUMP PROTONS

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1. Introduction

The most striking structural feature of the purple membrane (PM) of *Halobacterium halobium* is the arrangement of bacteriorhodopsin (BR) in a two-dimensional hexagonal lattice of protein trimers [2,3]. This raises the question whether this unusual state of aggregation is required for the function of BR as a light-driven proton pump. For other membrane transport proteins it is generally believed that only specific aggregates are functional. In order to answer this question for BR measurements were performed with a vesicle system [4], which is ideally suited to compare the properties of monomeric and hexagonally aggregated BR. By merely changing the temperature from below the lipid phase transition temperature to above, the state of aggregation of BR can be altered in a reversible manner from hexagonally aggregated to monomeric [4]. The results obtained show that monomeric BR itself is able to pump protons, most probably with an efficiency not significantly different from that of BR in the hexagonal array.

2. Materials and methods

BR was solubilized in the detergents Triton X-100 or octyl-β-D-glucoside [5] purchased from Packard Instrument Co. Inc. and Calbiochem, respectively. Using a detergent dialysis method, the BR monomers were incorporated into dimyristoylphosphatidylcholine (DMPC) vesicles according to [4] and into egg phosphatidylcholine (EPC) and asolectin vesicles. For DMPC vesicles the results were the same with both detergents. BR–EPC and BR–asolectin vesicles with normal spectroscopic properties and with the ability to generate a proton gradient could only be prepared with octylglucoside. The BR concentration was determined both spectroscopically using a molar extinction coefficient of 62 700 M^{-1} cm^{-1} at 568 nm [6] (vesicles bleached in the presence of hydroxylamine served as a reference) and by a modified Lowry method [7] corrected for a systematic error [6]. Lipid concentration was determined by phosphorus analysis [8]. Fluorescence depolarisation of the dye 1,6-diphenyl-1,3,5-hexatriene (DPH) as a function of temperature was measured using a Schoeffel RRS 1000 spectrofluorimeter. DPH (molar dye : lipid ratio of 1/800 to 1/4000) was excited at 360 nm and its fluorescence detected at 428 nm. Light-induced pH-changes of BR-lipid vesicles were measured in a thermostatted glass vessel equipped with a magnetic stirrer. A combined pH-electrode (Radiometer, Copenhagen, model GK 2321 C) was attached to a Radiometer pH meter model 51 connected to a W+W recorder 1200. The pH-changes were calibrated by the addition of small amounts (2–5 μl) of either 1 mM HCl or KOH. The temperature was continuously monitored in the chamber using a thermistor. Control experiments showed that the observed pH-changes are not due to light-induced changes in temperature (< 0.3°C). Circular dichroism measurements and flash photometric experiments were performed as in [9–11]. Further technical and experimental details are described in [12].

A preliminary report of this work was presented at the 23rd Annual Meeting of the American Biophysical Society [1]

3. Results and discussion

Pure DMPC vesicles exhibit a gel to liquid-crystalline phase transition at ~23°C. Figure 1 shows the lipid phase transition of BR–DMPC vesicles as monitored with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. Compared to protein-free DMPC vesicles the transition temperature has not changed. Due to the presence of BR the transition is broadened however, and the anisotropy of the fluorescence in the liquid-crystalline phase is increased. The exciton coupling effects in the visible circular dichroism spectrum (CD) can be used to distinguish between monomeric and aggregated BR [4,9,13]. In fig.1 the temperature dependence of the CD signal of BR–DMPC vesicles is plotted at the wavelength of maximal difference between the CD spectra of these two states of aggregation. This transition which is due to the change in protein aggregation clearly occurs at a

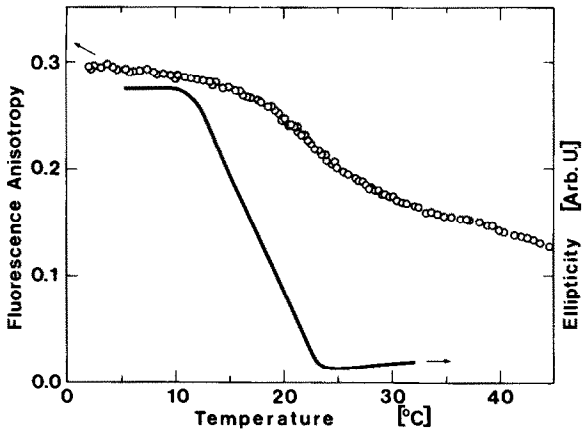

Fig.1. Thermotropic behaviour of BR–DMPC vesicles as monitored by fluorescence polarisation (open circles) and by circular dichroism (solid line). BR was solubilized using the detergent octylglucoside [5] and vesicles with a molar DMPC : BR ratio of 127 were prepared. The vesicles were suspended in 150 mM KCl (pH 6.4). Left ordinate: fluorescence anisotropy parameter, r, defined by:

$$r = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + 2F_{\perp}}$$

Molar DPH : DMPC ratio of 1/840. Right ordinate: ellipticity monitored at 610 nm. The temperature was increased at a scanning rate of 18°C/h.

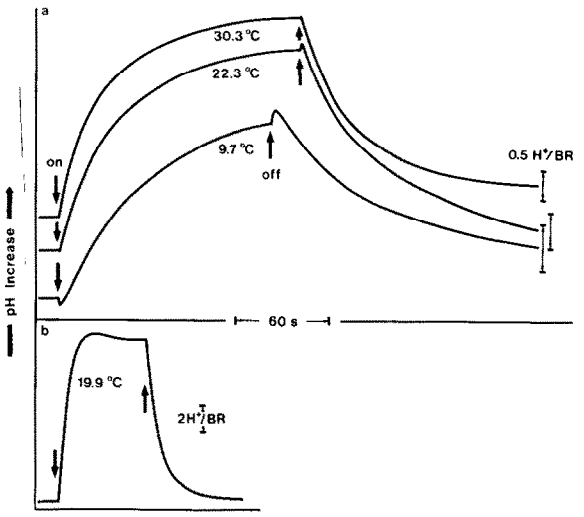

Fig.2. Light-induced pH-changes of BR–DMPC (a) and BR-asolectin (b) vesicles. Arrows depict onset and termination of the illumination period. Figures 1a,b have identical time scales. BR–DMPC vesicles (same vesicles as in fig.1) and BR–asolectin vesicles were suspended in 150 mM KCl (pH 6.4). Illumination conditions: 26 mW . cm^{-2}, 500 nm < λ < 800 nm.

lower temperature than the lipid phase transition. Furthermore, since plateau values are reached at the temperature extremes, the CD data indicate that no further change in the state of aggregation occurs below 11°C and > 23°C. Since above 23°C the CD spectrum shows no exciton features, all BR molecules are monomeric above this temperature.

Illumination of a suspension of these BR–DMPC vesicles results in a reversible alkalization of the external medium (fig.2a) i.e., in a light-induced proton translocation opposite to that observed in intact halobacteria. With increasing temperature (2–45°C) the magnitude of the overshoots decreases, the steady-state values of the alkalization, however, remain approximately the same (e.g., at 30°C ~3 H^+/BR, fig.2a).

In EPC and asolectin vesicles, which have the phase transition far below 0°C, BR is always in the monomeric state in the investigated temperature range above 2°C. This was substantiated by the absence of exciton bands in the CD spectrum and by the observation of fast rotational diffusion of BR [12]. The pH-recording of BR–asolectin vesicles at

20°C (fig.2b) also exhibits a large light-induced alkalization of the medium. The steady-state value corresponds to a net inward translocation of ~12 H^+/BR. Similar results were obtained with BR–EPC vesicles.

The light-induced alkalization is inhibited by proton-ionophores. This finding is in agreement with a transmembrane proton transport and rules out a simple association mechanism. Whereas the channel gramicidin D abolished the pH-gradient at all temperatures tested, the proton carrier carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) functioned only above the phase transition (see also [14]). In BR–DMPC vesicles CCCP almost completely inhibited the alkalization above 30°C. Lowering of the temperature below 30°C led in parallel with the lipid phase transition of the membrane (fig.1) to the reappearance of the light-generated proton gradient. The H^+/BR ratio observed below the transition was approximately the same as without the carrier. Since the measured H^+/BR ratios of up to 12 are much larger than those discussed for other possible mechanisms, e.g., stoichiometric and conformational protons [15], the protons must be pumped by BR. The H^+/BR ratios presented represent lower limits because they are calculated on the assumption that all BR molecules in the vesicles have the same orientation. Since this is most probably not the case the actual values of protons pumped per net oriented BR might be considerably higher. The population of misoriented BR molecules could explain the overshoot of the pH-response observable at lower temperatures after the onset of illumination ($\leq$0.1 H^+/BR, fig.2a). In BR–EPC and BR–asolectin vesicles, which generate a higher H^+/BR ratio, no overshoots occurred even at 2°C. The additional transient alkalization after termination of the illumination might be due to the final fast proton binding reaction of those BR molecules which were pumping into the vesicles.

