Protonation of a novel intermediate P is involved in the M→bR step of the bacteriorhodopsin photocycle

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A novel intermediate (P) of the bacteriorhodopsin (bR) photocycle, appearing between M412 and bR is described. Like bR, intermediate P shows an absorption maximum at 560–570 nm. However, the extinction coefficient of P is somewhat lower than that of bR. Moreover, there are some differences in spectra of bR and P at wavelengths shorter than 450 nm. The P→bR transition correlates with the absorption of H^+ from the water medium. The following conditions proved to be favourable for the detection of the new intermediate: a high salt concentration, low light intensity and low temperature (0.5°C). The P→bR transition is strongly decelerated by a small amount of Triton X-100. Illumination of P does not produce M412 before bR is formed. It is assumed that M412 converts to P when the Schiff base is protonated by a proton transferred from a protein protolytic group which participates in the inward H^+-conductivity pathway. Reprotonation of this group results in the conversion of P to bR. No more than 1 H^+ is transported per bR photocycle.

Bacteriorhodopsin *Photocycle* *Proton pump* *pH indicator* *Purple membrane* (Halobacterium halobium)

1. INTRODUCTION

The mechanism of H^+ transfer by bacteriorhodopsin (bR) is one of the key problems of membrane bioenergetics. In our group laser flash- induced fast kinetics of several essential parameters of this process have been measured, i.e. (i) formation and decomposition of the M intermediate of the photocycle; (ii) proton release and uptake; and (iii) electric potential generation by bR. It was concluded that H^+ transfer from the cytoplasmic membrane surface to the Schiff base is responsible for the generation of the major portion of $\Delta\psi$ [1,2]. In the present paper we report observations indicating that this step of the photocycle includes stages of H^+ transfer from a protein protolytic group to the Schiff base and the subsequent reprotonation of this group by H^+ of the bulk water phase. The new intermediate called P is identified between M and bR. In the P intermediate, the Schiff base, most probably, is already protonated, whereas H^+ is still not absorbed from the water medium.

2. MATERIALS AND METHODS

In all the experiments, purple sheets from *Halobacterium halobium* 353P strain were used. The methods for preparing the sheets and measuring the photocycle intermediates and pH changes were described elsewhere [1,2]. *p*-Nitrophenol was employed as a pH indicator to monitor fast light-induced pH changes. A Quantel YG-481 ND-YAG Q-switched laser with frequency doublers (λ = 532 nm, $t_{1/2}$ = 15 ns, 50 mJ) and a xenon flash lamp ($\lambda > 470$ nm, $t_{1/2}$ = 0.4 ms, 25 mJ) served as light source. HCl, NaOH and NaCl were of the OSCh grade; Triton X-100 and Mes were from Sigma.

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3. RESULTS AND DISCUSSION

Fig.1A shows a comparison of the kinetics of laser flash-induced light absorption changes at two wavelengths, namely, at 400 nm and 570 nm. The former signal monitors the level of M, whereas the latter is usually considered to reveal the level of the bR ground state. Under the conditions used, no O-intermediate formation was observed (see below), and so one could hope that the A_{570} increase kinetics would follow that of M decomposition. However, this assumption was incorrect. The A_{570} increase was slower than the drop of A_{400}. This difference became smaller at higher light intensities and lower salt concentration (not shown). On the other hand, the addition of small amounts of Triton X-100 enhanced the difference in 400 nm and 570 nm decay kinetics significantly (fig.1B).

