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The mechanism of H^+ transfer by bacteriorhodopsin

The properties and the function of intermediate P

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Regeneration of bacteriorhodopsin (bR) from the M intermediate via a new intermediate P has been studied. In the purple sheets treated with 0.015% Triton X-100 (the P → bR transition is suppressed), the α-maximum of absorption of P is located at 560 ± 5 nm, the extinction coefficient being equal to 0.7 ± 0.1 of that of bR. Besides, there is a small but measurable absorbance increase at 330–350 nm, which seems to be due to a β-maximum of 13-*cis*-retinal residue in P. The similarity of the α-maximum of P and bR suggests the Schiff base nitrogen to be protonated at the M → P transition. The kinetics of P → bR transition measured at the 335 nm absorbance decrease coincides with that of proton uptake accompanying bR regeneration after flash. A scheme is proposed assuming that H^+ absorption from the water phase is coupled to the 13-*cis* → all-*trans* isomerization of retinal residue in which the Schiff base is already protonated by a proton-donor group of the protein.

Bacteriorhodopsin; Photocycle; Proton pump; Purple membrane; *(Halobacterium halobium)*

1. INTRODUCTION

The H^+ transport by bacteriorhodopsin (bR) includes two main partial reactions, i.e. (i) deprotonation of the Schiff base due to the light-induced all-*trans* ⟶ 13-*cis* isomerization of the retinal residue, followed by a release of the H^+ into extracellular solution and (ii) reprotonation of the Schiff base by H^+ transferred from the cytoplasm. It is the stage (ii) that mainly contributes to the formation of the transmembrane protonic potential, the fact which may be explained by localization of retinal closer to the outer than to the inner membrane surface [1–5].

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Optically, stage (i) can be monitored by measuring a strong blue shift of the bR spectrum (formation of M intermediate). Stage (ii) i.e., conversion of M to the initial form of bR, is accompanied by a reversal of this shift. The latter process is usually assumed to occur via intermediate O_{640}. However, under certain conditions in vivo and in vitro, O_{640} does not seem to be involved in the bR photocycle. In our previous studies [6,7], it was shown that there is a novel intermediate (P) between M and bR in which the Schiff base is already protonated. It is believed that a proton-donor group of the protein, rather than the water phase, is the source of H^+ for the protonation reaction. The P ⟶ bR transition is accompanied by H^+ absorption from water. The P–bR differential spectrum was found to have a pronounced minimum in the region of the major band of bR.

In this paper, we shall report on the spectral

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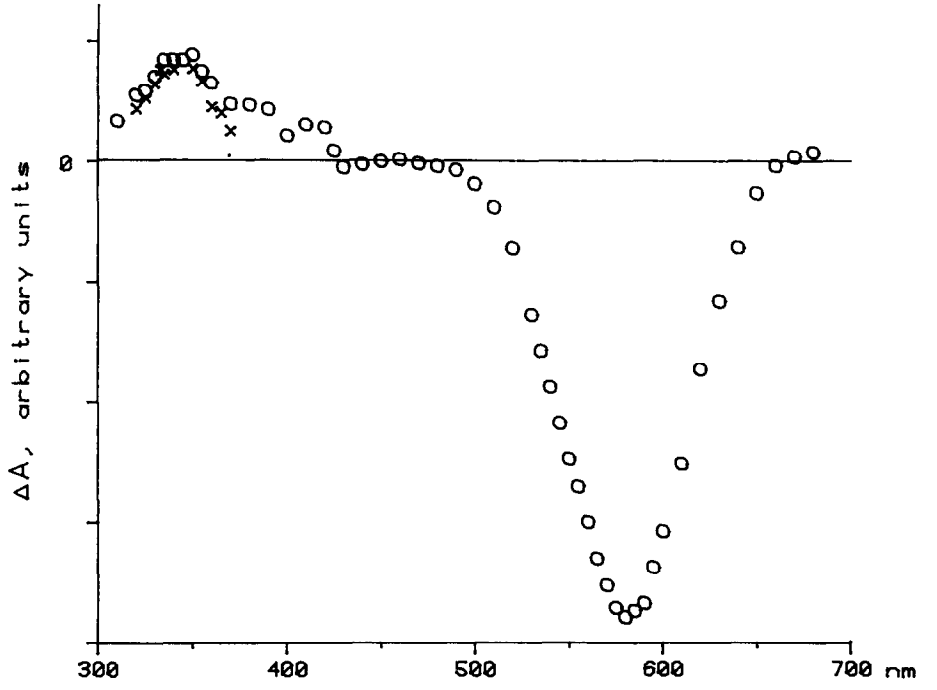

