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ENERGETICS AND CHRONOLOGY OF PHOTOTRANSIENTS IN THE LIGHT RESPONSE OF THE PURPLE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

WARREN V. SHERMAN*, RAFI KORENSTEIN** and S. ROY CAPLAN

Laboratory of Membranes and Bioregulation, Weizmann Institute of Science, Rehovot (Israel)

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SUMMARY

Arrhenius parameters for formation and decay of phototransients in suspensions of purple membrane fragments in H_2O and ^2H_2O have been determined in the temperature range 0–60 °C. Kinetic isotope effects are found which show that proton transfer steps are involved in both formation and decay of the two longest-lived transients absorbing at 410 nm and 660 nm, respectively. The results also suggest that these transients do not occupy a single pathway in the spontaneous deexcitation of bacteriorhodopsin within the purple membrane. Purple membrane undergoes a phase transition at 25–30 °C in both H_2O and ^2H_2O.

Fast processes in the light response of bacteriorhodopsin in the purple membrane of *Halobacterium halobium* have been intensively studied in several laboratories using conventional and laser flash photometry [1–5] and modulation [6] techniques. Subsequent to the primary light absorption event at physiological temperatures, a series of steps have been identified by the presence of phototransients with characteristic absorption maxima prior to the terminal relaxation of bacteriorhodopsin to the initial (light-adapted) state identified by the presence of a chromophore with a maximum at about 570 nm. While there are small discrepancies regarding the exact location of these transient maxima, there is general consensus that at room temperature, conversion of one transient to its successor occurs spontaneously (thermally) without the input of further light energy. After the formation of the primary 640 nm intermediate (< 1 μs) [3–5], there are three transients formed en route back to the initial state [1–3, 5, 6]. They are, using the convenient labelling of Lozier et al. [5], L(λ_{max} approx. 540 nm, decay half life approx. 10^{-4} s), M(λ_{max} approx. 410 nm, decay half life approx. 10^{-3} s), and O(λ_{max} approx. 640 nm, decay half life approx. 10^{-3} s). While M and O have similar decay times, O appears later than M. Additionally, a fourth transient, N(λ_{max} approx. 520 nm, decay half life approx. 10^{-3} s), has been tentatively identified by one group [5].

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[*] Permanent address: Department of Physical Sciences, Chicago State University, Chicago, Ill. 60628, U.S.A.

[**] Department of Structural Chemistry, Weizmann Institute of Science.

The principal function of the purple membrane in vivo appears to be to mediate between absorption of solar energy and phosphorylation of ADP [7]. It performs this by acting as a transmembrane proton pump transferring protons from the cytoplasm to the external medium. The proton gradient thus produced is energetically coupled to phosphorylation. In aqueous suspensions of the isolated purple membrane, protons are ejected from the protein interior to the medium. In considering the four or more phototransients formed during each light-driven cycle of the proton pump in vivo, it appears to us that the following questions are pertinent: (i) Do the phototransients occur sequentially in an unbranched chain, or are parallel (branched) sequences present? (ii) Since proton pumping is fundamental, at which point(s) of the chain(s) does proton transfer take place? In this paper we attempt to answer (at least partly) the first question by determination of Arrhenius parameters of the kinetics for formation and decay of M and O, and by comparison of rate constants in H_2O and ^2H_2O to identify proton-transfer steps. Additionally, the study reveals a break in the Arrhenius plots with a transition temperature corresponding to that previously observed for the microviscosity of membrane lipids of this species [8] and of *Halobacterium cutirubrum* [9].

Using conventional millisecond flash photometry techniques described fully elsewhere [1] utilizing a temperature-controlled sample compartment [10], appearance and decay profiles were analyzed at 410 nm and 660 nm for suspensions of purple membrane fragments (*H. halobium*, M_1) in H_2O and ^2H_2O having an initial light-

