Eur Biophys J (1985) 12: 223–228

European Biophysics Journal

pH and salt effects on the slow intermediates of the bacteriorhodopsin photocycle

A flash photolysis study

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Received December 3, 1984/Accepted in revised form May 3, 1985

Abstract. Purple membrane fragments of *Halobacterium halobium* were used to investigate pH and salt effects on the kinetics of M_{412}, O_{660} and BR_{568}. The flash-induced absorbance changes were measured in the 5–9 pH range, at low ionic strength and at 4 *M* NaCl. The results are consistent with a model which implies a branching in the last part of the bacteriorhodopsin photocycle.

Key words: Purple membrane, bacteriorhodopsin, pH effect, photocycle, flash photolysis

Introduction

Bacteriorhodopsin, the protein of the purple membrane of *Halobacterium halobium*, is a light-driven proton pump (Stoeckenius et al. 1979). Upon illumination at 568 nm, a reaction cycle is initiated and several intermediates are spectroscopically identifiable (*K, L, M, O*) (for a review, see Ottolenghi 1980). Until now however, the mechanism which links the intermediate formation to the proton transfer across the membrane has not been fully elucidated. A great number of studies have been devoted to the influence of various parameters on those processes. For several years, we have been interested in our laboratory in the effect of pH on the proton pump (Renard and Delmelle 1980) and on the photocycle (Renard and Delmelle 1981; Renard et al. 1983).

The quantum efficiency of proton release has been determined on intact cells by several groups. The most recent results suggest that more than one proton is pumped per cycle (Govindjee et al. 1980; Bogomolni et al. 1980; Renard and Delmelle 1980). We found, in addition, that the quantum yield decreases from 0.64 at pH 5.9 to 0.28 at pH 9.0. This effect is surprising. To investigate it further, we applied a photoacoustic method developed originally by Malkin and Cahen (1979) to derive some information about the pH influence on the energetics of the slow intermediates (Renard and Delmelle 1983; Renard et al. 1983). Our data were not interpretable on the basis of a linear photocycle. We were forced to conclude that branching occurs in the last part of the photocycle. We found moreover that the molar enthalpy change associated with the O_{660} intermediate is strongly pH-dependent.

The complexity of the photocycle has been outlined by several authors and particularly by Nagle et al. (1982). Neither a linear pathway nor a unidirectional model implying a simple branching straight back to BR_{568} is fully satisfactory for interpreting all flash photolysis data presently available. Few studies were concerned with pH. (Sherman et al. 1976, 1979; Lozier and Niederberger 1977; Ohno et al. 1981.) In fact, the data available in the literature are insufficient to establish a correlation between our photoacoustic data and the results which concern the pH dependence of proton release in cells. Therefore we had to investigate in some detail the pH and salt effects on M_{412}, O_{660} and BR_{568}; this is the purpose of the present paper.

Materials and methods

Purple membrane fragments from *Halobacterium halobium* (strain $R_1 M_1$) were isolated as previously described (Renard and Delmelle 1981). They were suspended in 66 m*M* phosphate or *Tris*-HCl buffer in the presence or absence of sodium chloride (4 *M*). The bacteriorhodopsin concentration was $1.2 \times 10^{-5} M$ as determined at 568 nm with a Perkin-Elmer 559 spectrophotometer equipped with an integrating sphere accessory ($\varepsilon = 63{,}000\ M^{-1}\ cm^{-1}$).

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For the flash photolysis measurements, the light-adapted membranes were flashed with the filtered light (cut-off Schott filter OG 530) from an electronic stroboscope (General Electric Strobolume Type 1540; half width 10 μs). The repetition frequency was 0.5 Hz. The analyzing light (Varian 300 W Xenon lamp VIX 300 UV) was passed through 2 cm of water and through a filter selected to isolate the intermediate under study: an Agfa-Gevaert interference filter ($\lambda_{max} = 405$ nm, bandwidth 26 nm) for the detection of M_{412}, a cut-off red filter ($\lambda > 628$ nm) for the study of O_{660} and a Schott interference filter ($\lambda_{max} = 575$ nm, bandwidth 25 nm) for the study of BR_{568}. Samples were contained in thermostatted quartz cells; the temperature was 23 ± 0.2 °C. The analyzing beam transmitted by the sample was detected by an RCA 1P28 photomultiplier after passage through a Bausch and Lomb monochromator (1200 grooves/mm, bandwidth 6 nm). This set-up protects the photomultiplier from the intense light of the flash. The transient absorption changes were recorded on a C-1024 CAT computer (Varian). Up to 520 flashes were summed in order to improve the signal-to-noise ratio. All the absorbance data were fitted by the least-squares method. The simulation procedure was performed on a TRS-80 microcomputer.

