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Time-course and stoichiometry of light-induced proton release and uptake during the photocycle of bacteriorhodopsin

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The kinetics and stoichiometry of light-induced proton release in purple membrane suspensions have been investigated using the pH-indicator dye pyranine and single-turnover flash spectroscopy at a time resolution of 0.1 μs. The number of protons detected by pyranine is inversely proportional to the buffering power of the medium and 1.1 ± 0.2 protons are released per photocycling bacteriorhodopsin molecule (pH 7.0, 20°C). At high ionic strength (250 mM KCl) and higher phosphate buffer concentrations (1 mM) the proton release lags slightly behind the formation of the intermediate M-412 and proton reuptake by the purple membrane parallels the slow decay process of M-412 or the decay of O-640.

Bacteriorhodopsin *Proton pump* *Purple membrane* *pH-indicator* *Pyranine* *Flash spectroscopy*

1. INTRODUCTION

Numerous investigations have been performed to determine the stoichiometry and transport kinetics for the light-driven proton pump bacteriorhodopsin (BR) in the purple membrane (PM) of halobacteria with the aim to understand its function at a molecular level (recent reviews [1–3]). Time-resolved flash spectroscopy in combination with optical pH-indicators has been applied to monitor the light-induced proton release and uptake by the PM and to correlate these events with the spectral changes during the photocycle of BR [4–10]. According to these experiments, the proton(s) appear(s) in the aqueous bulk phase surrounding the PM sheets after formation of the photocycle intermediate M-412 [3,5,6,8,10]. On the other hand, resonance Raman measurements show that the Schiff's base, the linkage between the chromophore retinal and the protein moiety, which is assumed to be part of the active center, is deprotonated during formation of M-412 [11]. To make both findings agree, either the Schiff's base proton is not the pumped one or a long delay exists between deprotonation of the active site and the release into the aqueous bulk phase. Also the number of protons translocated per cycling BR is controversial. The first findings yielded a value of 0.7–1 H^+ per intermediate M-412 [5,6], however, from subsequent measurements 1.6–4 H^+/M-412 have been obtained, at least at high ionic strength [8,9,12–15]. The higher stoichiometry led to the proposal of molecular models for BR's pumping mechanism, postulating other proton donating groups (e.g., tyrosine) in addition to the Schiff's base. Nowadays, a H^+ to M-412 ratio of 2–3 is generally accepted [3], although recently a stoichiometry of only 0.7 was stated, both at low and high ionic strength [16].

The present investigation convincingly demonstrates that at pH 7.0 one proton per cycling BR is released during the formation of M-412 and is subsequently taken up during the decay of this or the O-640 intermediate.

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2. MATERIALS AND METHODS

Purple membrane was isolated from strain ET1001 as described elsewhere [17]. Pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate, laser grade, Kodak), KCl, Na_2HPO_4, NaH_2PO_4 (p.a., Merck) were used without further purification. Bidistilled water was utilized for all preparations (standard: 10 μM PM, 50 μM pyranine, pH 7.0). Before measurement all samples were routinely sonicated in a bath-type sonicator for 30 s in order to disaggregate clusters of PM [14] and degassed afterwards at 0.1 Torr for 3 min, i.e., until there was no further significant development of air bubbles. After pH adjustment the fluorescence cuvettes containing the sample were tightly closed by a Teflon stopper and flash spectroscopic experiments were carried out at once (20°C). Following these measurements, pyranine containing samples were titrated by known amounts of HCl and KOH, and pH values as well as absorbance spectra were recorded. For flash spectroscopic experiments a home-built apparatus was used. Excitation of the BR photocycle was achieved by a Rhodamine 6G dye laser. Excitation pulse length was 5–10 ns (1 mJ) and excitation wavelengths had a broad distribution (20 nm FWHM) centered at 580 nm. Flash-induced transmission changes of the light-adapted sample were detected in 90° geometry and under magic angle polarization. Throughout the measurements the detection electronics were adjusted to an intrinsic time constant of 0.1 μs. Signals were then stored in an Iwatsu DM 901 transient recorder, gated by an external, logarithmic time base, spanning the time range of 0.1 μs to 2 s. 200 signals from flashes at a repetition rate of 0.1 Hz were averaged, and for each flash, transmission before the flash and laser intensity were recorded for normalization purposes. Excitation of PM did not exceed 10% and varied according to the flash intensity in a strictly proportional way. Transmission changes were converted to absorbance differences and up to 7 exponentials could be fitted to the time-dependent data by the program DISCRETE [18].

