CONTROL OF THE PHOTOCYCLE IN BACTERIORHODOPSIN BY ELECTROCHEMICAL GRADIENTS

A. T. QUINTANILHA

Membrane Bioenergetics Group, Energy and Environment Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720, USA

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1. Introduction

Bacteriorhodopsin, the retinal containing protein in the purple membrane of *Halbacterium halobium* functions as an electrogenic light-activated proton pump [1]. Illumination of suspensions of sub-bacterial particles or of reconstituted bacteriorhodopsin-containing liposomes leads to acidification or alkalinization respectively of the suspending medium [2]. Direct measurements of transient pH changes in purple membrane fragments using laser flash photolysis and indicator dyes [3] and time-resolved resonance Raman spectra [4] have indicated that proton release and uptake is associated with the formation and decay of the M_{412} intermediate of the photocycle. It is reasonable to suspect that any energy-requiring membrane-localized proton-pump will be influenced by the electrochemical proton-motive force ($\Delta\mu_{H^+}$) it can generate across the membrane. In fact, the decay kinetics of the O_{640} intermediate of bacteriorhodopsin photocycle has already been shown to be sensitive to $\Delta\mu_{H^+}$ [5–7]. Other systems can be found among the various reversible ATPase-synthetases in mitochondria [8], bacteria [9] and chloroplasts [10]. Here I show that the steady state concentration and decay kinetics of the M_{412} intermediate of bacteriorhodopsin in reconstituted liposomes depend on $\Delta\psi$, the electrical potential gradient, and to a lesser extent on ΔpH, the pH gradient established across these membranes.

2. Materials and methods

Purple membranes were prepared from *H. halobium* by standard procedures [11] and suspended in 0.2 M KCl, 5–10 mM phosphate buffer (pH 7) at 5–6 mg protein/ml (0.192–0.231 mM).

Asolectin containing 95% soy phosphatides was obtained from Associated Concentrates, Woodside, Long Island, and was partially purified by acetone extraction [12]. For the reconstitution of bacteriorhodopsin containing liposomes, 40 mg partially purified lipids were dried under a stream of nitrogen, redissolved twice in a small volume of ether and dried again under nitrogen for ~30 min. To this dried preparation, 2 ml 0.2 M KCl, 5 mM phosphate buffer (pH 7) was added and sonication of this suspension was performed in a bath-type sonifier (Laboratory Supplies Co., Hickville, NY) under nitrogen for ~5 min. To this milky suspension, 2 mg bacteriorhodopsin were added and the mixture was further sonicated under nitrogen for 10–20 min until it acquired the opalescent aspect typical of liposome suspensions. The photostationary state of M_{412} was measured in an Aminco DW-2 spectrophotometer, and the M_{412} concentration was calculated assuming $\Delta\epsilon_{412} = 23\,000\ M^{-1} \cdot cm^{-1}$ [3]. The photomultiplier was protected by a Beard Atomic interference filter (412 nm transmission maximum). A phase-R dye laser with rhodamine 575 (0.2 j/flash, 150 ns flash risetime) was used for the flash experiments; the rise and decay of M_{412} was measured in a flash photolysis apparatus, using a monochromatic measuring beam, and stored in a Biomation 1010 transient recorder interfaced to a PDP 11/34 computer. Usually, 10–20 transients were sampled and accumulated to improve the signal:noise ratio. For steady state illumination studies, a quartz iodide lamp was used through a Corning 3-67 low wavelength cut-off filter (light intensity at the sample ~40.5 mW/cm^2). Bacteriorhodopsin concentration for these studies was ~4 μM

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protein. Light-induced transmembrane pH gradients, electrical potentials and volume changes in the reconstituted liposomes were measured using spin probes as in [13–16]. A spin-labeled amine, tempamine [13–15] was used to measure the ΔpH, a spin-labeled phosphonium ion [16] was used to measure the $\Delta\psi$ and an uncharged spin probe (Tempol) was used to measure the volume changes [13]. The main advantage of spin probes for these types of measurements has already been discussed [13,15,16] and lies in the fact that membrane-bound and aqueous-free populations of the probe can be clearly distinguished and the signal from the external aqueous population can be broadened by $K_3Fe(CN)_6$ which is membrane impermeable. Bacteriorhodopsin concentration in these studies was ~40 μM, lipid concentration was ~1.8% (w/v), and the spin probe concentration was ~50 μM and did not interfere with the photocycle. Ferricyanide, when used, was at 90 mM. Tempamine was obtained from Aldrich Chemical Co., the phosphonium spin label was a gift from Dr W. Hubbell and the uncharged spin probe was a gift from Dr R. J. Mehlhorn.

