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A possible protein motion during the bacteriorhodopsin photocycle detected by combined photothermal beam deflection and optical detection

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Abstract

Photothermal beam deflection of bacteriorhodopsin in the time range from 1 to 200 μs resolved a new process with a time constant of approx. 20 μs at 20°C in addition to the K and L decays with time constants of 1.3 and 90 μs. The latter time constants are concordant with those obtained by nanosecond flash photolysis with optical detection on the same samples. The additional process may be ascribed to a protein conformational change during the decay of L → M, i.e., concomitant with the starting of the proton transfer.

Key words: Bacteriorhodopsin; Photocycle; Protein motion; Photothermal; Beam deflection (*H. halobium*)

1. Introduction

The light-induced photocycle of the membrane protein bacteriorhodopsin (BR) from *H. halobium* is the key process for its function as a light-driven proton pump. This proton transport capability across the cell membrane enables the bacterium to create a proton gradient and exploit it as an alternative energy source [1]. Several intermediates (named alphabetically J, K, L, M, N, and O) can be spectrally discerned during the course of the photocycle. They result from changes of the conformation and/or protonation state of the retinal chromophore and closely adjacent amino acids. The first intermediates, J and K, are formed within picoseconds as products of a photoisomerization of the chromophore, which is covalently bound via a Schiff base to the protein. The following thermally formed intermediates (L, M, N, and O) have much longer lifetimes [2] and are due to de- and re-protonation of the Schiff base (L → M and M → N conversions, respectively) and the thermal re-isomerization of the retinal chromophore.

Whereas formerly a linear succession of the intermediates was proposed, back reactions (i.e., the formation of equilibria), branching pathways, and independently running, parallel photocycles have been introduced [2–5]. In addition to the reactions of the chromophore and the closely adjacent amino acids, conformational changes of large protein domains were proposed to support a unidirectional proton transport [2,6–8].

We now report a flash photolysis study of the photocycle of BR in the time range from several hundred nanoseconds to microseconds with time-resolved optical and photothermal beam deflection (PBD) detection. Photothermal methods have been shown as useful to investigate conformational changes of photoreactive compounds [9]. As an extension of our studies with laser induced optoacoustic spectroscopy (LIOAS) [10], we now apply PBD to the BR system, since decays on a longer time scale than those reached with LIOAS can be studied following the refractive index change in a solution. This makes it possible to obtain information about enthalpy and volume changes taking place during the steps involved in the proton transfer (e.g., L → M).

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2. Materials and methods

BR was isolated as described [11]. Light adapted samples (15 μM, $A_{568} = 1.0$) were investigated in the temperature range from 0 to 35°C.

The PBD experiment was realized by focusing a pulsed excitation laser beam and a parallel continuous wave (cw) probe beam onto a 1 cm sample cuvette. The excitation beam was generated by a dye laser (pulse width 8 ns, rhodamine 6G, 555 nm) pumped by a frequency doubled Nd:YAG laser ($\lambda = 532$ nm, Spectron Laser SL8000). The cw probe beam at approx. 825 nm (Philips CQL 44 A/D laser diode) was deflected by the gradient of the refractive index which arose in the sample upon absorption of the excitation pulse. Similar to the formalism applied to acoustic detection in which the pressure wave arises from a molecular movement in addition to a heating of the solution, we adapt now this concept to the case of monitoring the refractive index change [9,12]. The complete mathematical treatment will be published elsewhere. The angle of probe beam deflection, ψ, was detected by a double photodiode (Centronic LD2–5T) placed behind a filter (RG 780) that absorbed the pump beam. The resulting voltage difference was stored after amplification by a transient recorder (Gould Biomation 8100). The signals were averaged 100 times to improve the S/N ratio. For small ψ values, the signal is proportional to ψ [13], and therefore also proportional to the amount of released energy and additional volume changes. The validity of this proportionality was tested by the linearity of the signal of a calorimetric reference (bromocresol purple) with the excitation energy. Within a time range of 100 ns to about 200 μs there was no change in the signal of the reference. This indicates that heat conduction is negligible in this time range. For both reference and sample, the energy of the excitation beam was varied by a neutral density filter and controlled by an energy meter. The maximum repetition rate of the excitation pulse was 2 Hz in order to guarantee a full thermal relaxation of the sample as well as completion of the photocycle of BR (approx. 10 ms at room temperature). The maximal excitation energy was set to 2 μJ, corresponding to less than 1 photon per molecule and ns.

