Correlation of photochemical cycle, H^+ release and uptake, and electric events in bacteriorhodopsin

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The kinetics of 3 photoinduced responses of bacteriorhodopsin have been compared: spectral changes, pH shifts in a suspension of open purple membrane sheets and electric potential generation by the sheets incorporated into a lipid-impregnated collodion film. In the presence of a pH-buffer, the H^+ release by bacteriorhodopsin was shown to correlate with the formation of the M412 intermediate and the microsecond phase of the potential generation. The H^+/M412 ratio is equal to 0.7 ± 0.1 if the ionic strength of the solution is high. In the absence of the buffer, the H^+ release proved to be much slower than spectral and electric responses. The kinetics of H^+ uptake by bacteriorhodopsin is close to M412 decay and to the electrogeneous millisecond phase in both the presence and absence of the pH buffer. The bacteriorhodopsin-induced proton release phase accounts for about 20%, and the uptake phase for about 80% of the overall potential. This is compatible with the model assuming that the proton start-out point – possibly, the protonated Schiff base connecting lysine 216 with retinal – is closer to the outer rather than the inner (cytoplasmic) surface of the bacterial membrane.

Bacteriorhodopsin *Retinal location pH indicator* *Photocycle* *Proton pump* Halobacterium halobium

1. INTRODUCTION

Bacteriorhodopsin is the light-driven H^+ pump of halophilic bacteria. It is the object of intense investigations involving most diverse physicochemical techniques [1,2]. Our group has evolved a direct method of monitoring bacteriorhodopsin electric activity [3–6]. Using a system obtained as a result of association of purple membrane sheets or bacteriorhodopsin proteoliposomes with a phospholipid-impregnated collodion film, we have studied the electrogeneous phases of bacteriorhodopsin photocycle. Three phases have been detected. The first, 'negative', phase, small in amplitude and opposite in direction to the others, is due to bacteriorhodopsin's conversion to intermediate K. We suggested that this phase is due to an intramembrane translocation of the charged (protonated) Schiff base caused by retinal isomerization [5]. The second (microsecond) and third (millisecond) 'positive' phases in the first approximation correlate with the formation and decay of the intermediate M412. They may be explained respectively by proton release from the Schiff base and by proton uptake from the opposite surface of the membrane with subsequent protonation of the Schiff base. Since the amplitude of the second phase is a fourth of the amplitude of the third phase, it was suggested that retinal is localized closer to the outer surface of the membrane where H^+ release takes place; otherwise, an additional supposition had to be advanced that the dielectric constant should be very heterogeneous throughout the membrane's thickness.

Other authors, using similar model systems [7–9] and a suspension of electric-field oriented purple membranes [10], found photoelectric

Abbreviations: Mes, 4-morpholineethanesulfonate; NP, *p*-nitrophenol

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response phases similar to those previously obtained in our group. On the other hand, the adopted schemes of bacteriorhodopsin polypeptide chain packing in the membrane [11,12] assume that the 216th lysine to which retinal is bound is localized closer to the inner membrane surface on which the proton is taken up; also, according to [13,14], the proton appears much later in the medium than the intermediate M412. Hence it was suggested [10] that a greater part of the millisecond phase might be due to proton release from bacteriorhodopsin on formation of intermediate O 640.

To resolve this discrepancy, we performed a series of experiments by monitoring (i) the bacteriorhodopsin spectral changes simultaneously with the pH changes in the suspension of purple membranes and (ii) the photopotential in the purple membrane-colloidal film system in response to a short laser flash.

2. MATERIALS AND METHODS

Purple membranes were isolated from the strain *Halobacterium halobium* 353-P as in [15]. For direct measurements of the bacteriorhodopsin-caused generation of electric potentials, the purple membranes were associated with a colloidal film impregnated with a decane solution of lecithin (70 mg/ml) in a medium containing 100 mM NaCl, 1 mM Mes, pH 6.8. The techniques for measuring electric photoresponses and photochemical cycle have been described [3–6]. Fast pH changes in the purple membrane suspension were monitored mainly as in [16]. Photoinduced changes in the absorbance of the purple membrane suspension at 400 nm were determined (with 100–300 single curves averaged) in the absence and presence of the pH indicator *p*-nitrophenol and then, by subtraction, the spectral response of the pH indicator itself was found. The pH responses were calibrated with small additions of HCl. All measurements were taken on light-adapted bacteriorhodopsin preparations. A Quantel Y6-481 Nd-YAG Q-switched laser with frequency doublers (532 nm, 15 ns, 50 mJ) was used as a light source.