Valinomycin was obtained from Sigma and Nigericin was a gift of Dr W. E. Scott (Hoffmann-LaRoche Inc, Nutley, NJ). Unless otherwise indicated, all the results were obtained at 20°C.

3. Results

The absorption spectra of bacteriorhodopsin and bacteriorhodopsin reconstituted liposomes are shown in the inset of fig.1 at the same protein concentration. Under steady state illumination, the maximum pH gradient measured by the uptake of the spin-labeled amine in reconstituted liposomes was 0.6–1 with a half-time rise of ~10–30 s and was biphasic.

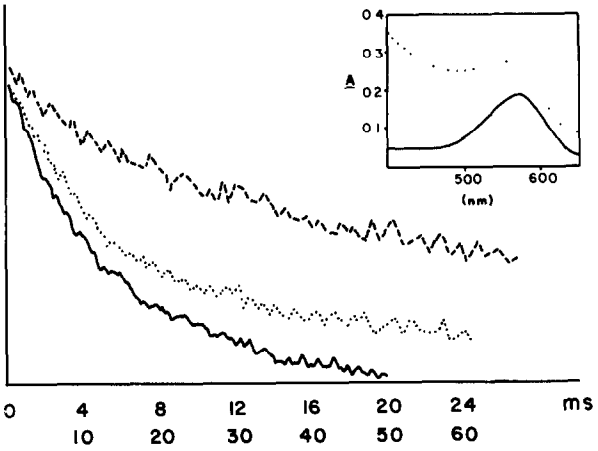

Fig.1. Decay curves for the M_{412} intermediate measured as in section 2 using flash photolysis: (——) purified bacteriorhodopsin (upper scale in steps of 4 ms); (• • •) reconstituted bacteriorhodopsin without steady state illumination; (– – –) reconstituted bacteriorhodopsin under steady state illumination (the latter two are plotted using the lower scale in steps of 10 ms); protein 0.2 mg/ml (~8 μM); temp. 17°C. Inset: Absorption spectra of purified (——) and reconstituted (• • •) bacteriorhodopsin: protein 0.125 mg/ml (~5 μM). In the reconstituted preparation, lipid was ~2.5 mg/ml.

Under the same conditions, the maximum $\Delta\psi$ measured by the change in partitioning of the spin-labeled phosphonium probe was 60–80 mV with a half-time rise which could not be resolved but was certainly <0.5 s. For the calculation of $\Delta\psi$, the ratio of internal to external volumes is needed. Measurements with the uncharged spin probe gave ~1/104. Volume changes under steady state illumination required for the determination of ΔpH were also measured with the uncharged spin probe, and found to be negligible.

The M_{412} steady state values for bacteriorhodopsin and reconstituted bacteriorhodopsin are shown in table 1. The half-time for the rise in M_{412} steady state

Table 1
M_{412} steady state concentration in purified and reconstituted bacteriorhodopsin

	Control	Valinomycin (0.4 μM)	Nigericin (0.3 μM)	Nigericin (1 μM) + valinomycin (0.2 μM)
Bacteriorhodopsin[a]	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
Reconstituted bacteriorhodopsin[a]	0.32 ± 0.06	0.06 ± 0.02	0.38 ± 0.06	0.03 ± 0.01

[a] The bacteriorhodopsin concentration in these studies was always 4 μM and the lipid concentration in the reconstituted system was 1.8% (w/v). Measurement of M_{412} steady-state was as in section 2 at 20°C

is probably instrument-limited but cannot be >1–2 s. It is clear that the steady-state value of M_{412} in the reconstituted bacteriorhodopsin liposomes is at least one order of magnitude larger than in purified bacteriorhodopsin. In the absence of swelling, this large value of steady-state M_{412} in the reconstituted system could not be mistaken for light scattering changes. Furthermore, swelling would be expected to follow the kinetics of ΔpH generation, which is slow.

Flash photolysis measurements of the half-time for rise of M_{412} indicate that it is ~3-times faster in the reconstituted system (0.02 ms) than in the purified system (0.07 ms) at the same temperature (20°C). The decay kinetics of M_{412} are often biphasic. Typical decay curves are shown in fig.1; as is clearly seen, the decay of M_{412} in the reconstituted system is much slower than in the pure bacteriorhodopsin system.