The data obtained on the BR containing vesicles cleary indicate that the ability to pump protons is independent of BR aggregation state. Apparently light-driven proton transport can be performed both by BR molecules which are immobilized in a hexagonal lattice and by monomeric BR incorporated in an artificial more 'fluid' lipid environment. Preliminary results obtained on BR–DMPC vesicles indicate that the photochemical cycle of BR (which drives the proton pump) is qualitatively the same in the aggregated and monomeric state. This is in accordance with experiments on solubilized monomeric BR [5]. In the vesicles, both for aggregated and monomeric BR, the formation rate of the intermediate ^{411}T (M) is ~3-times faster as compared to BR in aqueous PM suspensions and its decay is slowed down ~10 times (pH 5.0). Under conditions of weak excitation intensities (< 7% excited BR molecules/flash) no pronounced break in the Arrhenius plot of the rate constant for decay of ^{411}T occurred between 5–50°C (K. D. Kohl, W. Sperling, N.A.D., M.P.H., unpublished). Recent experiments performed with continuous light excitation indicate that BR activity may be modulated by excited neighbouring BR molecules [16].

The similar photocycle kinetics of aggregated and monomeric BR (see above and [5]) may be regarded as an indication that the pumping efficiency is independent of BR aggregation state as well. This cannot be concluded from data such as those of fig.2a, since the rate and extent of proton-pumping are not only determined by the efficiency of the pump but may also be influenced by the membrane permeability and by the protonmotive force which may both in turn depend on the temperature. Although at 22°C the turnover-number of the photocycle in these vesicles is about twice as large as at 10°C the measured steady-state values of the alkalization are similar (fig.2a). The increase in the rate of back-diffusion of protons at higher temperatures, as indicated by the pH-traces after termination of the illumination (fig.2a), might at least partially explain this result.

The results presented also seem to exclude a requirement for specific lipids in the pumping and in the photochemical cycle, although the rate of at least one step in the cycle (i.e., ^{411}T decay, see above) is probably affected by the lipid environment (cf. [17,18]). Since the native PM lipids were not or only in part removed during preparation of the vesicles, this statement is not unambiguous. The native lipids, however, amount to < 10% of the total lipid content.

The conclusion that BR is monomeric above the lipid phase transition is based on CD measurements. Additional support comes from calculations of the size of the rotating particle derived from rotational diffusion measurements of BR in the same vesicle system [12,19]. The exciton CD effects will also disappear when extensive chromophore loss occurs. On the basis of absorption measurements and because the

exciton bands do occur below the phase transition, this explanation can be ruled out for our experiments. In [18] PM–phospholipid vesicles were subjected to prolonged sonication, which was accompanied by the disappearance of the exciton bands. Since the proton pump remained operational it was suggested that monomeric BR molecules are functional. This conclusion is only valid, however, if the disappearance of the exciton bands was due to the formation of monomers rather than to chromophore destruction which is well known to occur upon severe sonication [20].

It may be argued that either short-lived transient aggregates of BR formed by collisions or a very small number of permanent dimers or trimers are responsible for the observed pH-changes above the phase transition. Such aggregates would not be detected in the CD measurements. Each BR in the aggregate, however, would have to translocate an unreasonably high number of protons to create the observed pH-changes. In the case of transient aggregates the probability of collisions increases with increasing BR/lipid ratio and therefore the rate of proton translocation should increase too. This was not observed in our experiments.

Whereas assembly into specific aggregates seems to be a prerequisite for the function of other membrane transport proteins, monomeric BR itself can effectively pump protons. The photosensory function of BR was also shown to be independent of the crystalline structure of the PM [21]. The finding that monomeric BR is the functional unit may well be relevant for a further understanding of the molecular mechanism of this proton pump.

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