The amount of the photoproduct K formed within the pulse duration of the excitation laser was calculated using a model including the excitation of K and its photochemical back reaction to BR (see Fig. 1). According to this model, only the ground states of BR and K but not the J state become significantly populated during the duration of the excitation pulse. Thus, excitation of J was neglected for the formalism. No significant change resulted upon varying the rate constant k_5 (back reaction from excited K to BR) between 1 and 10 ps. Since L is formed after more than 1 μs, it is not affected by the laser pulse. The absorption

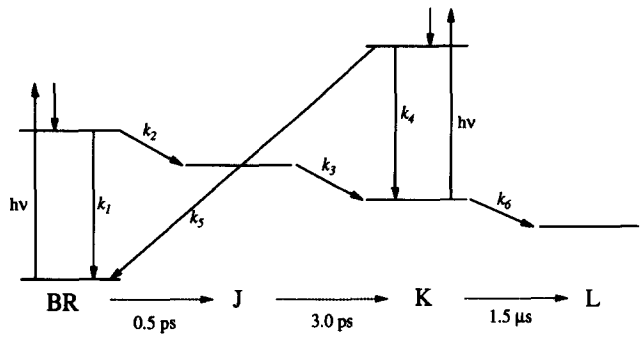

Fig. 1. State and reaction diagram of the first steps of the BR photocycle after absorption of a nanosecond laser pulse [13]. k_i ($i = 1,\ldots,6$) indicate the rate constants for the different decays. The excited state of J was neglected (see text).

cross-sections of BR and K at 555 nm were determined as 2.343 and $1.610 \cdot 10^{-16}/\text{cm}^2$, respectively, after extracting the spectra of the pure components BR and K from a difference spectrum. The quantum efficiency for the process BR → K was taken as 0.6 [10,14]. For the back reaction K → BR, values between 0.6 [10] and 1.0 [14–16] were applied, yielding concentrations of K vs. the initial BR concentration at the end of the laser flash between 45 to 60% and 40 to 47%, respectively, for the examined energy range.

The absorption flash photolysis experiment was previously described [17,18]. In Ref. 18 the S/N ratio conditions are described in detail. The repetition rate as well as the photon density did not exceed those from the PBD experiment. A sum of exponential functions was fitted to the signal $S(t)$ for both experiments:

$$S(t) = a_0 + \sum_i a_i(1 - \exp[-k_i t])$$

Independent of the kinetic model used to explain the mechanistic implications of the values of a_i [18], these preexponential terms contain different information depending on the detection employed. While for PBD the amplitudes a_i contain the enthalpy as well as the volume and optical changes per molecule of the intermediates (the separation of these factors is currently investigated), for absorption detection the amplitudes contain the absorbance.

For optical detection, the photocycle of native BR was excited by laser flashes (pulse length 15 ns) at 568 nm. The absorption changes were monitored at wavelengths which are indicative for the K, L, and M intermediates, i.e., 590, 550, and 412 nm, respectively. The decay kinetics were followed between 0 and 35°C. All measurements were performed with samples dissolved in pure water (pH 7.2) or with KCl addition to a final concentration of 150 mM. In all cases the salt-containing samples showed slightly faster decays than the pure water preparations, but still exhibited mono-

exponential behaviour and an identical temperature dependence as preparations without KCl. The same holds true for buffered solutions.

3. Results and discussion

At 20°C, the K → L conversion proceeded with a time constant of 1.4 ± 0.2 μs. Since both intermediates, K and L, absorb significantly around 550 nm, the absorption changes at that wavelength became undetectably small and no L formation could be followed. The L → M conversion had a lifetime of 80 ± 10 μs at both wavelengths (see Table 1). In all cases a mono-exponential function yielded a sufficient fit to the data.