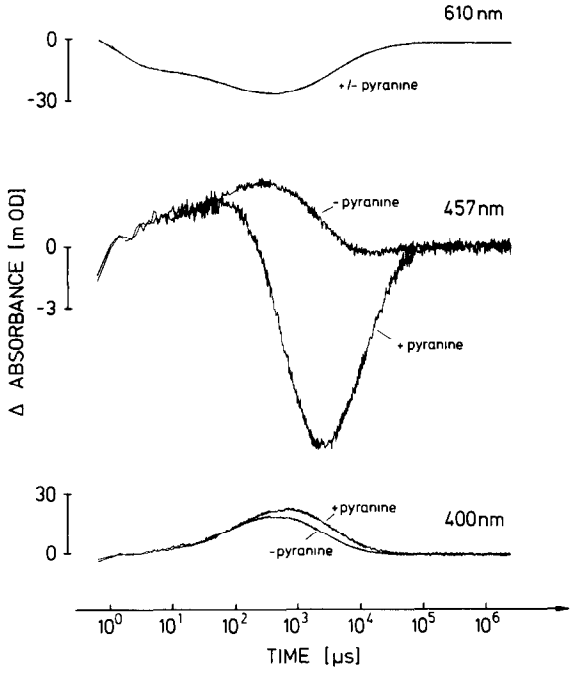

Fig.1. Flash-induced absorbance changes (optical pathlength 1 cm) at three different wavelengths of 10 μM PM, 250 mM KCl solutions with (+) and without (−) 50 μM pyranine (pH 7.0, 20°C).

3. RESULTS AND DISCUSSION

3.1. *Stoichiometry of proton release per cycling BR*

In order to elucidate the kinetic correlation between M-412 intermediate and pH changes in the medium, and to determine the stoichiometry of protons translocated per cycling BR, PM suspensions have been investigated by means of flash spectroscopy using the pH-sensitive dye pyranine. This dye is found to be an excellent pH probe for such a purpose [19,20]. The absorption maximum of its unprotonated form (pK 7.2) is at 457 nm, a wavelength where absorbance changes due to the BR photocycle for times longer than 1 μs are minimal, i.e., this wavelength is near a quasi-isosbestic point between M-412 and the ground state all-*trans* BR-568 [4,5,21]. The absorption maximum of protonated pyranine is at 402 nm. As the protonation reaction is responsible for absorbance changes of this dye, its intrinsic response to pH changes in the medium is very fast (k_{prot} = 1.8 × 10^{11} $M^{-1} \cdot s^{-1}$) [22]. For net negatively charged membrane systems like PM, pyranine is localized in the aqueous bulk phase due to its 3–4 negative charges. Flash-induced absorbance changes of aqueous PM suspensions in the presence and

absence of pyranine were followed at three different wavelengths (fig.1): 610 nm (decay of K-610, formation and decay of L-550, formation and decay of O-640, recovery of ground state BR-568; no absorbance changes due to pyranine), 457 nm (quasi-isosbestic point of M-412 and ground state BR-568; decay and formation of deprotonated pyranine) and 400 nm (decay of K-610, formation and decay of L-550, formation and decay of M-412, formation and decay of protonated pyranine). At 610 nm no difference in absorbance changes of samples with and without pyranine is detectable. The signals at this wavelength could be fitted by 5 exponentials. The apparent absorbance increase for times longer than 1 ms is due to the formation and decay of O-640 as well as to the recovery of the ground state BR-568. Nevertheless, in the sum of the amplitudes of exponentials with time constants longer than 1 ms up to the end of the photocycle, the contributions of O-640 should cancel because the formation as well as the decay of this intermediate happen within this time range. Therefore this sum was taken as a measure for the number of BR molecules that had undergone photocycle. An extinction coefficient of 31000 $M^{-1} \cdot cm^{-1}$ for ground state all-*trans* BR-568 at 610 nm was taken from ground state absorption spectra ($\epsilon_{BR,568}$ = 63000 $M^{-1} \cdot cm^{-1}$ [23]). At 457 nm strong absorbance changes are observed in the presence of pyranine corresponding to the disappearance and reappearance of the deprotonated dye. This feature is reversed in sign at 400 nm due to the formation and decay of the protonated pyranine. Absolute absorbance differences of samples with and without pyranine, however, are 1.6-times larger at 457 nm, since the molar extinction of deprotonated pyranine is larger. This information can be used as an independent control in order to discriminate between spectral contributions at 400 nm due to formation of M-412 or to pyranine protonation. Pure pyranine signals corresponding to the transient protonation of the medium (and the deprotonation of the PM) are calculated as the difference of absorbance changes in samples with and without pyranine. These changes are normalized by the described sum of preexponential terms at 610 nm and corrected for slight variations of excitation laser power. Such signals (fig.2) are directly proportional to the number of protons detected