Fig.1. Differential spectrum of the P intermediate (○) and of the fast component of the flash-induced absorbance increase in ultraviolet (×). Incubation mixture contained 1 M NaCl, 5 mM KH_2PO_4, 0.015% Triton X-100 (pH 7). $t°$ = 4°C.

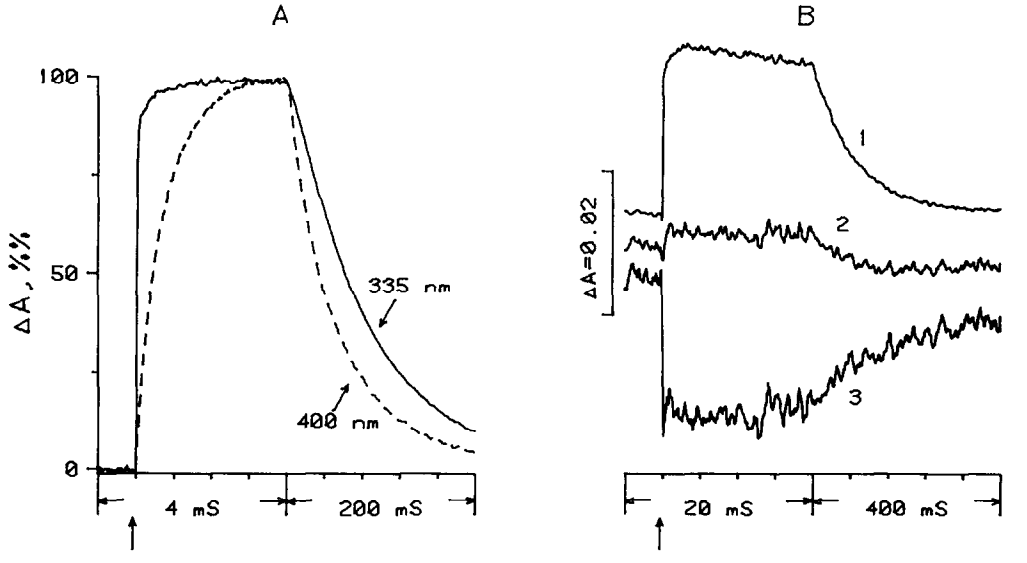

Fig.2. Kinetics of the 335 nm absorbance changes caused by a laser flash. (A) In comparison with the 400 nm kinetics. (B) 335 nm responses of the light-adapted (curve 1) and dark-adapted (curve 2) purple membranes. Curve 3, the 335 nm response of 13-*cis* bR, calculated from curves (1) and (2), using the assumption that light-adapted bR contains 100% all-*trans*-retinal and dark-adapted bR contains 50% all-*trans*- and 50% 13-*cis*-retinal. Conditions as in fig.1 but without Triton X-100. Here and below, vertical arrows indicate the laser flash.

characteristics of the P intermediate and the correlation between the optical and pH shifts related to the P ⟶ bR transition.

2. MATERIALS AND METHODS

In this study, purple sheets from the *Halobacterium halobium* ET 1001 strain were used. Methods of isolation of the sheets and measurements of the flash-induced spectral and pH responses were described elsewhere [1,2,6,7]. All the experiments were performed at 4°C. 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 lamp (λ > 470 nm, $t_{1/2}$ = 0.4 ms, 25 mJ) served as light sources.

