BRANCHING REACTIONS IN THE PHOTOCYCLE OF BACTERIORHODOPSIN

Rafi KORENSTEIN*, Benno HESS and Dietrich KUSCHMITZ
Max-Planck-Institut für Ernährungsphysiologie, Rheinlanddam 201, 4600 Dortmund 1, FRG

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1. Introduction

The purple membrane of *Halobacterium halobium* acts as a light-driven proton pump [1]. It contains a single protein to which a retinal is bound via a protonated Schiff-base [2,3]. This protein–retinal complex, bacteriorhodopsin (BR), undergoes a reaction cycle involving several intermediates after light absorption. The photocycle can be described by the following scheme showing the time sequence of intermediates of BR in the purple membrane as defined in [4], the numbers giving their approximate wavelength maxima [4–6]:

$$\underset{\text{all-}trans}{BR^{570}} \rightarrow K^{590} \rightarrow L^{550} \rightarrow M^{412}\ (O^{660})$$

Although the scheme describes the photocycle in terms of consecutive reactions the possibility of branching reactions has already been raised in the early studies of the photocycle. Polarization studies utilizing modulation excitation spectrometry [7] had shown the possible existence of two species absorbing at 412 nm, though they could not be kinetically resolved. Biphasicity in the decay kinetics of M was observed as a function of the ionic strength [8]. Moreover photobleaching of M had revealed two forms of M due to a difference of 0.3 in their photoconversion yields [9]. Recently [10] a photochemical equilibrium between two forms of M was shown to exist at 77 K. Here we present a study which shows that a branching in the thermal reactions of the cycle occurs and demonstrate the existence of two conformations of M^{412}, which are in thermal equilibrium.

* Present address: Department of Membrane Research, The Weizmann Institute of Science, Rehovot, Israel

2. Materials and methods

Purple membrane was isolated from *H. halobium* (mutant NRL R_1M_1) [2]. Purple membrane (20 μM) was suspended in an ethylene glycol–water 1:1 mixture, 50 mM phosphate buffer and pH 7.2. The kinetics as well as the absorption difference spectra were measured by the experimental set up shown in fig.1a. The sample was excited by a 400 W W-I_2 lamp (LS_1) whose light was filtered by a neutral density filter (F_1) and a cut-off Eppendorf filter > 500 nm (F_2) or by a flashlamp-pumped rhodamine 6G dye laser (so that M^{412} was not excited by the light). The measuring light from a 400 W W-I_2 lamp (LS_2) passed a Bausch and Lomb monochromator (M), was focussed onto the cuvette and then filtered by a 412 nm interference filter (F_3) (8 nm band width) on the multiplier (PM) (EMI 9634 QR). The output of the multiplier was amplified and passed through a variable RC filter both to a slow speed recorder and to a digital scope (Nicolet 1090). All experiments were performed at −20°C in order to obtain a high conversion of BR^{570} into M^{412}, under continuous light irradiation.

3. Results and discussion

A typical formation kinetics of M^{412} is shown in fig.1b. The kinetics were analysed in terms of a sum of exponentials. The initial values for the rate constants and their relative amplitudes were calculated by a computer program, based on a nonlinear approximation (K. H. Müller and T. Plesser, unpublished results). These values were optimized by a least square program (Harwell Subroutine Library, VCO5A). The analysis of the formation kinetics gave best fit when

analysed as a sum of two exponentials, yielding rates and amplitudes (in brackets) of $k_1 = 2.87 \pm .95$ ms^{-1} (0.14 ± .03) and $k_2 = 0.19 \pm .01$ ms^{-1} (0.85 ± .04) ($n = 10$, where n is no. expts). The reliability of the kinetic analysis is based on a construction of a contour map of the sum of squares shown in fig.2. The contours in fig.2 show the possible variation of the two rates, whenever performing an optimization of the rates by least squares. The variation of the rates will depend on its form and on how shallow or deep is the minimum. Each point on the contour curves (representing a constant value of sum of squares) was calculated by fixing the rates and fitting the amplitudes linearly by least squares. The percentages represent the probability of finding the minimum in the corresponding contour of sum of squares, found by a statistical analysis using the F-test. Thus the possible variation of the two rates in the formation of M^{412} is shown in fig.2a.