3.1. *The effect of ionophores and permeable ions*

The absorption spectrum of bacteriorhodopsin and reconstituted bacteriorhodopsin containing liposomes did not change appreciably upon addition of μM levels of nigericin (K^+/H^+ exchange) or valinomycin (K^+ ionophore) or mM levels of NH_4Cl or KSCN.

Valinomycin, at ≲0.4 μM and under steady state illumination, gradually collapsed Δψ to ~10% of the control value. Under the same conditions the half-time for the rise in ΔpH was much smaller (~5 s) but the actual ΔpH was only 10–20% greater than the control value. Changes in the steady state value of M_{412} followed very closely the changes in Δψ (fig.2a). No change in the half-time for the rise of M_{412} in the flash photolysis experiments was detected by the presence of valinomycin, but the half-time for the decay was shorter.

Nigericin, at ≲0.3 μM, and under steady state illumination, increased Δψ by ~10–20% of the control value and collapsed the ΔpH to almost zero. At higher concentrations of nigericin, Δψ decreased. Changes in the steady state value of M_{412} followed very closely the changes in Δψ up to 0.5 μM nigericin and decreased steadily at higher concentrations (fig.2b). Again, no change in the half-time for the rise of M_{412} (under flash photolysis) was detected in the presence of nigericin (<0.3 μM) and the half-time for the decay also did not seem to be affected.

In the presence of both nigericin (1 μM) and valinomycin (0.2 μM), under steady state illumination, both Δψ and ΔpH were reduced to <20% of the control value. The steady state value of M_{412} was <10% of the control (close to the value measured in isolated bacteriorhodopsin) and no change in the half-time for the rise of M_{412} (under flash photolysis) was detected, whereas the half-time for the decay, was shorter.

Ammonium chloride has often been used to collapse pH gradients [17]. At 100 mM NH_4Cl, under

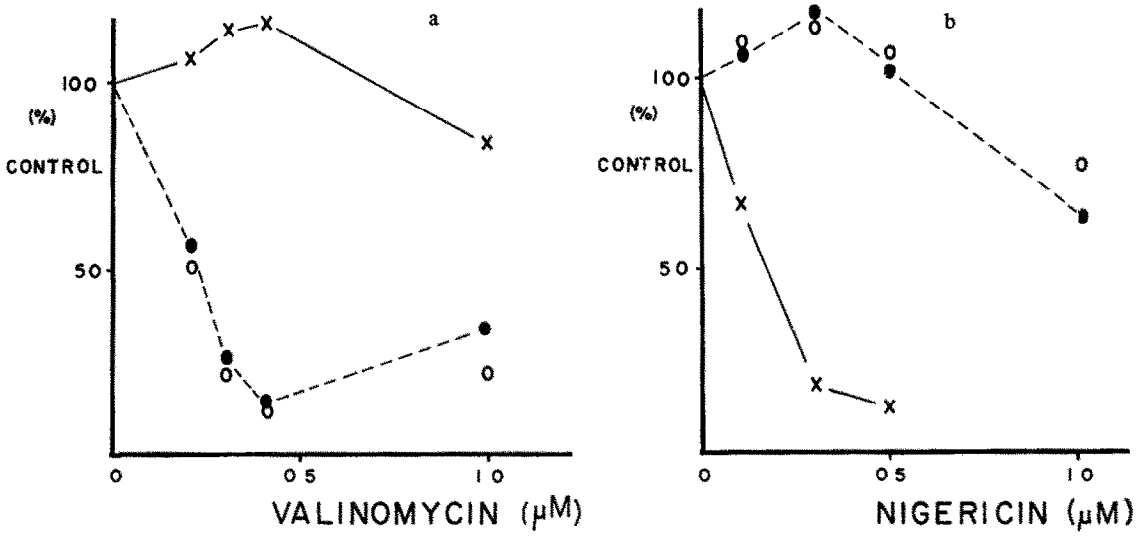

Fig.2. Steady state values of M_{412} (•) transmembrane potential, Δψ (○) and pH gradients, ΔpH (X) as a function of (a) valinomycin and (b) nigericin concentration. Measurements were as in section 2.

steady state illumination, the formation of a ΔpH could not be detected, whereas $\Delta\psi$ and the steady state levels of M_{412} remained at control levels.