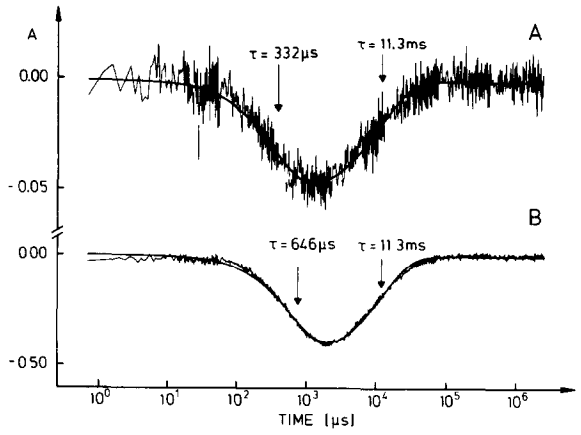

Fig.2. Pure pyranine absorbance changes at 457 nm, calculated as the difference of samples with and without pyranine. Signals are normalized to the ground state BR-568 recovery amplitudes at 610 nm. Smooth lines depict biexponential fits to the data points. (A) 10 μM PM, 250 mM KCl, 500 μM phosphate; (B) 10 μM PM, 250 mM KCl.

by pyranine per cycling BR molecule. Whereas fig.2B shows an experiment where no phosphate buffer was used, the sample of fig.2A includes 500 μM phosphate. Obviously in the buffered case the concentration of protonated pyranine is smaller. Additionally, an acceleration of the detection by pyranine of the released protons is observed. All pyranine signals are fitted by only two exponentials corresponding to the formation and decay of protonated pyranine. If there is a protolytic equilibrium between deprotonated PM and the surrounding medium in the millisecond time range, one expects that the number of additionally protonated pyranines, i.e., the amplitude of the pyranine signal should be inversely proportional to the buffering power of the PM suspension. This assumption was tested by variation of the phosphate content (0–2 mM) of the sample (fig.3). Experimental points were approximated by a straight line fit and the data are plotted in a double logarithmic way. Obviously the amplitude of the pyranine signal has a finite value at zero phosphate concentration. This corresponds to the rest buffering power of the suspension due to the PM, added dye, and water.

From the slope of the straight line fit a calculation of released protons per cycling BR (as

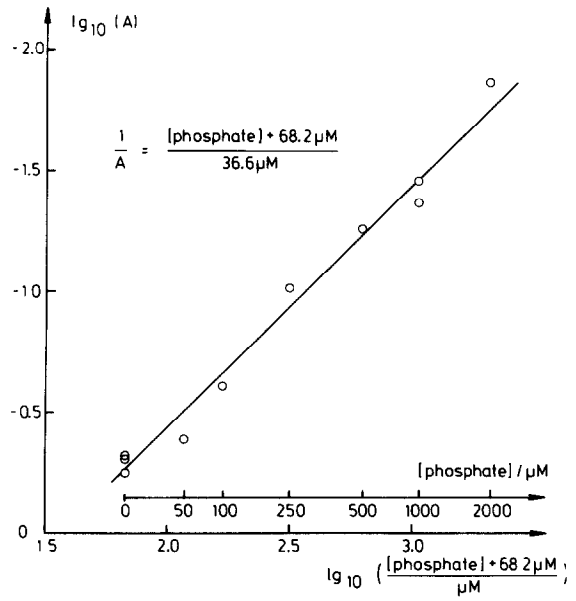

Fig.3. Dependency of pyranine signal amplitudes at 457 nm (fig.2) on the phosphate buffer content of the samples. Data were approximated by a straight-line fit (offset $-68.2\ \mu M$, slope $(36.6\ \mu M)^{-1}$).