3. RESULTS AND DISCUSSION

As seen from fig.1, under certain conditions the

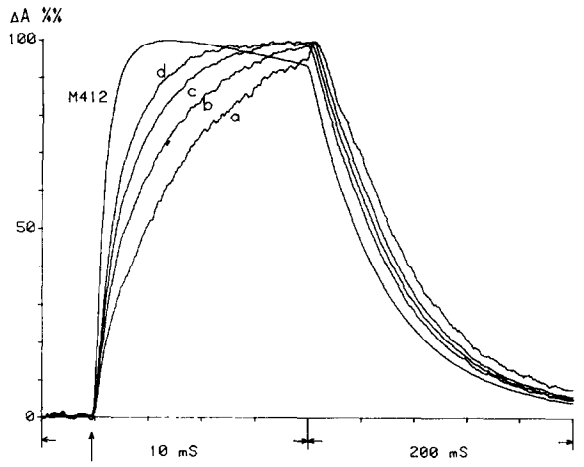

Fig.1. The effect of *p*-nitrophenol (NP) concentration on the rate of photoinduced pH changes in a purple sheet suspension. Incubation medium: 8 μM bacteriorhodopsin, 25 mM NaCl, pH 6.8, 5°C. M412 curve, formation and decay of M412. Curves a–d, pH changes (H^+ release and uptake): (a) 25 μM NP, (b) 50 μM NP, (c) 100 μM NP, (d) 200 μM NP. Here and in figs 2 and 4, the vertical arrow indicates the laser flash. All the measurements were taken at 400 nm. Here and in figs 2 and 4, the M412 curve is for the increase, and the pH curve for the decrease in absorbance at 400 nm.

kinetics of photoinduced release of H^+ monitored with *p*-nitrophenol in the suspension of open purple membrane sheets is slower than the kinetics of formation of M412, as noted in [13,14,16]. The conditions in question are low concentration of the pH indicator and absence of pH buffers in the medium. An increase in the pH indicator concentration leads to a decrease in the halftime of the pH-response rise (fig.1). Various substances which act as pH buffers, i.e., acetate, Mes, NH_2OH, phosphate, etc. (fig.2) have a similar effect. Yet, neither the buffers nor the indicator affect the M412 formation and relaxation kinetics. There is little likelihood that the hydrophilic pH buffers could in any way speed up proton movement within a bacteriorhodopsin molecule. It is more probable that they accelerate movement of H^+ from the membrane surface to the pH indicator in the water phase. Apparently such acceleration is essential if the measured pH changes occur on the microsecond time scale. On the millisecond scale, response of the pH indicator is not limited by H^+ diffusion in the water solution and pH buffers

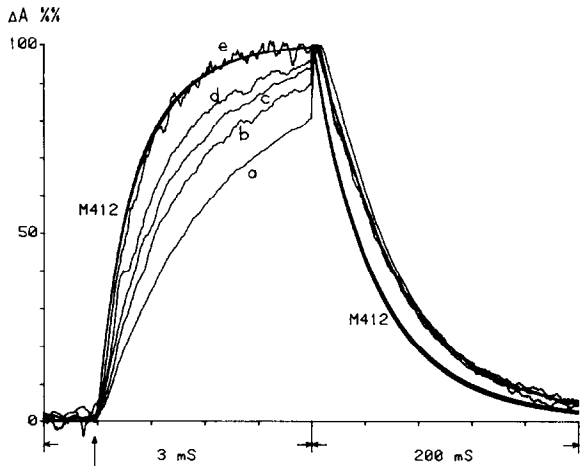

Fig.2. The effect of pH buffer (Mes) concentration on the pH change rate in the purple sheet suspension. Incubation medium: 8 μM bacteriorhodopsin, 100 mM NaCl, pH 6.8, 5°C. M412 curve, formation and decay of M412 (the curves are for different Mes concentrations, 0–4 mM). Curves a–e, pH changes (100 μM NP used in all cases): (a) without Mes, (b) 200 μM Mes, (c) 400 μM Mes, (d) 1 mM Mes, (e) 4 mM Mes.