3. RESULTS AND DISCUSSION

Fig.1 shows a differential P–bR spectrum measured at 320–680 nm. In the experiment, the rate of the P ⟶ bR transition was decreased by a low Triton X-100 concentration (0.015%) as found earlier [6]. One can see a pronounced minimum at 580 nm and a small but measurable maximum at 330–350 nm.

In a previous paper [6], we suggested that the short wavelength maximum of the P intermediate is due to the 13-*cis* configuration of the retinal residue. We therefore studied in detail the kinetics of the 335 nm absorbance change. As one can see in fig.2A, the flash-induced 335 nm absorbance increase is composed of (i) a large fast phase (kinetics not resolved in the figure) accounting for 85–90%

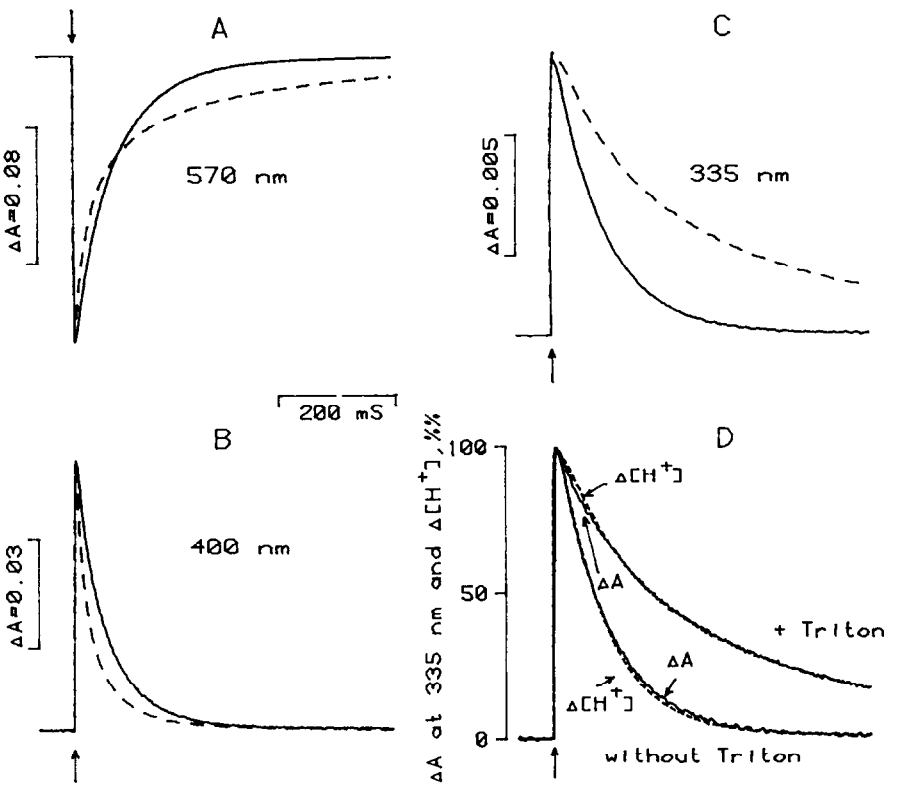

Fig.3. Effect of Triton X-100 upon photocycle and the bR reprotonation after a laser flash. (A–C) Absorbance changes at 570, 400 and 335 nm, respectively. Solid and dashed lines, without and with 0.015% Triton X-100. Other conditions, as in fig.1. (D) Comparison of kinetics of flash-induced changes in the 335 nm absorbance (A) and in $[H^+]$ with and without Triton. Incubation mixture, 1 M NaCl, 0.5 mM KH_2PO_4, pH = 7.2, $t°$ = 4°C. 0.1 mM pyranine was added for the pH measurements.

