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PHOTOACOUSTIC CALORIMETRY OF BACTERIORHODOPSIN PHOTOCYCLE

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Résumé - Le cycle photochimique de la bactériorhodopsine a été étudié par photolyse par flash et par spectroscopie photoacoustique. Les spectres photoacoustiques de fréquence ont été simulés sur base d'un cycle branché, afin de déterminer les variations d'enthalpie associées aux intermédiaires les plus lents.

Abstract - Flash photolysis and photoacoustic spectroscopy were used to study the bacteriorhodopsin photocycle. Kinetics and the enthalpy changes associated with the slow intermediates of bacteriorhodopsin were evaluated from photoacoustic frequency spectra. Simulation of frequency spectra was performed on basis of a branched photocycle.

INTRODUCTION

Bacteriorhodopsin is the protein-pigment complex found in the purple membrane of *Halobacterium halobium* (1). Absorption of a photon by the light-adapted form of bacteriorhodopsin initiates a photocycle during the course of which protons are translocated across the purple membrane. The photocycle takes about 10 msec and several intermediates have been identified. Initially, the scheme was thought to be linear ($\text{BR}(570) \rightarrow \text{K}(590) \rightarrow \text{L}(550) \rightarrow \text{M}(412) \rightarrow \text{O}(660) \rightarrow \text{BR}(570)$) (2). However several papers have provided experimental data which support a branched model with a pathway going from $\text{M}(412)$ to $\text{BR}(570)$ bypassing $\text{O}(660)$ (Fig. 1) (3,4). This paper describes the pH effect on the slow intermediates of the photocycle. Flash photolysis experiments were carried out to investigate the pH and salt effect on intermediate $\text{O}(660)$.

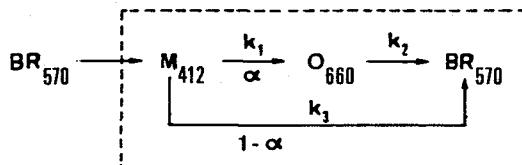


Figure 1. Branched pathway model of the slow part of the photocycle of bacteriorhodopsin. α represents the fraction of $\text{M}(412)$ molecules which decay via $\text{O}(660)$. $k(1)$, $k(2)$ and $k(3)$ are first order rate constants. Intermediates $\text{K}(590)$ and $\text{L}(550)$ were not considered in the model.

Photoacoustic (PA) frequency spectra were measured in the 5-420 Hz range at three pHs. Kinetics and energy variations associated with

the intermediate formation were investigated. The pH effect on the frequency spectra is similar to that observed by flash spectrophotometry. PA frequency spectra were simulated assuming a branched photocycle. The enthalpy changes associated with M(412) and O(660) intermediates were evaluated.

MATERIALS AND METHODS

Purple membrane fragments from *Halobacterium halobium* were isolated as previously described (5). They were suspended in buffers with or without 4 M NaCl.

For flash photolysis measurements, membrane suspensions (12 μM) were illuminated with a flash tube ($\lambda > 530 \text{ nm}$). The flash-induced absorbance changes were followed with an analytic light beam ($\lambda > 628 \text{ nm}$) provided by a Varian 300 W Xenon lamp. After passage through a monochromator, it was detected by a photomultiplier and stored in a CAT computer. Usually 70 flashes were added. All measurements were performed at 23°C.

The PA spectrometer was described elsewhere (6). PA signals were measured on purple membrane suspensions (0.75 mM) using a monochromatic light beam which corresponded to the bacteriorhodopsin absorbance maximum (570 nm). The chopping frequency was varied between 5 and 420 Hz. The photoinactive reference sample was a bleached suspension mixed with black ink in order to match the optical and thermal properties of the photoactive sample (6,7). The lock-in amplifier was adjusted at each chopping frequency in order to obtain the maximal in-phase signal with the reference sample. This phase setting was used for recording the signal from the photoactive sample. Simulations were performed as described elsewhere (6).

RESULTS AND DISCUSSION

Flash-induced absorbance changes were measured on purple membrane suspensions at 660 nm in the 5-9 pH change. This wavelength corresponds to the maximum absorption of the intermediate O(660).