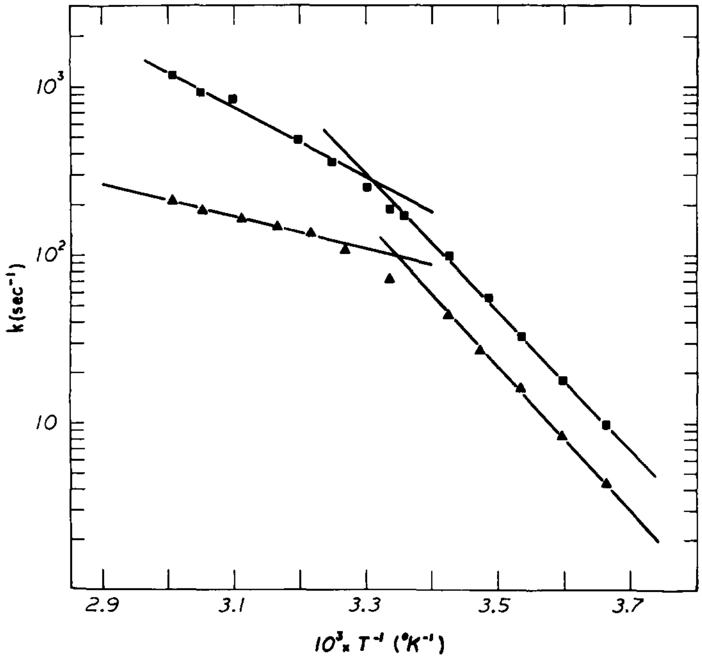


Fig. 1. Arrhenius plot for decay of phototransient M at 410 nm in H_2O (squares) and in ^2H_2O (triangles) in purple membrane fragments suspended in water. Bacteriorhodopsin concentrations approx. 3 μM based [3] on $\epsilon_{570} = 63\,000\ M^{-1} \cdot cm^{-1}$. Experimental methodology is as previously described [1].

adapted maximum absorbance at 570 nm of about 0.2 (approx. 3 μM) over the temperature range 0–60 °C. As indicated previously [1], the decay of absorbance at 410 nm and 660 nm and recovery of the ground state chromophore at 570 nm followed good first order kinetics for which rate constants were calculated. The grow-in times of M and O however do not follow simple first order kinetics, the former owing to photomultiplier fatigue at the exciting-flash tail, and the latter since it is superimposed on the long-wavelength end of the 570 nm ground state depletion [1]. However, relative rate constants for formation are conveniently estimated by comparing the reciprocal of the total grow-in times, i.e. time between the exciting flash and maximum development of phototransient absorbance. The values thus obtained are approximations which assume that attenuation of maximal product yields due to decay may be neglected. An Arrhenius plot of the data for decay of M in H_2O and ^2H_2O is shown in Fig. 1. This clearly bimodal character for the dependence of log k on T^{-1} with mode transition at 25–30 °C was also found to be characteristic of the formation of this transient, and of the formation and decay of O. Arrhenius parameters calculated for the linear regions of these plots are summarized in Table I. Also given, for purposes of comparison, are relative constants in H_2O and ^2H_2O at representative points in the low temperature (10 °C) and high temperature (40 °C) regions. The complete kinetic data and the implications of the kinetic isotope effect for possible mechanisms of proton transfer within the purple membrane will be dealt with elsewhere (Korenstein, R., Sherman, W. V. and Caplan, S. R., unpublished), but as was previously pointed out [12], the relatively small entropies of activation indicate that

TABLE I

ARRHENIUS PARAMETERS AND REPRESENTATIVE RATE CONSTANTS FOR APPEARANCE AND DECAY OF PHOTOTRANSIENTS IN AQUEOUS SUSPENSIONS OF PURPLE MEMBRANE

	Phototransient M at 410 nm				Phototransient O at 660 nm			
	Formation		Decay		Formation		Decay	
	Temperature region: Low	High	Low	High	Low	High	Low	High
k^{H} (ms^{-1})[a]	6.3[b]	—	$3.3 \cdot 10^{-2}$	0.49	$6.7 \cdot 10^{-2}$[b]	0.56[b]	$4.6 \cdot 10^{-2}$	0.45
$k^{^2\mathrm{H}}$ (ms^{-1})[a]	1.2[b]	8.9[b]	$1.6 \cdot 10^{-2}$	0.15	$3.3 \cdot 10^{-2}$[b]	0.21[b]	$1.9 \cdot 10^{-2}$	0.14
$k^{\mathrm{H}}/k^{^2\mathrm{H}}$	5.2	—[b]	2.0	3.4	2.0	2.6	2.4	3.2
E_a^{H} ($\mathrm{kcal \cdot mol^{-1}}$)	12	—[c]	18[e]	9.1	14	6.6	16	3.7
$E_a^{^2\mathrm{H}}$ ($\mathrm{kcal \cdot mol^{-1}}$)	15	4.2	19	4.0	14	3.5	15	2.2
log A^{H} (s^{-1})	—[d]	—[d]	16[e]	9.0	—[d]	—[d]	14	5.2
log $\mathrm{A}^{^2\mathrm{H}}$ (s^{-1})	—[d]	—[d]	16	4.9	—[d]	—[d]	13	3.7
S_a^{H} (entropy units)	—[d]	—[d]	6.4[e]	−6.5	—[d]	—[d]	2.5	−15
$S_a^{^2\mathrm{H}}$ (entropy units)	—[d]	—[d]	6.4	−16	—[d]	—[d]	0.48	−18

[a] Representative values at 10 °C for low temperature region and 40 °C for high temperature region.