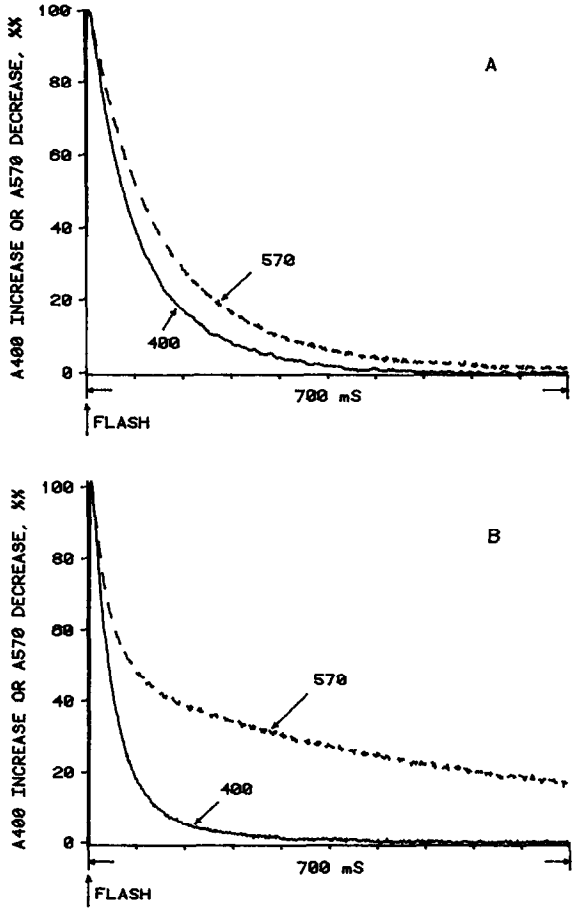

Fig.1. Comparison of the laser flash-induced kinetics of the light absorption changes at 400 nm and at 570 nm. The incubation mixture contains bacteriorhodopsin sheets (0.2 mg protein·ml^{-1}), 1 M NaCl, 0.5 mM Mes, pH 7.0, 0.5°C. The laser intensity is 1 mJ. In B the mixture is supplemented with 0.04% Triton X-100.

In fig.2A and B, the light minus dark difference spectra were measured 3 ms and 1 s after the flash, respectively. 0.02% Triton X-100 was present. In the former case, a typical spectrum with an absorption increase at 410 nm and absorption decrease at 570 nm is seen. In the latter case, however, no 410 nm maximum is revealed in spite of the fact that the 570 nm minimum remains quite measurable. Note a new minimum at 430 nm and an absorption increase at $\lambda \leqslant 390$ nm. No peaks at wavelengths longer than 570 are seen in both spectra, indicating the absence of the O intermediate of the photocycle.

The obtained data may be explained by assuming that under the conditions used, M converts to bR via an unknown intermediate which has (i) a major absorption maximum at 560–570 nm like bR but a somewhat lower molar extinction coefficient and (ii) the above mentioned peculiarities at $\lambda < 450$ nm.

If the new intermediate cannot enter another turnover of the photocycle before converting to bR, the second flash added after the first one must

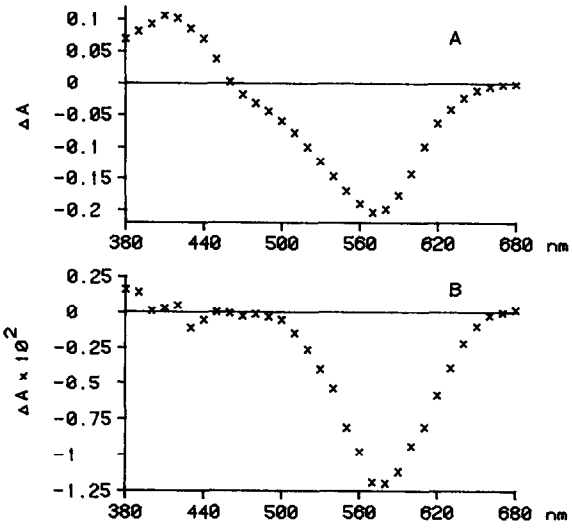

Fig.2. Light minus dark differential spectra of the bacteriorhodopsin sheets, measured 3 ms (A), and 1 s (B) after the laser flash. Conditions as in fig.1, but the light intensity is 25 mJ. 0.02% Triton X-100 is present.

be ineffective in M formation within the time interval corresponding to the life-time of this intermediate. This prediction was confirmed in an experiment shown in figs 3 and 4. Fig.3 illustrates the typical 400 nm responses to the first illumination by a saturating light flash of the xenon lamp or by a laser, and to the second laser illumination. In fig.4 the magnitudes of the 400 nm responses to the second laser flash are plotted against the time interval between the two flashes (upper curve). For comparison, the 400 nm and 570 nm kinetics of response to the first saturating flash are shown. The smaller difference between the two latter curves as compared to that in fig.1 is due to the higher light intensity in fig.4.