Potassium thiocyanate has also been used to collapse $\Delta\psi$ [18]. At 100 mM KSCN, under steady state illumination, the formation of $\Delta\psi$ could not be detected, but ΔpH was also considerably reduced (40% of the control value). The steady state level of M_{412} was ~5% of the control and close to the value measured in isolated bacteriorhodopsin.

3.2. *The effect of steady-state light-induced electrochemical gradients on the kinetics of rise and decay of M_{412}*

In the presence of steady-state illumination (i.e., in the presence of $\Delta\mu_{H^+}$), the kinetics of the rise and decay of the M_{412} intermediate were followed in the flash photolysis apparatus. No alteration on the rise of M_{412} could be detected, whereas the decay was considerably slower (fig.1). The effect of ionophores (valinomycin and/or nigericin) was also studied. At 0.3 μM, nigericin had only a small effect on the decay kinetics, whereas at the same concentration, valinomycin decreased the half-time for decay by ~30%. Together, valinomycin (0.2 μM) and nigericin (1 μM) decreased the half-time for decay even further (>50%). We were not able to detect any changes in the half-time for the rise of M_{412} under any conditions.

4. Discussion

When illuminated, our reconstituted bacteriorhodopsin generated a $\Delta\psi$ ~60–80 mV and a ΔpH ~0.6–1.0 (acidic and positive inside) depending on the sample. To specifically collapse $\Delta\psi$ and ΔpH, valinomycin and nigericin were used, respectively, because they were effective at very low concentrations (≤0.4 μM); together, at <1 μM they were used to abolish $\Delta\mu_{H^+}$. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S_{13}) and 3,5-di-*tert*-4-hydroxybenzilidene malonitrite (SF 6847) were not used as protonophores, because large concentrations (>50 μM) have to be used to collapse $\Delta\mu_{H^+}$ (unpublished; [7]). Ammonium chloride effectively abolished ΔpH, but potassium thiocyanate, in our hands, decreased both $\Delta\psi$ and ΔpH. The observation that the steady-state level of M_{412} is reached in a few seconds, whereas the half-time for the rise in ΔpH is 10–30 s, suggests that it is the $\Delta\psi$ (rise-time < 0.3 s), which principally controls the accumulation of M_{412}; the fact that, in the presence of ammonium chloride, ΔpH ~ 0, but both the $\Delta\psi$ and the steady-state levels of M_{412} remained at control values, further supports our view. However, under conditions where $\Delta\psi$ is virtually undetectable, a collapse in ΔpH will further slightly accelerate the photocycle and decrease the steady-state of M_{412} to the levels measured in purified unreconstituted bacteriorhodopsin.

The greater accumulation of M_{412} in the reconstituted system suggests a faster rise time and a slower decay time for this intermediate. In fact, the rise kinetics of the M_{412} were ~3X faster in the reconstituted system, but were not affected by the presence of steady-state illumination or ionophores. The decay kinetics of M_{412} were also much slower in the reconstituted system, but could be substantially slowed down in the presence of steady-state illumination and accelerated in the presence of ionophores which collapsed $\Delta\psi$ or $\Delta\mu_{H^+}$.

The decay kinetics of the O_{640} intermediate, which arises after the M_{412} in the photocycle, is slower under steady-state illumination [5] and can be accelerated by nigericin + valinomycin or S_{13} [7]. Although our results are compatible with these observations, we find that the major controlling mechanism can be ascribed principally to an effect of $\Delta\psi$ on the decay kinetics of M_{412} (i.e., the reprotonation of the Schiff base).

The absence of an effect of $\Delta\psi$ or ΔpH on the rise kinetics of M_{412} may not be surprising, since the quantum yield of M_{412} is known to be independent of salt concentration [19] and not very sensitive to pH in the range pH 7 ± 1 [1]. The relatively small effect of ΔpH on the decay kinetics and on the steady-state level of M_{412} may also not be surprising; unpublished observations (S. Tristram and P. Sullivan, personal communication) suggest that the steady-state levels of M_{412} in the purified bacteriorhodopsin vary by <10–20% as the pH is increased from 6–8. The decay kinetics of this intermediate are also known [1] to be only slightly sensitive to pH in the above range. The strong effects of $\Delta\psi$ on the decay kinetics of M_{412} (usually associated with the reprotonation of the Schiff base) could be ascribed to an increase in the activation free-energy for the reprotonation, which may be due to the fact that an electric field could: (a) repel protons away from the Schiff base; or (b) produce conformational changes

in the protein thereby altering the relative distances between specific groups that seem to be important for this process [20].

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