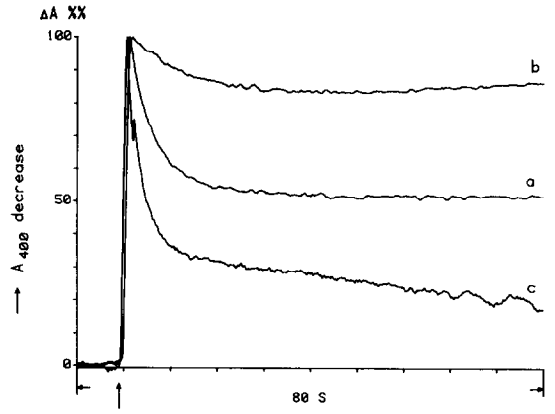

Fig.3. Change in absorbance at 400 nm in response to the addition of a small amount of HCl in a medium containing 8 μM bacteriorhodopsin, 500 mM NaCl, 25 μM NP, pH 6.8, 25°C (curve a). In the case of curve b, medium without bacteriorhodopsin was preboiled; in the case of curve c, 100 μM $NaHCO_3$ was added. Vertical arrow, addition of 6×10^{-6} M HCl.

have little, if any, effect on it. This interpretation is consistent with the mechanism proposed in [17] to explain the facilitating effect of pH buffers on H^+ diffusion in water. In this context, it may be noted that the acoustic method of pH response measurement showed even a somewhat higher rate of H^+ release than of M412 formation. The point is that using this technique the authors carried out measurements at high concentration of the pH buffer in the medium [18].

One should pay special attention to the nature of the calibration curve (fig.3). The pH indicator response to addition of small amounts of HCl or KOH follows a complex pattern: after a rapid change in the absorption at 400 nm, a relatively slow ($t_{1/2} \approx 4$ s at room temperature) partial relaxation of the response is observed. Analysis of the nature of these changes led us to the conclusion that the response observed is due to real pH changes in the suspension, but not to the artifacts of measurement. The proportion of the relaxation phase decreases with an increase in the concentration of the pH buffer or indicator itself. This relaxation is due to the presence of the carbonic acid buffer and the slow rate of equilibration between carbonic acid and dissolved CO_2. The relaxation amplitude is decreased significantly upon removal of CO_2 by boiling the incubation medium (fig.3b) and is sharply increased with a higher concentration of the carbonate buffer (fig.3c). Since the pH responses of bacteriorhodopsin are on the millisecond scale, it becomes obvious that the rapid response component only has to be taken into account in calibration.

Calibrating pH responses as described above and extrapolating the amplitude of pH and spectrum responses of bacteriorhodopsin to zero time, we determined the H^+/M412 ratio in 100–500 mM NaCl to be no higher than 0.7 ± 0.1 (molar extinction coefficient for M412 at 400 nm taken as 30000 $M^{-1} \cdot cm^{-1}$ [16]).

The ratio did not depend on the buffer concentration and excitation light intensity. The fact that the H^+/M412 ratio is less than 1 does not rule out the possibility of one bacteriorhodopsin molecule transporting two and even more protons if we assume that there are bacteriorhodopsin photoconversions which include intermediate M412 but which are not connected with proton transport. On the other hand, one should be very cautious about H^+/M412 values higher than 1: one cannot exclude errors in calibrating pH indicator responses without considering the CO_2 effect; in other cases, complications may arise because of the

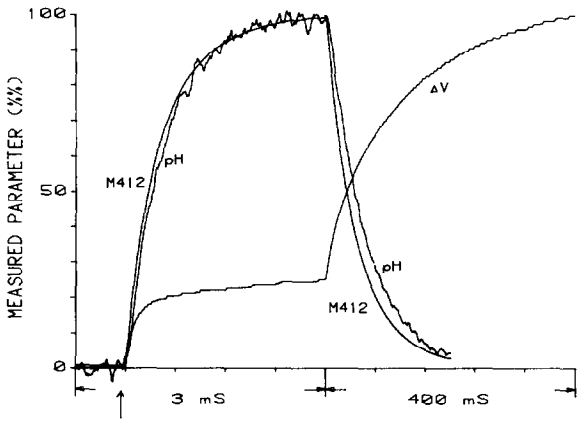

Fig.4. A comparison of the kinetics of the 3 photoinduced processes: formation and decay of intermediate M412, pH changes in the purple sheet suspension and electric potential generation (ΔV) in the collodion film–purple membrane system. Incubation medium: 100 mM NaCl, 1 mM Mes, 8 μM bacteriorhodopsin, pH 6.8, 5°C. 100 μM NP was added in the case of the pH curve.

aggregation of purple membranes in a high ionic strength medium.