estimated from the 610 nm ground state depletion) is possible according to eqn 1:

$$\frac{[\Delta H^+]}{[BR]_{cycl.}} = \frac{d[phosphate]}{d(A^{-1})} \times \frac{\epsilon_{BR,610}}{[pyranine]} \times \frac{dpH}{d\epsilon_{pyr,457}} \times \frac{dB_{phosphate}}{d[phosphate]} \quad (1)$$

where A represents the normalized (with respect to ground state recovery) pyranine signal amplitude at 457 nm (figs 2,3). A value of $11\,100\ M^{-1}\cdot cm^{-1}\cdot(pH\ unit)^{-1}$ for $d\epsilon_{pyr,457}/dpH$ was determined by titration of the pyranine containing PM suspensions with known amounts of KOH and HCl, and a buffering power per phosphate molecule ($dB_{phosphate}/d[phosphate]$) of $0.56\ (pH\ unit)^{-1}$ is taken at pH 7.0 [24]. The error of the slope in fig.3 is 5%. Assuming a maximal error of 10% for all other values in eqn 1, the number of protons per cycling BR is calculated as 1.1 ± 0.2. It should be noted that at low phosphate concentrations, the buffering power of the sample as determined by titration varied strongly (smd = 27%, $n = 4$) with the CO_2 content of the suspension (see also [16]), whereas the pyranine amplitude in the flash experiments was much less affected (smd = 11%). This indicates that in the millisecond regime there is no exchange of physically and chemically solved CO_2, whereas in the longer time range of statical titrations this exchange leads to an additional buffering power resulting in an overestimation of pumped protons in the millisecond time range.

Since for all samples also the formation and decay of M-412 was followed at 400 nm, it is possible to normalize the induced pH changes also with respect to the spectral changes resulting from this intermediate. For this purpose the sum of the amplitudes of the two exponentials necessary for the description of the M-412 decay was used. From the ratio of this sum with respect to the 610 nm normalization sum a differential extinction coefficient for M-412 at 400 nm of $29000\ M^{-1}\cdot cm^{-1}$ is calculated. Within the assumed errors this value is in the range of published data (23000 [5] to $30000\ M^{-1}\cdot cm^{-1}$ [8]). It is therefore concluded that also 1.1 protons per M-412 intermediate are released. This value has to be compared to published values of 1.6–4 [8,9,12–15] on the one hand and values of 0.7 [16] and 1 (at high ionic strength and light saturation [9], and for envelope vesicles [15]) on the other.

3.2. *Kinetic correlation of pH changes and photocycle*

For the complete description of the 400 nm BR signal a sum of five exponentials was satisfying. The most rapid process (1–2 μs) is ascribed to the decay of the K-610 intermediate. Fig.4B depicts in a logarithmic scale time constants of the 4 slower exponentials coupled with the formation ($\tau_{3,4}^-$) and decay ($\tau_{1,2}^+$) of M-412, and the two time constants of protonation (τ_2^+) and deprotonation (τ_1^-) of pyranine in the presence of 250 mM KCl. No dependency on the bulk buffer concentration of the photocycle lifetimes (0–10 mM) and of the deprotonation time constants of pyranine is observed (0–1 mM). The deprotonation process of pyranine (reprotonation of PM) (τ_1^- = 11.6 ± 2.3 ms, n = 12) is strongly correlated to the slow phase (30% amplitude) of M-412 decay (slow phase τ_1^+ = 11.4 ± 2.6 ms, fast phase τ_2^+ = 3.0 ± 0.3 ms, n = 14). It cannot be ruled out, however, that proton reuptake by the PM nevertheless oc-

curs during the decay of O-640. A biexponential decay of this intermediate was monitored at 660 nm with a fast, predominant τ of 8.3 ms and a second slow decay constant of 20.1 ms (250 mM KCl, 5 mM phosphate). The preceding protonation of pyranine (monitoring the release of protons by the PM) is accelerated in the presence of phosphate buffer (τ_2^+ = 646 μs, no phosphate; τ_2^+ = 260 μs, 1 mM phosphate), which obviously facilitates the exchange of protons between the membrane and the dye molecules in the aqueous bulk phase [16,22]. The apparent proton release by the PM lags slightly behind the formation of M-412 (fast phase (56% amplitude) τ_4^- = 54 μs, slow phase (44% amplitude) τ_3^- = 173 μs) in the strongly buffered case. It cannot be decided, if at still higher phosphate concentrations the apparent proton release would completely match the formation of M-412 or even be faster [25], since at these buffering powers the pyranine signals become too small for the correct evaluation of time constants.