Analysis of the decay kinetics of M^{412} (fig.1c, curve 1) gives similarly to the formation kinetics a best fit when analysed as a sum of two exponentials (see table 1). Again the possible variation of the rates was analysed by a contour map (see fig.2b). Thus the fact that M both forms and decays as a sum of two exponentials suggests the existence of two forms of L^{550} which decay by parallel paths yielding two conformations of M^{412} which then decay to BR^{570}.

The occurence of an equilibration process between the two proposed conformations of M^{412} was studied by measuring the decay kinetics of M as a function of the time duration of the light pulse (pulse width) [11]. Since the decay kinetics depend on the ratio of M^{412}/BR^{570} (R. K., B. H. and M. Markus, in preparation), the intensity of the longer light pulses was decreased by means of neutral filters (fig.1a) so that the decay was measured always for the same concentration level of M^{412}. Similar to results obtained at

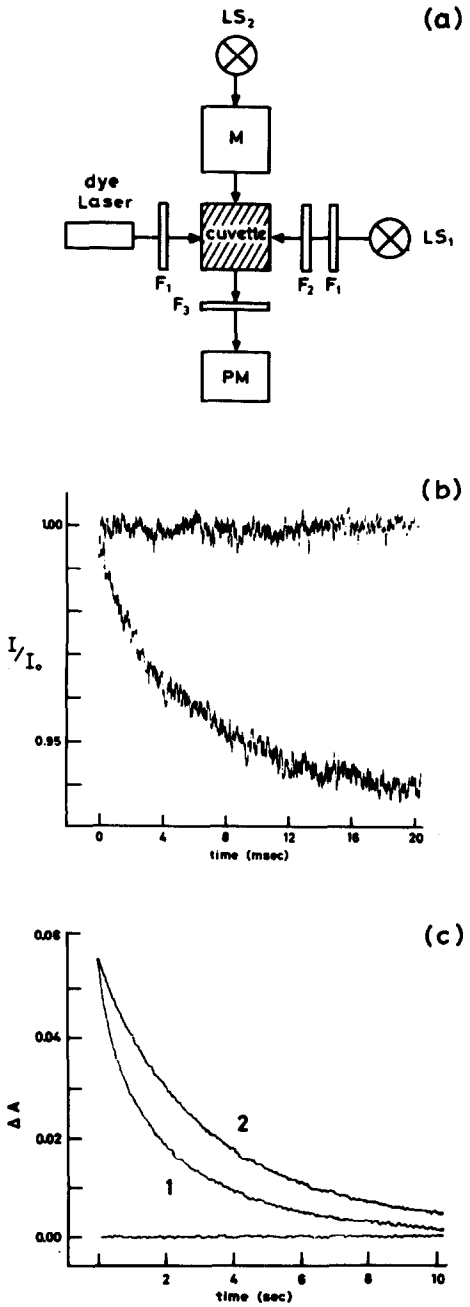

Fig.1. (a) Block diagram of the experimental set-up.

(b) The formation kinetics of M^{412} measured at 412 nm. The sample ([BR] 2×10^{-5} M in ethylene glycol–water 1:1 mixture 50 mM phosphate buffer pH 7.2) was excited by a linearly polarized light pulse from a rhodamine G6 laser, at −20°C.

(c) The decay kinetics of M^{412}. Curve 1: decay of M^{412} formed by a pulsed light (3 μs pulse width). Curve 2: decay of M^{412} formed by a continuous light (∞ pulse width). Both decay kinetics were measured at −20°C with the same sample as in (b).