of its magnitude, and (ii) of a small slow phase, the rate of the latter being comparable with the M intermediate formation. The fast phase, in its turn, proved to be biphasic. Its fast and slow components were shown to correlate with the formation of K and L intermediates, respectively (not shown in fig.2). The fast phase was previously studied by Kuschmitz and Hess [8]. It was found that its differential spectrum resembles that of bR dark adaptation. It was concluded that these spectral changes were due to all-*trans* ⟶ 13-*cis* retinal isomerisation. Later using the same logic, Lanyi [9] concluded that there is an isomerization of the all-*trans* retinal in halorhodopsin at an early stage of the photocycle. A short-wavelength band is shown to be inherent in different *cis*-retinal derivatives in solution, being absent from all-*trans* derivatives [10].

A differential spectrum of the fast phase of the flash-induced response in the ultraviolet region is shown in fig.1 (×). It clearly resembles the P–bR differential spectrum, hence suggesting that the retinal maintains *cis*-conformation from a very early stage of the bR photocycle up to the P intermediate. A further indication proving that the 335 nm absorbance increase is due to the 13-*cis* retinal was obtained when the photocycle of the dark-adapted bR was studied. It was found that illumination of the 13-*cis* bR component of dark-adapted purple membrane causes maximal decrease in absorbance at 330–350 nm (fig.2B). The response was shown to correlate with the formation and decomposition of the batho-intermediates of the 13-*cis* bR photocycle. Apparently it is connected with isomerization of 13-*cis*-chromophore group.

In fig.3 it is seen that 0.015% Triton X-100 accelerates decomposition of the M intermediate and decelerates that of the P intermediate. The latter phenomenon is believed to contribute a slow phase to the absorbance increase at 570 nm and to the absorbance decrease at 335 nm (fig.3A–C). The 335 nm response is the most demonstrative since in this region, M practically does not contribute to the spectral change.

As previously shown by our group [6,7], neither

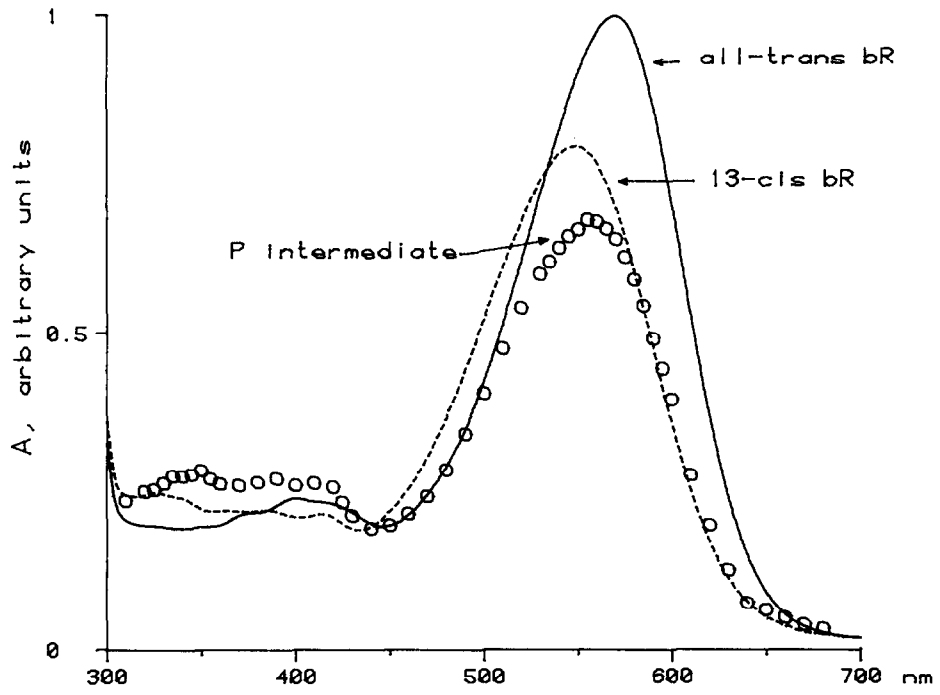

Fig.4. The comparison of calculated absolute spectrum of P intermediate with the experimental spectrum of the light-adapted bR and calculated spectrum of 13-*cis*-bR (the same assumption as in fig.2). To reduce the light scattering, the mixture (see fig.1) was supplemented with 1.5 M sucrose.