Results

Flash-induced absorbance changes were measured on purple membrane suspensions in the 5–9 pH range, in the presence and absence of NaCl. The absorbance variations were measured at 412, 568 and 660 nm.

Study of the M_{412} intermediate

Figure 1 A (left) shows the flash-induced absorbance change at 412 nm (pH 5.94; 4 *M* NaCl). The absorbance increases rapidly and then it decays to its initial value. Figure 1 A (right) shows that the decay is not monophasic. Indeed, it can be described by the two exponential function:

$$A(t) = A_f^M \exp(-k_f^M t) + A_s^M \exp(-k_s^M t), \quad (1)$$

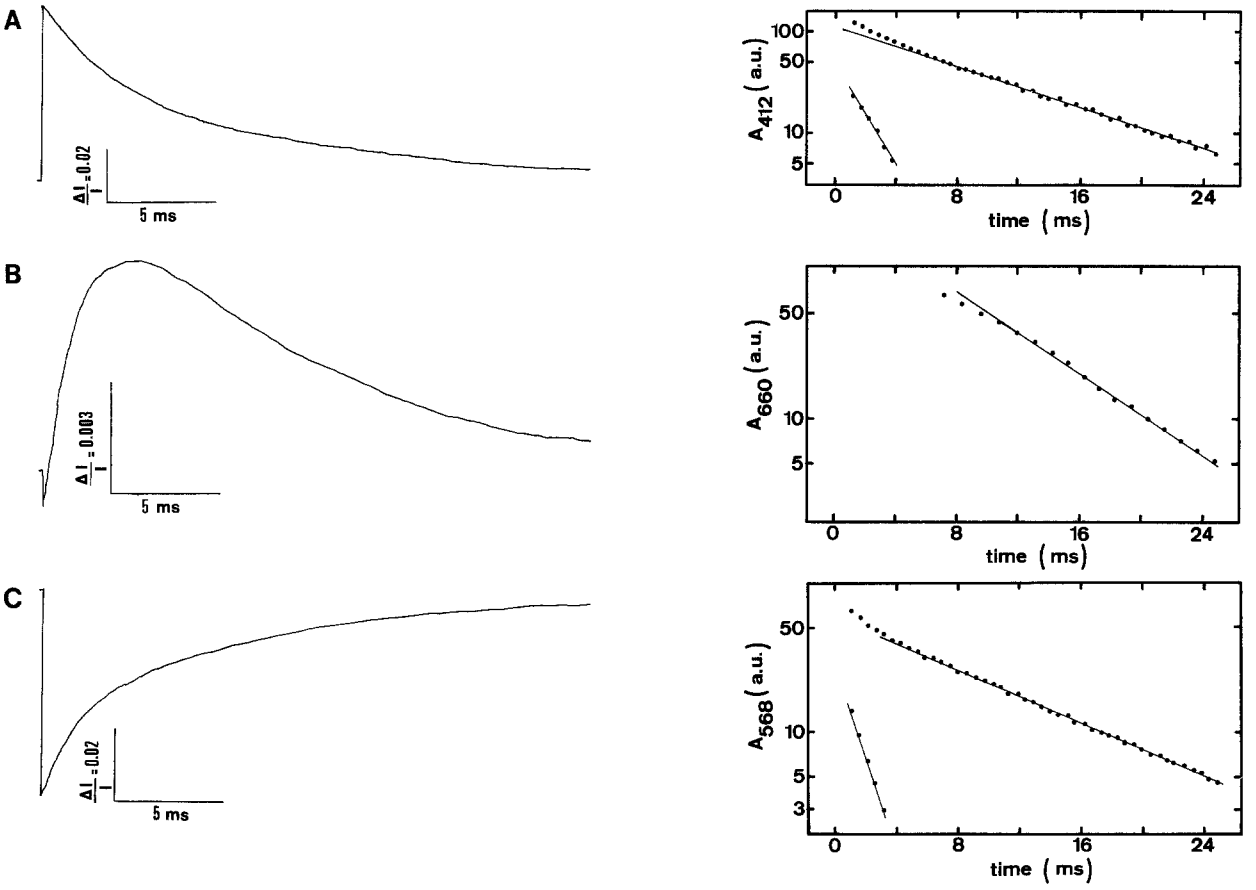

Fig. 1 A–C. *Left:* Flash-induced absorbance changes observed on purple membrane suspensions. **A:** at 412 nm (pH = 5.94; 4 *M* NaCl). 300 flashes; **B:** at 660 nm (pH = 6.42; 4 *M* NaCl). 460 flashes; **C:** at 568 nm (pH = 7.60; 4 *M* NaCl). 150 flashes. *Right:* Semi-logarithmic plots of the absorbance changes. In **A** and **C**, the lower line represents the difference between the experimental points and the fitted slow component