As there are conflicting reports on the salt dependence of the stoichiometry of proton release [8,9,12,13,16] by PM, this question has been reexamined by the pyranine method (fig.4A). Apparently the M-412 decay times are shortened in the presence of higher amounts of KCl, whereas M-412 formation times do not seem to be affected. In parallel to this process, the amplitude of the slow M-412 decay increases relatively to the fast decay process (18% at 25 mM KCl, 30% at 250 mM KCl), and no such alteration is observed for the amplitudes of M-412 formation. The detection of the released protons by pyranine (τ_2^+) is accelerated at higher ionic strengths, probably as the Debeye length decreases in the examined ionic strength range of 10 to 250 mM from about 3 to 0.6 nm at 20°C. This results in a closer approach of pyranine to the negatively charged PM surface. However, also a slowing down of the disappearance of protons from the bulk (reuptake by BR, τ_1^-) is observed at higher ionic strengths, and only at these salt concentrations this process parallels the slow decay phase of M-412. Since in the millisecond range of proton uptake by PM, the proton exchange between PM surface and pyranine does not seem to be the rate limiting step [22], this slowing down by salt of the apparent proton uptake by the PM should be an intrinsic property of the BR proton pump cycle. On the other hand, the number of protons released per cycling BR molecule is not strongly affected by the variation of ionic strength (0.94, 0.98, 1.02, 1.20 for 10, 25, 100, 250 mM KCl, 100 μM phosphate). These values are derived by titration of the completely degassed samples taking into account the pK change of pyranine at lower ionic strengths (e.g., pK 7.7 at 10 mM KCl, 20°C).

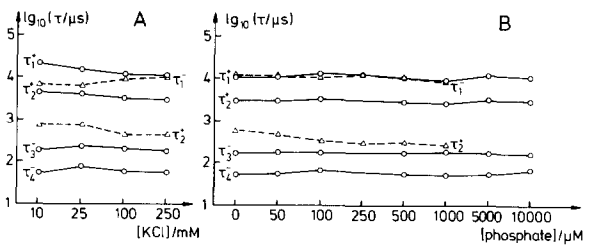

Fig.4. Dependency of time constants on salt (A) and phosphate (B) content. Solid lines: kinetics of M-412 followed at 400 nm (τ_1^+, τ_2^+ decay), (τ_3^-, τ_4^- formation). Broken lines: kinetics of pyranine protonation (τ_2^+) and deprotonation (τ_1^-) followed at 457 nm. (A) 10 μM PM, 100 μM phosphate; (B) 10 μM PM, 250 mM KCl.

It should be noted that the stoichiometry of released protons per cycling BR was not affected either by variation of the exciting laser power (3.8–10% excitation of BR).

For the present it cannot be decided if the protons detected by pyranine are actually pumped protons or if they are – at least in part – Bohr protons released and subsequently taken up by the same PM side due to light-induced pK changes of surface groups. The value of 1.1 ± 0.2 released protons would then represent an upper limit for the number of pumped protons per cycle. On the other hand, in localized chemiosmotic theories, it could be argued that not all pumped protons necessarily have to leave the PM surface and enter the bulk water phase before being utilized for other purposes. This possibility, however, seems rather improbable in the case of the open membrane system PM in the observed time range. An apparent exchange time for protons between the bulk and the surface of Brij 58/phosphatidylserine micelles in the order of 10^{-4} s was determined by Gutman [22]; this process should therefore be completed before reprotonation of PM (11.6 ms) takes place.

4. CONCLUSIONS

Our results can be summarized as follows:
(i) Upon excitation of BR, appearance and disappearance of the proton in the medium correlate with the formation of M-412 and decay of M-412 or O-640.
(ii) Diffusion of the released proton to the pH-indicator pyranine in the aqueous bulk is accelerated by pH-buffers.
(iii) One proton per cycling BR is released at pH 7.0.
(iv) The stoichiometry of released protons is independent of the ionic strength of the solvent and the excitation intensity.
(v) The proton is released in the reaction pathway that includes the M-412 intermediate.

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