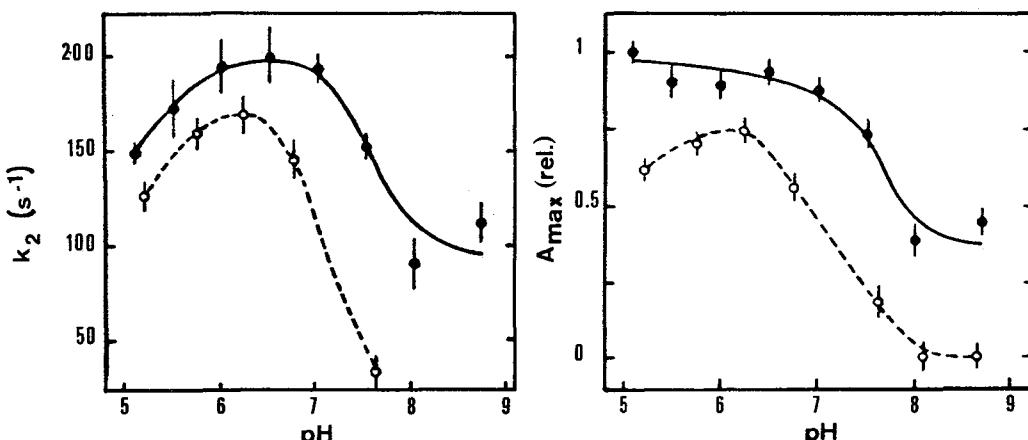


Figure 2. Effect of pH on O(660). Dependence of the decay rate constant (k_2) (left) and of the maximum absorbance (A_{max}) (right). ● buffer, ○ buffer with 4 M NaCl. The A_{max} value at pH 5.1 (no salt) was used for normalization.

Responses were similar to those previously published (8): the absorbance reaches a transient maximum some 5 msec after the flash and then decays exponentially. Fig. 2 shows the pH effect on the decay rate constant ($k(2)$) and on the maximum absorbance ($A(\max)$). In absence of NaCl, $k(2)$ peaks at pH 6.5 ($t_{1/2} = 3.5$ msec) and then decreases sharply at pHs higher than 7.0. $A(\max)$ remains constant up to pH 7.0 and then decreases with an apparent pK at 7.7. High salt concentration reduces both $k(2)$ and $A(\max)$ by 15-25 % in the 5-6.5 pH range. The apparent pK is shifted to 7.1 and above pH 8.0, $O(660)$ becomes undetectable. The results of Fig. 2 extend those of Sherman *et.al.* (5) who compared at low ionic strength the yield and kinetics of $O(660)$ at pHs 5, 7 and 9 and found a sharp reduction at alkaline pHs.

It seems reasonable to conclude that high pH and high salt concentration inhibit the $O(660)$ formation. Since the total amount of $M(412)$ is independent of these two parameters, the results suggest the existence of a branching in the bacteriorhodopsin photocycle. At present, neither an unidirectional unbranched model nor an unidirectional with simple branching is completely satisfactory for the interpretation of all the experimental data presently available (9). The scheme of Fig. 1 represents an approximation. However it provides a tool for discussing the pH effect on flash photolysis and photoacoustic data. Our PA experiments were performed at frequencies ranging from 5 to 420 Hz. Intermediates $K(590)$ and $L(550)$ were undetectable since their lifetimes fall in the μ sec range. Only the reactions enclosed in the Fig. 1 dashed box were considered. They were assumed to be first order reactions with rate constants $k(1)$, $k(2)$ and $k(3)$. $M(412)$ should be considered as a generic name for the two states $M(1)$ and $M(2)$. Several reports have indeed shown that $M(412)$ decays biphasically (10, 11). Actually α describes the equilibrium between $M(1)$ ($=\alpha M$) and $M(2)$ ($= (1-\alpha)M$). $M(1)$ decays straight to $O(660)$ whereas $M(2)$ goes back directly to $BR(570)$. As shown below α is pH dependent.

PA spectroscopy was used to derive information on thermodynamic and kinetic properties of the intermediates. Three transitions were detected at 22 Hz, 77 Hz and 215 Hz in the PA frequency spectra (6). This last transition was interpreted as resulting from a conformational change. The two lowest transitions coincide with the formation of $O(660)$ and $M(412)$. An increase in pH reduces the amplitude of the 22 Hz transition. At pH 8.9 it becomes undetected. The 77 Hz transition is pH independent. These findings should be compared with those of Fig. 2. Both sets of data can be interpreted with a branched model.