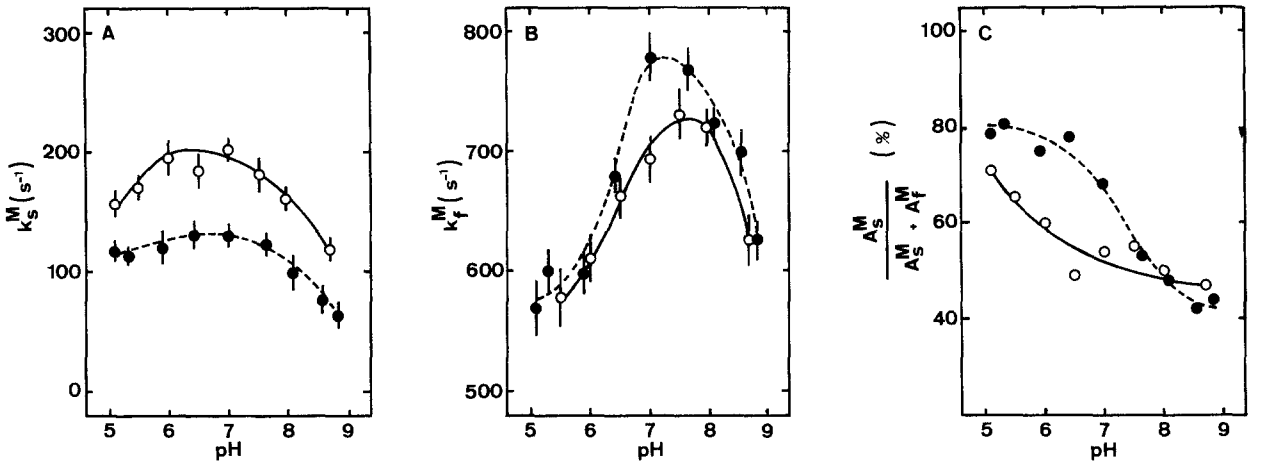

Fig. 2 A–C. **A** and **B:** Effect of pH on the rate constants of the decay at 412 nm. **A** slow component k_s^M; **B** fast component k_f^M. **C:** Effect of pH on the relative amplitude of the slow component. ○ without NaCl; ● with 4 *M* NaCl

where *f* and *s* denote the fast and slow components respectively. k_f^M and k_s^M are the corresponding rate constants. A_f^M, k_f^M, A_s^M and k_s^M vary with pH but the total concentration of the M_{412} intermediate ($A_{\text{total}}^M \equiv A_t^M = A_f^M + A_s^M$) remains constant in the whole pH range investigated. Figures 2A and B show the effect of pH on k_f^M and k_s^M, at low and high ionic strengths. The rate constants reach maximum values at about pH 7.0 but they decrease sharply above pH 8.0. High salt concentration reduces k_s^M by 25% to 40%. Figure 1C describes pH and salt effects on the relative amount of A_s^M. Depending upon the ionic strength, A_s^M represents in the acid range some 60% to 80% of the total concentration of M_{412}. At alkaline pH's, A_s^M amounts to 40%–50% of A_t^M.