400 nm nor 570 nm kinetics correlates with that of the H^+ uptake by the purple sheets at the terminal step of the photocycle. On the other hand, fig.3D shows an excellent correlation (in fact, coincidence) of the 335 nm response and the H^+ uptake. This is the case in both Triton-treated and non-treated preparations. As we have found earlier [6,7], the H^+ uptake correlates also with bR regeneration which was evaluated with the help of double-flash method. This method reveals the kinetics of the regeneration of bR competent in entering a new photocycle after the flash.

Fig.4 shows an absolute spectrum of the P intermediate. It was calculated using the absolute spectrum of bR and the P–bR differential spectrum and taking into account (i) the contribution of the slow phase to the 570 nm absorption increase, and (ii) the portion of bR participating in the P-mediated pathway (according to the 340 nm or pH measurements). It has an α-peak at 560 ± 5 nm, the extinction coefficient being 30 ± 10% lower than that of the bR α-peak. At $\lambda < 370$ nm the P intermediate has higher absorbance than bR, probably due to the 13-*cis* conformation of retinal.

In 1978 Shkrob and Rodionov [11] disclosed a long-lived intermediate of the bR photocycle at high pH. Its differential maximum was similar to that of the P intermediate described in this paper. Later this observation was confirmed and extended by Dancshazy et al. [12] who also studied the photocycle in alkaline media. Apparently, this intermediate is identical to P which reveals itself due to deceleration of proton uptake when $[H^+]$ lowers. In fact, 0.015% Triton and high pH modify the terminal step of photocycle in a similar way. Besides, alkalinization causes the appearance of long-lived M derivatives. It is noteworthy that these derivatives appear when the Triton concentration becomes $\geqslant$0.05%. Fig.5 shows that at high pH the 400 nm absorbance decrease is composed