Malkin and Cahen (12) have provided a theoretical analysis of the PA frequency spectra in terms of the energy variations associated with intermediate formation in photochemical reactions. The ratio of total heat dissipated in the photoactive sample to that dissipated in a photoinactive sample is given by the signal ratio between the photoactive sample and the reference :

$$\rho/\rho_{ref} = 1 - \{ \sum_i \phi(i) \cdot \Delta E(i) / Nhv \} \quad (1)$$

By applying the theory of Malkin and Cahen (12) to the branched model of Fig. 1, one obtains Eq. 2 where $\Delta E(M)$ and $\Delta E(O)$ are the enthalpy changes associated with $M(412)$ and $O(660)$ respectively :

$$\left[\frac{\rho}{\rho_{ref}} \right]_{in} = 1 - \phi \frac{\Delta E_M}{Nhv} + \phi \frac{\Delta E_M}{Nhv} \frac{(ak_1 + (1-\alpha)k_3)^2}{\omega^2 + (ak_1 + (1-\alpha)k_3)^2} \quad (2)$$

$$- \frac{\Delta E_O}{Nhv} \frac{\phi ak_1 \omega^2}{\omega^2 + k_2^2} \frac{ak_1 + (1-\alpha)k_3 + k_2}{\omega^2 + (ak_1 + (1-\alpha)k_3)^2}$$

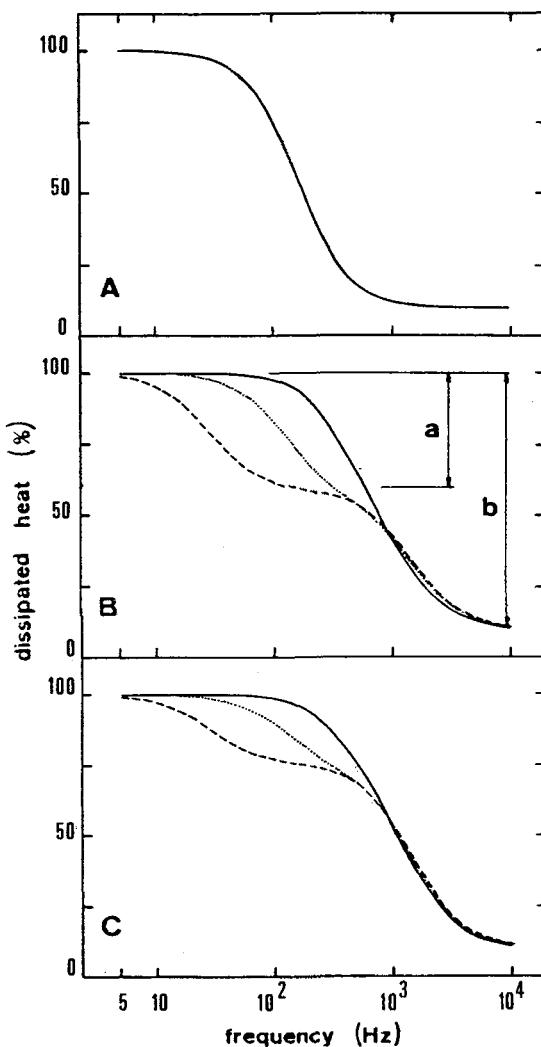


Figure 3. PA simulated frequency spectra computed in three hypothetical models. The photochemical quantum yield ϕ was assumed equal to 1.

A : unbranched model $M(412) \rightarrow BR(570)$ ($\alpha = 0$).

$$\Delta E(M)/N_{hv} = 0.9 ; k(3) = 1000 \text{ (1/sec)}.$$

B : linear model $M(412) \rightarrow O(660) \rightarrow BR(570)$ ($\alpha = 1$).

$$\Delta E(M)/N_{hv} = 0.9 ; \Delta E(O)/N_{hv} = 0.4 ; k(1) = 9000 \text{ (1/sec)}.$$

Three values of $k(2)$ were considered : $k(2) = 180 \text{ (1/sec)}$ (---), $k(2) = 800 \text{ (1/sec)}$ (....), $k(2) = 3000 \text{ (1/sec)}$ (—).

a and b represent respectively $\Delta E(O)/N_{hv}$ and $\Delta E(M)/N_{hv}$.

C : branched model (see Fig. 1) ($\alpha = 0.6$).

$$\Delta E(M)/N_{hv} = 0.9 ; \Delta E(O)/N_{hv} = 0.4 ; k(1) = k(3) = 9000 \text{ (1/sec)}.$$

Three values of $k(2)$ were considered : $k(2) = 180 \text{ (1/sec)}$ (---), $k(2) = 800 \text{ (1/sec)}$ (....), $k(2) = 3000 \text{ (1/sec)}$ (—).

This equation can be used to simulate PA frequency spectra.