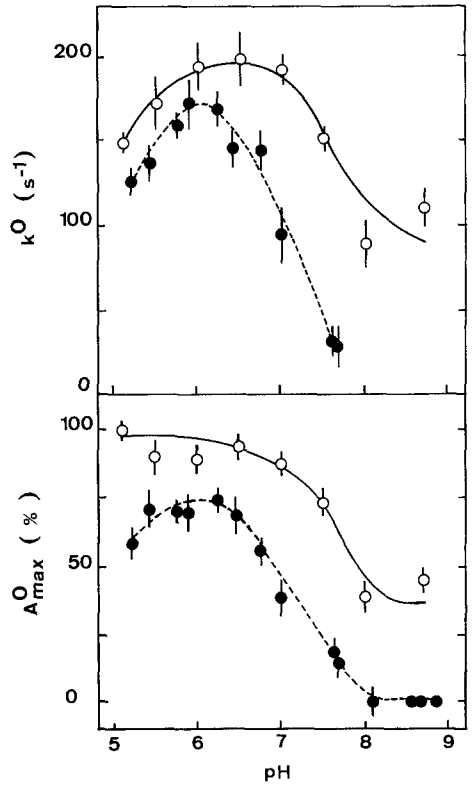

Fig. 3. Effect of pH on the O_{660} kinetics (○ without NaCl, ● 4 *M* NaCl). *Upper part:* dependence of the rate constant k^O. *Lower part:* dependence of the maximum absorbance A_{max}^O. The A_{max}^O value at pH 5.1 (no salt) was used for normalization

Study of the O_{660} intermediate

Figure 1B (left) shows a flash-induced absorbance change at 660 nm. Absorbance reaches a maximum some 3.5 ms after the flash. The overshoot occurring at the flash onset is in part due to the transient bleaching of bacteriorhodopsin. The right side of Fig. 1B demonstrates that in the latter half the absorbance decays monophasically. This particular measurement was performed at pH 6.42 and 4 *M* NaCl. Under these conditions, the rate constant k^O is equal to 145 s^{-1} ($r = 0.998$). Actually, a monophasic decay was observed in the whole pH range investigated, at both low and high ionic strengths.

Figure 3 presents the pH and salt effects on k^O and on the maximum absorbance value A_{max}^O. Both k^O and A_{max}^O decrease sharply at alkaline pH's. Salt concentration reduces k^O and A_{max}^O by some 15% to 20% as compared to their values at low ionic strength. Interestingly, at 4 *M* NaCl, O_{660} becomes undetectable at pH 8 and higher.

Study of the BR_{568} regeneration

The regeneration of BR_{568} observed at pH 7.6 and 4 *M* NaCl is shown in Fig. 1C (left). Under these

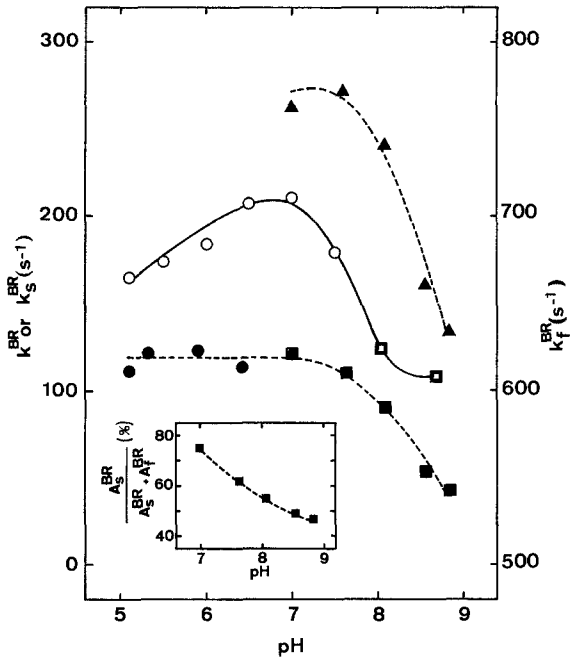

Fig. 4. Effect of pH on the generation of BR_{568}. White and dark symbols correspond to experiments performed respectively in the absence and in the presence of salt (4 *M* NaCl). Monophasic kinetics (k^{BR}) (*dots*). Biphasic kinetics: slow component (k_s^{BR}) (*squares*), fast component (k_f^{BR}) (*triangles*). *Inset.* Effect of pH on the relative amplitude of the slow component (4 *M* NaCl)

Table 1. Comparison of the experimental and simulated values of the rate constants relative to M_{412} and O_{660}