It can be seen from fig.4 that during the first several milliseconds, the curve outlines the fast increase in the second flash-induced M production. This phenomenon contributes 30% for the overall M production amplitude. It may be due to superposition of the first (saturating) flash and the second (laser) flash, as well as to the photoconversion of L intermediate to bR [3] or to some other phenomenon which is outside the scope of the present study, since it is much faster than the M ⟶ bR transition. The major increase in the second flash-induced M production (65–70% of the

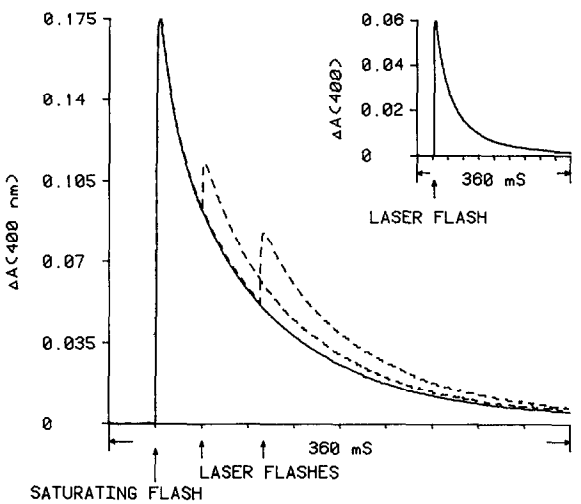

Fig.3. Double-flash method allowing one to monitor the regeneration of bR in M formation. Xenon flash lamp is used to obtain the first (saturating) flash. The inset shows a response of the system to the first laser flash. Conditions as in fig.2.

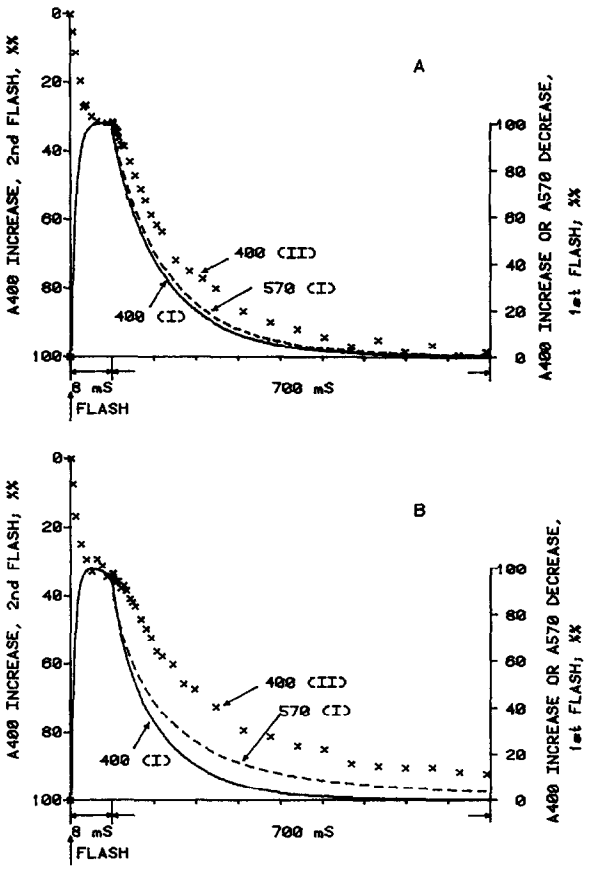

Fig.4. Regeneration kinetics of bR in the second laser-flash induced formation of M. Conditions as in fig.2, but in A Triton X-100 is omitted.

overall response) proved to be somewhat slower than the decay of the M intermediate appearing in response to the first flash. It was also slower than the regeneration of 570 nm absorption after the first flash if we take into account the total 570 nm changes. This phenomenon was particularly pronounced in the presence of Triton X-100 (fig.4B). However, in both cases the τ value of the slow phase of the 570 nm absorption change proved to be similar to the τ value of the slow phase of increase in the second flash-induced M formation. In the presence of Triton X-100 (fig.4B) these τ values were found to be approx. 600–700 ms.

In fig.5 three parameters were simultaneously measured, namely, pH and the 400 nm and 570 nm absorption changes. The light flash intensity was intermediate between those in figs 1 and 4.