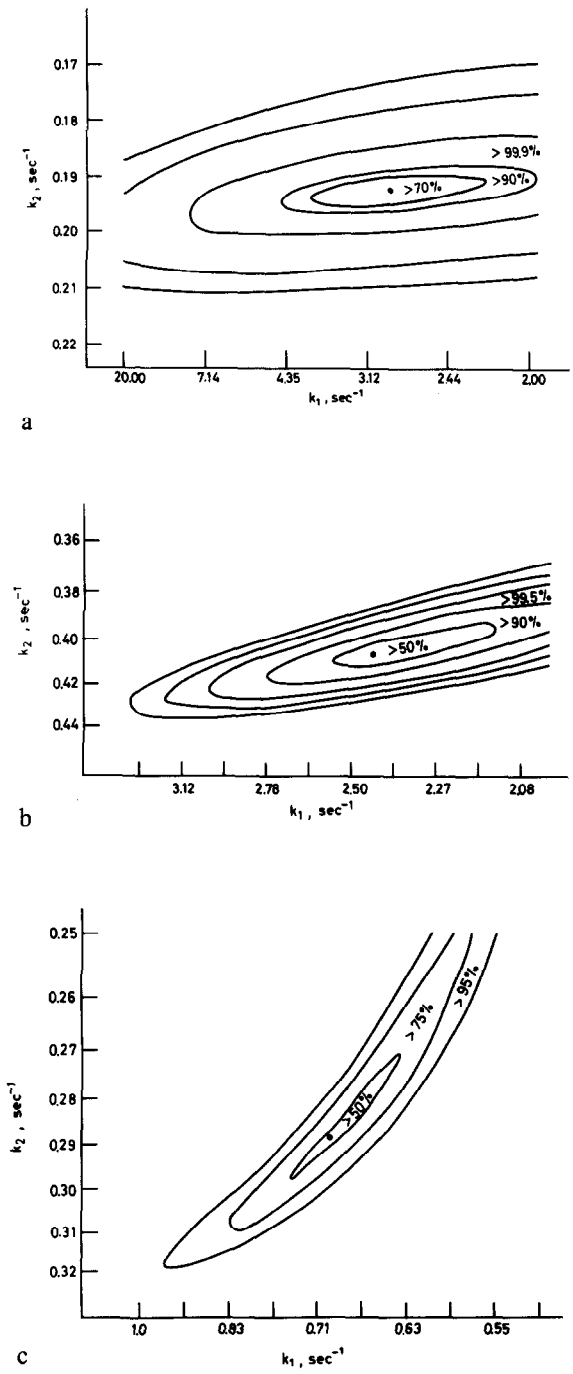

Fig.2. Sum of squares contours as a function of the variation of the two rates found in the formation and decay kinetics of M^{412}. (a) Formation of M^{412}. (b) Decay of M^{412} produced by a pulsed light. (c) Decay of M^{412} produced by a continuous light.

higher temperatures and high ionic strength [11], a pronounced difference in the kinetics is observed when the sample was excited by a laser light pulse (3 μs pulse width) or by continuous light of a lamp (∞ pulse width) as shown in fig.1c. Again here the decay of M^{412}, produced by continuous light was analysed in terms of two exponentials and a contour map of sum of squares was constructed for the rates of its decay (fig.2c). The analysis of the dependence of the decay rate constants on the light pulse width is given in table 1. It is evident from table 1 that the bigger rate constant varies strongly with the change of the light pulse width while the other rate does not change much.

In order to establish whether the change of the rate constants is connected to a possible equilibration process between the two conformers of M^{412}, two different spectra of M^{412} were produced by pulsed and continuous light (fig.3). From comparison of both it is evident that the photostationary difference spectrum of M^{412} is blue-shifted relative to the difference spectrum produced by pulsed light. This spectral shift can be attributed to a change in the composition mixture of the two conformers due to the existence of equilibration process between them. This process can explain the dependence of the rate constant on the light pulse width due to the conversion of the conformer which decays at a high rate into a conformer which decays at a low rate (where the rates shown in table 1 are apparent rates). This suggestion

Table 1
The decay[a] of M^{412} as a function of pulse width of the exciting light

Pulse width	k_1, s^{-1}	k_2, s^{-1}
3 μs	2.32 ± .09 (0.35 ± .02)[b]	0.40 ± .01 (0.65 ± .01)[b]
125 ms	2.39 ± .05 (0.35 ± .02)	0.40 ± .01 (0.65 ± .01)
1 s	2.29 ± .17 (0.22 ± .01)	0.42 ± .01 (0.79 ± .01)
2 s	1.70 ± .33 (0.16 ± .01)	0.39 ± .01 (0.85 ± .02)
4 s	1.39 ± .21 (0.16 ± .03)	0.37 ± .01 (0.84 ± .03)
6 s	0.97 ± .20 (0.26 ± .07)	0.33 ± .02 (0.74 ± .07)
∞[c]	0.68 ± .22 (0.26 ± .07)	0.30 ± .05 (0.74 ± .07)