Ionic strength	pH	M_{412}		O_{660}		R
		k_f^M(exp) [s^{-1}]	k_{sim}^M [s^{-1}]	k^O(exp) [s^{-1}]	k_{sim}^O [s^{-1}]	
Without	5.1	616	560	150	130	0.965
4 *M* NaCl	7.0	694	660	193	180	0.982
	8.7	627	570	112	110	0.979
With	5.1	570	530	127	120	0.995
4 *M* NaCl	6.42	680	560	145	130	0.996
	7.63	770	900	37	65	0.960

experimental conditions, the regeneration is biphasic as demonstrated in Fig. 1 C (right). The corresponding rate constants are labeled k_f^{BR} and k_s^{BR} respectively. However, the regeneration of BR_{568} is not always biphasic (Fig. 4). In fact, monophasic regenerations were observed at acid pH's in the presence of salt. In the absence of NaCl, the regenerations could be considered as monophasic from pH 5.0 up to pH 8.0. Under these conditions, the kinetics are described by a single rate constant labeled k^{BR}. Figure 4 indicates that the three rate constants k_f^{BR}, k_s^{BR} and k^{BR} decrease sharply when the medium becomes alkaline.

The Fig. 4 inset describes the pH effect on the relative amplitude of the slow component at 4 *M* NaCl. The importance of the slow pathway decreases when the pH is alkaline.

Simulation

In order to verify whether one of the components of the M_{412} decay leads to the formation of O_{660}, the following model was considered:

$$M_{412} \xrightarrow{k_{sim}^M} O_{660} \xrightarrow{k_{sim}^O} BR_{568}.$$

Equation (2) describes the dependence of the O_{660} concentration with respect to time

$$[O_{660}]_t = [M_{412}]_0 (\exp(-k_{sim}^M t) - \exp(-k_{sim}^O t)) \times \frac{k_{sim}^M}{k_{sim}^O - k_{sim}^M}. \quad (2)$$

$[M_{412}]_O$ represents the initial total concentration of the intermediates absorbing at 412 nm. These intermediates are assumed to be formed immediately after the flash. Equation (2) was used to fit the experimental data. Table 1 shows that k_{sim}^M fits within 10% the values of k_f^M. On the other hand, the decay of the O_{660} concentration is satisfactorily simulated by k_{sim}^O. In Table 1, R characterizes the quality of the fit between the experimental and the simulated curves.

Discussion

The present work describes an extensive study concerning pH and salt effects on the kinetics of the slow intermediates of the bacteriorhodopsin photocycle.

We observed a biphasic decay at 412 nm under all our experimental conditions. Therefore we hypothesize the existence of two absorbing species, M_{412} and M'_{412}, characterized respectively by the rate constants k_f^M and k_s^M. This hypothesis is supported by previous observations. Slifkin and Caplan (1975) detected two intermediates with slightly different polarizations and lifetimes. Dencher and Wilms (1975) interpreted their results on the basis of a branched kinetic model with a direct pathway going from M_{412} to BR_{568}. The same model was also favoured by Sherman et al. (1976, 1979). Actually, a biphasic decay at 412 nm was observed by several groups (Lozier et al. 1976; Ort and Parson 1978; Ohno et al. 1981) but the existence of two absorbing species could result from a branching occurring earlier in the cycle (Korenstein et al. 1978). More recently, Parodi et al. (1984) suggested an alterna-

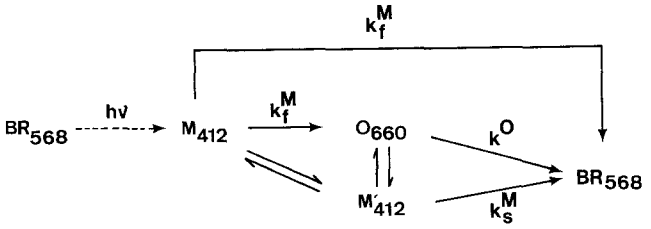

Fig. 5. Photocycle model

tive interpretation of the biphasicity based on a backreaction between O_{660} and M_{412}. We feel however that our previous photoacoustic results (Renard and Delmelle 1983; Renard et al. 1983) support a kinetic model involving a branching at the M_{412} level.

Our experiments demonstrate that the total concentration of the species absorbing at 412 nm are pH and ionic strength independent. This is in agreement with the results of Rosenbach et al. (1982), Kalisky and Ottolenghi (1982) and Govindjee et al. (1980). However since A_f^M, k_f^M, A_s^M and k_s^M are influenced differently by pH, a pH-dependent equilibrium is hypothesized between M_{412} and M'_{412} (Fig. 5).