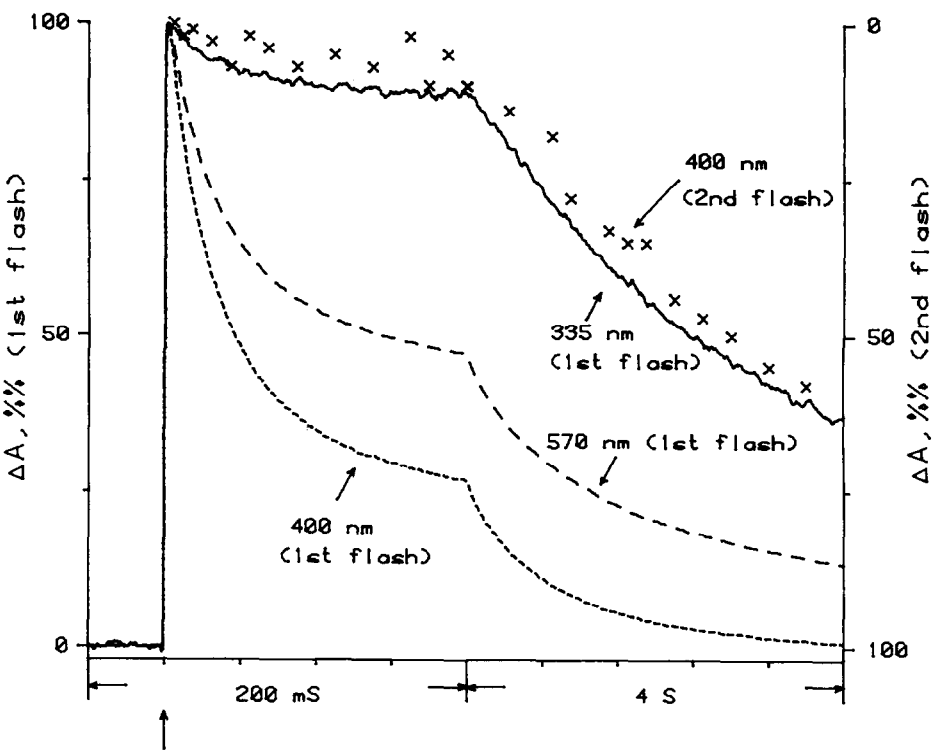

Fig.5. The laser flash-induced responses of the purple membranes at pH 10.5. Incubation mixture, 15 mM phosphate-borate buffer, $t° = 4°C$. Upward deflection of the trace means the absorbance increase at 400 and at 335 nm and the absorbance decrease at 570 nm. (×) Kinetics of regeneration of the 400 nm response amplitude to the second flash as described previously [6,7].

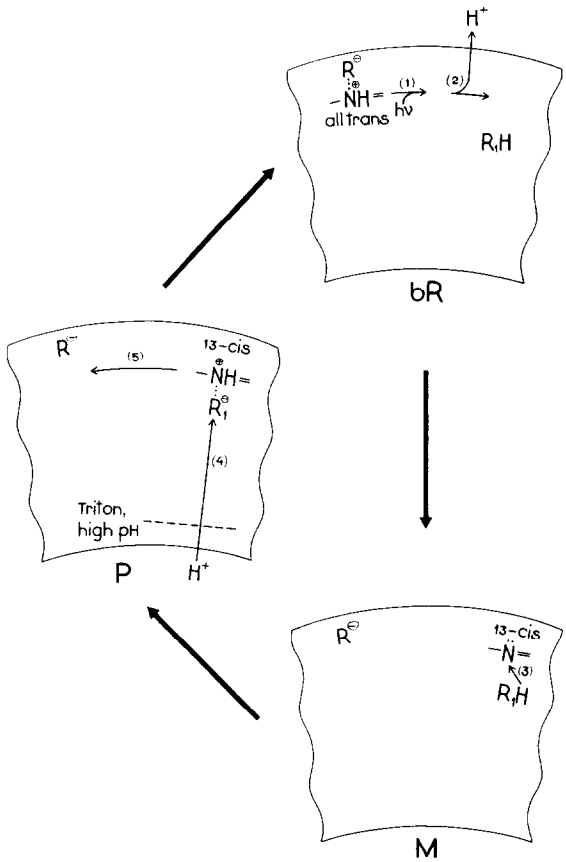

Fig.6. Mechanism of H^+ transfer by bR. For explanations, see text.

of at least two phases developing in the millisecond and second scales. Biphasic behaviour is also inherent in the 570 nm increase. On the other hand, the 335 nm decrease or regeneration of bR measured using the double flash techniques, appears mainly or exclusively in the second scale. The 10% fast component of the 335 nm decrease is due to the contribution of the M intermediate at this wavelength.

The data discussed above are summarized in fig.6. Absorption of photon causes the all-*trans* ⟶ 13-*cis* isomerization of the retinal residue and, as a consequence, removal of the protonated Schiff base from an anion group R^- (reaction 1). This strongly decreases pK of the Schiff base, resulting in its deprotonation and release of H^+ to the outer medium (reaction 2). In this way, M is formed. In M, an H^+ transfer from a proton-donor group R_1H to the Schiff base takes place (reaction 3), so that P appears. Reprotonation of R_1 by cytoplasmic protons (reaction 4) initiates 13-*cis* ⟶ all *trans*-isomerization (reaction 5) and, hence, regeneration of bR. It is reaction 4 that is inhibited by alkalinization (maybe simply because H^+ is its substrate) and by Triton. The role of the O intermediate which is absent in the scheme and its relation to P are now under investigation.

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