Fig.4 combines the curves of the 3 processes: formation and decay of M412, proton release and uptake by open purple sheets and bacteriorhodopsin-induced generation of the membrane potential in response to a short laser flash. All the curves were measured in a medium with a buffer concentration which makes it possible to register fast kinetics of pH responses sufficiently well. It is clear that the process of M412 formation and of H^+ release from bacteriorhodopsin is accompanied by a generation of the potentials accounting for about 20% of the overall photoelectric response. The main portion of potential (about 80%) is generated under M412 relaxation and uptake of the proton by bacteriorhodopsin. The data shown in fig.4 were obtained at 5°C. Similar results were also observed at room temperature. However, because of the higher rates of the processes, the same correlation between the rates of M412 formation and H^+ release can be achieved only at higher concentrations of the buffer which lead to a decrease in the amplitudes of pH responses and to a tangible lowering of the signal-to-noise ratio.

The scheme depicted in fig.5 may furnish an in-

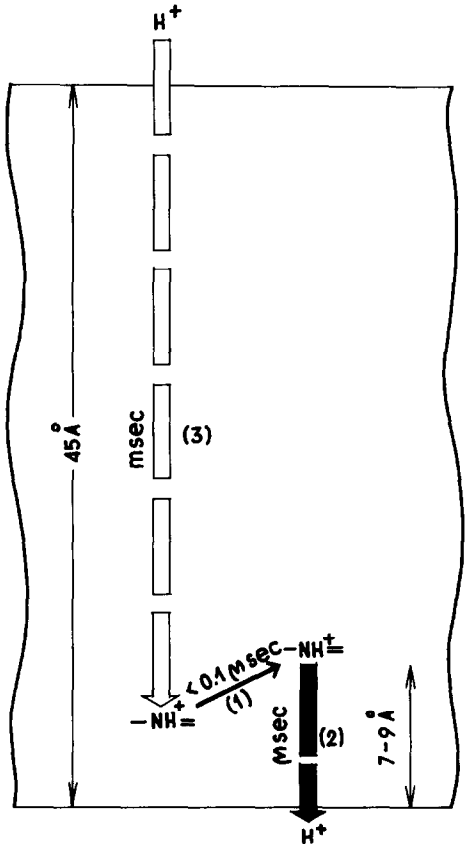

Fig.5. The proposed scheme for retinal location and generation of the main electrogenic phases of a bacteriorhodopsin photoresponse. (1) Photoisomerization of retinal resulting in the translocation of the protonated Schiff base toward the cytoplasmic surface of the purple membrane. (2) H^+ transfer from the protonated Schiff base toward the outer membrane surface. (3) H^+ transfer from the membrane cytoplasmic surface to the deprotonated Schiff base.

sight into the mechanism of the phenomena observed. Here we proceed from the concepts of one-proton transfer. It is presumed that the absorption of a quantum of light induces the isomerization of retinal; the concomitant intermembrane translocation of the positively charged Schiff base toward the cytoplasmic surface of the purple membrane is monitored as a negative phase of the photoelectric response. The Schiff base shifts into a new environment favorable for proton detachment. In other words, the translocation of the Schiff base in the membrane

results in an acid shift of its pK. The deprotonation of the Schiff base is accompanied by a strong spectral change (formation of intermediate M412). The released proton is monitored by the pH indicator, while the intramembrane dipole formed as a result of the proton transfer is measured as a microsecond phase of the potential generation. The protonation of the Schiff base by H^+ transported from the purple membrane cytoplasmic surface is responsible for the millisecond phase of the membrane potential generation. The 4-fold lower microsecond phase amplitude is due to the retinal being positioned 4-times closer to the outer surface of the purple membrane than to the cytoplasmic one.

Studies on fluorescent energy transfer [19–21] also testify to the asymmetric position of retinal at a distance which does not exceed 10 Å from one of the purple membrane surfaces. The surface to which the retinal is closer has not been determined [19,20] and the conclusion about its location closer to the cytoplasmic surface does not appear unequivocal [21]. So far we have no indications of the high heterogeneity of the dielectric constant throughout the thickness of the purple membrane. It should also be noted that our conclusion about retinal's location being closer to the outer surface of the purple membrane is in good agreement with a scheme of the bacteriorhodopsin structure [22] according to which Lys-216 is localized in the vicinity of the outer surface of the membrane.

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