[b] Reciprocal of total rise time, see text.

[c] Rise time not completely resolved from tail of exciting flash.

[d] Arrhenius parameters calculated from total rise times. Hence only E_a may be calculated.

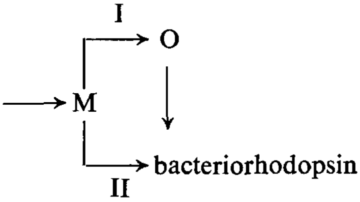
[e] These values differ from those previously determined [12]. The latter were based on a narrower temperature range, and lie between the high and low temperature values given here.

significant conformational changes are not involved in the proton transfer steps. Additionally we find that below the transition temperature the first order rate constants for recovery of the 570 nm chromophore are identical to those for decay of M. Above the transition temperature the former lie somewhere between the M decay rates and those for decay of O.

The significant isotope effects observed in the formation and the decay of both M and O clearly show proton transfer to be involved in all of these four processes. Lewis et al. [11] have concluded on the basis of resonance Raman data that unexcited BR and transient M are respectively protonated and deprotonated at the Schiff base linkage connecting the retinylidene prosthetic group and the opsin protein. Our present findings therefore are consistent with these conclusions, and in addition show that O is also intimately involved in the proton pump. However, we must be cautious and point out that although our data could be construed to show that a proton is ejected from bacteriorhodopsin on formation of M and/or O and returns on the conversion of M and/or O to initial bacteriorhodopsin, these isotope effects are also consistent with internal proton shifts within the protein prior to and after proton extrusion into the external medium. It seems probable that the proton pump involves a series of proton transfer steps (proton hopping or proton diffusion) within the purple membrane [1, 5, 12, 13], in addition to proton ejection into the medium and uptake from the cytoplasm. These manifest themselves by producing transient electronic absorption spectra at the site of the long-wavelength absorption band of bacteriorhodopsin. This model is consistent with the pore or channel mechanism proposed by Racker and Hinckle [14], for proton pumping by bacteriorhodopsin in reconstituted phospholipid vesicles. The temperatures at which we observe changes in Arrhenius parameters coincide with those found for changes in mobility of probes penetrating to the lipid region bordering the purple membrane protein in *H. halobium* [8] and the related *H. cutirubrum* [9].

Finally we address ourselves to the question of the chronological relationship of M and O and the regeneration of bacteriorhodopsin. Stoeckenius and his collaborators favor an unbranched chain M → O → bacteriorhodopsin [5, 13]. However, our results clearly contradict this being the exclusive pathway for disappearance of M. First, we find that at all temperatures studied the rate constants for decay of M are significantly lower than for appearance of O. For an exclusively unbranched sequence clearly these two rate constants must be identical. Further, we see (Table I) that activation energies for decay of M and formation of O at comparable temperature regions in H_2O and in ^2H_2O are significantly different in all cases.

We have previously shown that on valinomycin treatment M and O respond differently; the appearance of O can be completely inhibited while the yield of M is little changed [1]. Hence, the appearance of O is not a requirement for the decay of M. This same conclusion was of course implicit in the first-published account of the existence of O at physiological temperatures [6]. The latter species, while readily identifiable at room or higher temperatures, was overlooked when the system was previously examined at low temperatures [15]. The yield of M, as we confirm here, is only slightly affected by temperature. The possibility remains however that while the intermediacy of O is not essential for the relaxation of M to bacteriorhodopsin it is a competing minor pathway, i.e.



Such a scheme was proposed previously [6]. Since an increase in yield of O is observed with increasing temperature, route I becomes more important at elevated temperatures. Consistent with this scheme is the observation that the difference between the activation energies for decay of M and formation of O is smaller at the high temperature region and therefore thermodynamic control becomes less unfavorable to route I. However, balanced against this we see that the actual rate constants differ considerably at the higher temperatures, and there is also no evidence for any break in the good first order kinetics for the decay of M (Korenstein, R., Sherman, W. V. and Caplan, S. R., unpublished).

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