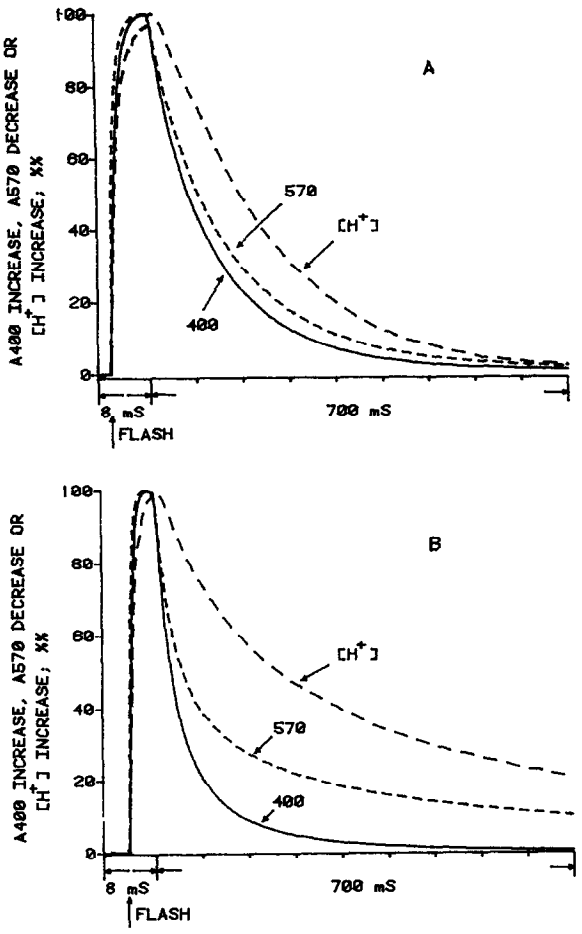

Fig.5. Kinetics of the laser flash-induced [H^+], 400 and 570 nm absorption changes. (A) Without Triton X-100; (B) 0.01% Triton X-100 is added. Other conditions as in fig.2.

It is found that the slow phase of H^+ reabsorption by illuminated purple sheets in the presence of Triton X-100 (τ = 700 ms) is much slower than the slow phase of M decay (τ = 120 ms), being similar to those of (i) the slow phase of the 570 nm absorption increase, and (ii) the slow phase of the second flash-induced M formation. From these data one may conclude that the conversion of the new intermediate to bR is accompanied by the H^+ uptake. As measurements showed, one proton is absorbed from the water medium per regenerated bR. These relationships are illustrated by the scheme depicted in fig.6.

It should be emphasized that the existence of the

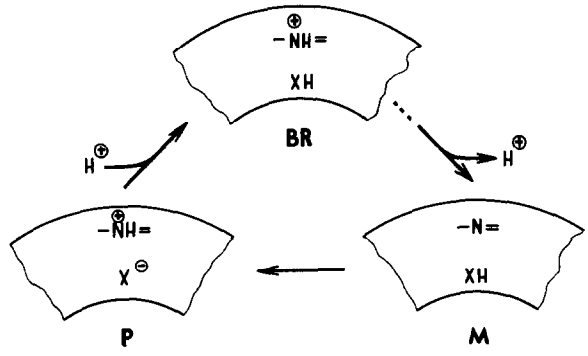

Fig.6. The place of intermediate P in the bacteriorhodopsin photocycle.

P intermediate, which produces bR upon protonation, invalidates any conclusions about participation of various forms of M in the H^+ transport based only upon the comparison of the rates of H^+ absorption and M intermediate decay.

The reason for the observed spectral differences between intermediate P and bR remains unclear. It may be due to the effect of an intramolecular dipole $\underline{x}\ldots = NH^{\cdot+}$-. Moreover, it seems possible that in state P, 13-*cis* chromophore (M) is still not converted to all-*trans* (bR). It is known that the short wavelength region of the bacteriorhodopsin spectrum is sensitive to the isomeric state of retinal [4].

Another problem awaiting solution is the relationship of the intermediate P with pseudobacteriorhodopsin postulated in 1978 by Gillbro [5]. The molar extinction coefficient of this precursor of bR in the photocycle, also abbreviated as P, was assumed to be higher than that of bR. The opposite relationships are inherent in the P intermediate described in our experiments.

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