[a] Purple membrane (2×10^{-5} M) was suspended in ethylene glycol–water 1:1 mixture (50 mM phosphate buffer, pH 7.2) and the decay kinetic was measured at −20°C
[b] Amplitudes are shown in parentheses
[c] Continuous light

is compatible with the decrease of the relative amplitude of the higher rate constants with an increase of the light pulse width (table 1). From the change of the rates as function of the pulse width, the relaxation time constant for the equilibration can be estimated to be of the order of 1–3 s (at –20°C). The photocycle of bacteriorhodopsin can be thus summarized by fig.4. It is interesting to note that the recent study of the rate of proton uptake also yielded a dependency of the time constant as a function of the pulse width [11]. The present study puts only a lower limit to the reaction step where branching occurs, since the decay of K^{590} was unresolved under our experimental conditions. The possible existence of heterogeneity in the retinal sites [12], may also contribute to the multi-exponential kinetics of the photocycle.

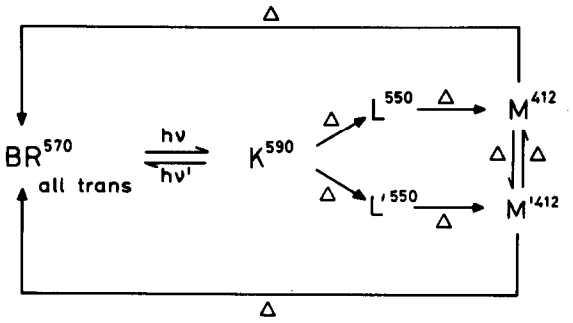

Fig.4. Scheme of the photocycle of bacteriorhodopsin (Δ represents thermal reactions).

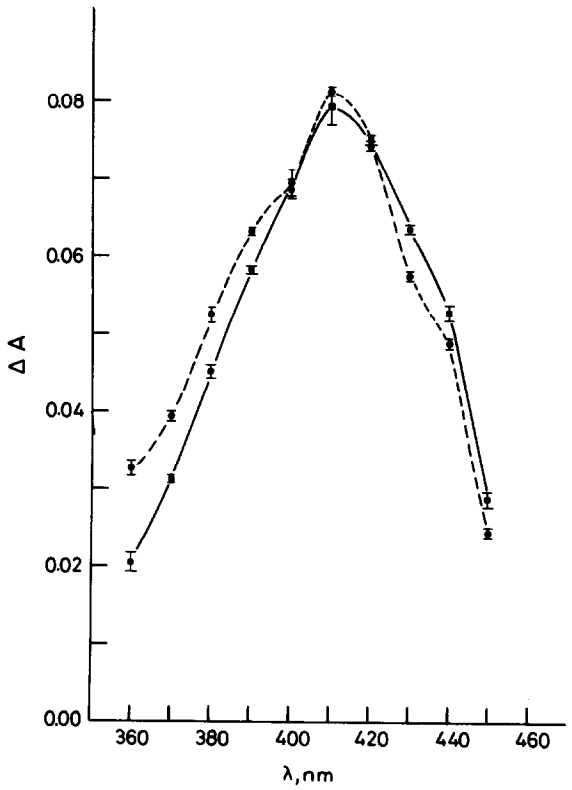

Fig.3. Difference spectra of $M^{412}-BR^{570}$ obtained by excitation of light-adapted bacteriorhodopsin by a pulsed and continous light. The full curve (circles) was obtained by a pulsed light, whereas the broken curve (squares) was obtained by a continuous light. Bars represent standard deviation in the measurement of ΔA. The spectra were normalized at 400 nm. The normalization was accomplished by changing the intensity of the continuous light till the absorption change by the continuous light, at 400 nm, was equal to the absorption change produced by the laser pulse at the same wavelength. The difference spectra were measured by the experimental set up shown in fig.1a except the filter F_3 which was interchanged with a prism Zeiss monochromator. Other experimental conditions were identical to those in fig.1b.

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