Similar kinetics were observed for M'_{412} and O_{660} in the whole pH range investigated, especially at low ionic strength (Figs. 2A and 3). On the other hand, at alkaline pH's, the concentration of O_{660} becomes very small and at high ionic strength this intermediate is undetected at pH $\geqq$ 8. These observations provide an argument in favour of another pH-dependent equilibrium between M'_{412} and O_{660} (Fig. 5). Large pH effects on O_{660} were also reported by other groups. Lozier and Niederberger (1977) observed a reduction of the O_{660} concentration when the pH becomes alkaline at low ionic strength. Sherman et al. (1979) measured a decrease of the O_{660} decay rate constant between pH 7 and 9. The results reported in the present paper extend these previous findings. Ort and Parson (1978) observed that k^O decreases linearly between pH 5.8 and 7.8 but these results were obtained at 3.4 °C and the O_{660} concentration is very small under these conditions.

The simulation carried out in the present work suggests that M_{412} decays to O_{660} with the rate constant k_f^M. With respect to the regeneration of BR_{568}, two situations have to be considered. When the regeneration is monophasic, the kinetics match the decays of O_{660} and of M'_{412}. Alternatively, when the regeneration is biphasic at high pH's, the fast regeneration component follows the M_{412} kinetics (Figs. 2 and 4). Moreover a relationship exists between the relative concentrations of M_{412} and of M'_{412}, on the one hand, and the relative importance of the two components of the BR_{568} regeneration, on the other hand.

This discussion leads to the conclusion that two pathways seem to be involved in the slow part of the photocycle. At low ionic strength and at pH's below 8.0, the pathway $O_{660} - M'_{412}$ leads to a monophasic regeneration of BR_{568}. The other pathway $M_{412} - BR_{568}$ becomes significant at alkaline pH's. In the presence of NaCl, this latter pathway is already detected at pH 7.0. The Fig. 5 model is similar to the one proposed by Ottolenghi (1980); however, this model takes into account the N_{520} intermediate. We were unable to detect this intermediate under our experimental conditions and its existence is also challenged by others (Gilbro 1978; Lozier et al. 1978).

Most of the kinetic models which have been proposed for the bacteriorhodopsin photocycle rely on the assumption that the other intermediates do not interfere with the measurements made at a given wavelength. This approximation was questioned by Nagle et al. (1982) and Parodi et al. (1984). These authors performed careful investigations at many wavelengths and they discussed several models. Up to now however, these models do not seem completely satisfactory. Nevertheless the discrepancies that we observed between our experimental and simulated data, might indicate interference between several intermediates at a given wavelength.

The present results may be compared with the measurements reported earlier concerning the effect of pH on the proton release quantum yield in intact cells (Renard and Delmelle 1980). The curve which describes the pH effect on the proton quantum yield, $QY(H^+)$, is very similar to the one which describes the pH effect on the O_{660} concentration in 4 M NaCl. This suggests that O_{660} plays a key role in the pumping mechanism of a second proton. Several papers have shown that a regulation mechanism of the photocycle is performed by the electrochemical potential difference for protons, $\Delta\mu_{H^+}$, and its electrical component, $\Delta\psi$ (Hellingwerf et al. 1979; Quintanilha 1980; Westerhoff and Dancshazy 1984). Recently, Dancshazy et al. (1983) and Groma et al. (1984) have studied the coupling between the bacteriorhodopsin photocycle and the protonmotive force, in cells and in cell envelope vesicles. However, under our experimental conditions in bacteria, the electrochemical potentiel, $\Delta\mu_{H^+}$, as well as the electrical potential, $\Delta\psi$, are virtually constant between pH 6 and 8 (Michel and Oesterhelt 1976). It tends to demonstrate that the pH effect on $QY(H^+)$ does not result from $\Delta\mu_{H^+}$ or $\Delta\psi$ variations. Our results show that a regulatory role for pH has to be considered in the pumping mechanism of bacteriorhodopsin.

Note added in proof. During the review of this manuscript, an important paper was published by Li

et al. (Proc Natl Acad Sci USA (1984) 81:7079–7082 on the correlation between proton pumping and the bacteriorhodopsin photocycle. Effects of pH and temperature on flash-induced proton pumping and the photo-intermediates O_{660} and M_{412} were measured. Results indicate that: i) a branching occurs at the M_{412} level; ii) the O_{660} concentration declines very rapidly at pH's greater than 6.5; iii) the proton pumping is coupled through the slow branch of the photocycle. These results are in very good agreement with the kinetic model discussed in the present work.

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