Fig. 3 shows the simulated frequency spectra obtained from Eq. 2 in three hypothetical cases. For simplicity, the quantum yield is chosen equal to 1. Fig. 3A relates to an unbranched model ($\alpha = 0$). M(412) is supposed to decay directly to BR(570). If the rate constant is set arbitrarily to 1000 (1/sec), the transition is detected at 159 Hz. If the enthalpy change associated with the M(412) decay represents 90% of the light energy absorbed, the transition represents 90% of the dissipated heat. In Fig. 3B, M(412) is assumed to decay via the O(660) pathway. $k(1)$, $\Delta E(M)/Nhu$ and $\Delta E(O)/Nhu$ are set arbitrarily to 9000 (1/sec), 0.9 and 0.4 respectively. The three curves correspond to various values of $k(2)$ (180 (1/sec) dashed curve, 800 (1/sec) dotted curve and 3000 (1/sec) solid curve). When $k(2) = 180$ (1/sec), the two transitions are well resolved and the amount of dissipated heat are clearly measurable. If $k(2)$ falls in the same order of magnitude than $k(1)$ only one transition is detected and the enthalpy determination is very difficult. Fig. 3C introduces a branching in the photocycle ($\alpha = 0.6$). The main effect concerns the position of the plateaus. Their determination is rather difficult but surprisingly the reaction rate constant determination is not significantly perturbed by the branching.

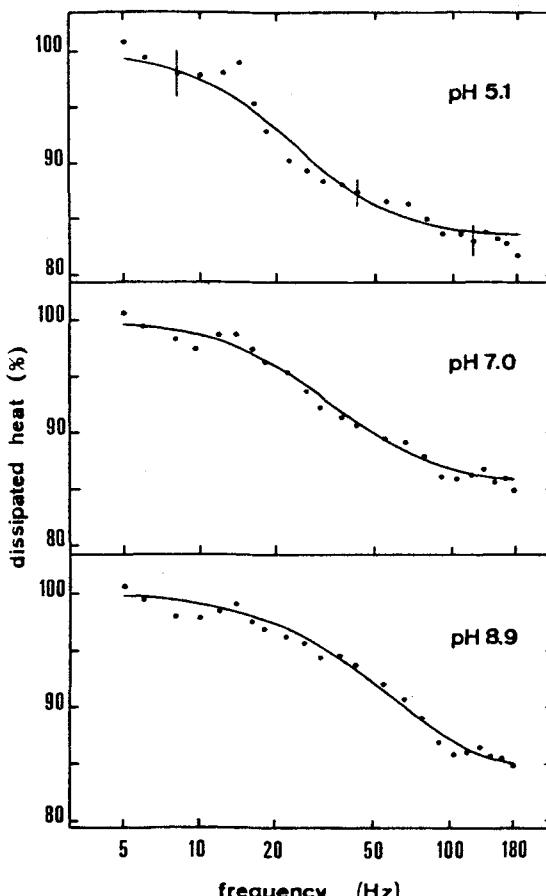


Figure 4. Comparison between the experimental and simulated frequency spectra of purple membrane suspensions at pHs 5.1, 7.0 and 8.9.

Simulation of the experimental frequency spectra was performed on basis of a branched photocycle, taking into account the flash photolysis data. Fig. 4 compares the simulated curves to the experimental points measured at pHs 5.1, 7.0 and 8.9 between 5 and 180 Hz. The parameter values adopted for the simulation are given in Table 1, as well as the evaluation of the enthalpy changes. The best fit is obtained at pH 8.9. Only one transition is detected in the simulated curves and this is understandable on basis of the Fig. 3 results, $k(1)$ and $k(2)$ falling within the same order of magnitude. The fit is satisfactory in terms of the pH influence. The discrepancy results probably from the simplicity of the model and from the imprecise evaluation of the parameters. $\Delta E(M)$ is unmodified by pH variations but $\Delta E(O)$ becomes smaller when pH increases (Table 1).

	pH 5.1	pH 7.0	pH 8.9
Φ	0.3	0.3	0.3
α	1	0.85	0.41
$k(1)$ (1/sec)	503	506	225
$k(2)$ (1/sec)	150	193	112
$k(3)$ (1/sec)	0	0	450
$\Delta E(M)$ (Kcal/mole)	26.7	24	26.5
$\Delta E(O)$ (Kcal/mole)	19.6	11.8	9.3

Table 1. Parameter values used and enthalpy variations evaluated in the simulation of the PA frequency spectra.

In conclusion, flash photolysis and photoacoustic data demonstrate the importance of pH on the bacteriorhodopsin photocycle. Our results are in qualitative agreement with those reported by Sherman et.al. (4) and by LeGrange et.al. (13). The interpretation based on a branched model can provide an insight into the understanding of the pH effect on the proton pumping mechanism (14).

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