In order to allow for a comparison between both detection methods, the BR samples without KCl investigated by absorption flash photolysis were also used for PBD. Since the time resolution for PBD is in the μs range, the observed signal is expected to be a composite of all heat emitting and/or volume changing processes occurring within this time range. A typical PBD signal for 555 nm excitation is shown in Fig. 2 for the time interval from approx. 100 ns to 200 μs together with the resulting residuals of two fit procedures. It is clearly seen that the three-component fit exhibits good accordance between measured and simulated kinetics. Time constants of approx. 1.3 ± 0.1 μs, 20 ± 5 μs, and 90 ± 10 μs with relative amplitudes of approx. 35–40, 10, and 50–55% were derived. Attempts to fit only two exponentials to the data resulted in a worse agreement between data and fit. This becomes obvious from an inspection of the residuals (see insets of Fig. 2). Thus, in addition to the decay of the K- and the L-intermediates which are in good accordance with the absorption flash photolysis data, a third component is required for a sufficient description of the time-dependent refractive index changes (see Table 1). PBD data obtained with BR in D_2O-based solutions (unpublished data) support the present assignment of the longest-lived decay to the L → M transition involving the proton transfer.

The temperature dependence of the kinetics for both sets of data is shown in Fig. 3. The slopes for the

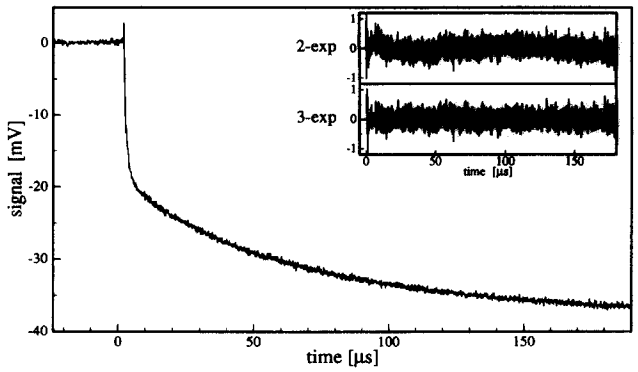

Fig. 2. Beam deflection signal of BR (T = 20°C, λ_{exc} = 555 nm, A_{555} = 0.93, E_{exc} = 0.75 μJ) and residuals of a 2- and 3-exponential fit (inset).

optical data obtained at 590 and 412 nm yielded an activation energy of $E_a^K = 37$ (K → L) and $E_a^L = 57$ (L → M) kJ/mol, respectively. The corresponding values from PBD are $E_a^K = 48$ and $E_a^L = 58$ kJ/mol, respectively. The relative error of the decay time for the additional process measured by PBD is obviously larger than the other processes due to its small amplitude. A linear fit to the data for this transient yields an activation energy of 35 kJ/mol. The activation parameters E_a and $\log A$ are listed in Table 1.

The value of E_a^K measured in the absorption experiment is in good accordance with data from Varo and Lanyi (38 kJ/mol) [5], while the value obtained by PBD is closer to the data from Beece et al. (52 kJ/mol) [19]. The value for the activation energy for the process L → M was the same for both methods and within the discussed range for $E_a^L = 46$ to 66 kJ/mol [5,19,20]. The values of $\log A$ determined from both methods are similar within the experimental error. In particular the $\log A$ values measured by PBD are in accordance with literature data [19].

A comparison of the amplitudes from both methods is not straightforward, since, as mentioned above, they contain different information. In the case of PBD, the small amplitude of the 20 μs component may be due to the small enthalpy difference between the intermediates. The a_i for optical detection depend on absorb-

Table 1
Lifetimes (at T = 20°C) and activation parameters (T = 0–35°C) for the early steps of the BR photocycle detected by PBD and optical detection

T = 20°C				T = 0–35°C			
PBD	optical detection			PBD		optical detection	
τ/μs	τ/μs	λ_{obs}		E_a/kJ/mol	$\log A$	E_a/kJ/mol	$\log A$
1.3 ± 0.1	1.4 ± 0.2	590 nm	K → L	48 ± 5	14.5 ± 1.0	37 ± 5	12.4 ± 1.0
20 ± 5	[a]			35 ± 8	10.9 ± 2.0	[a]	[a]
90 ± 10	80 ± 10	550, 412 nm	L → M	58 ± 3	14.5 ± 0.5	57 ± 3 [b]	14.3 ± 0.5 [b]

[a] Not observed (see text).
[b] λ_{obs} = 412 nm.

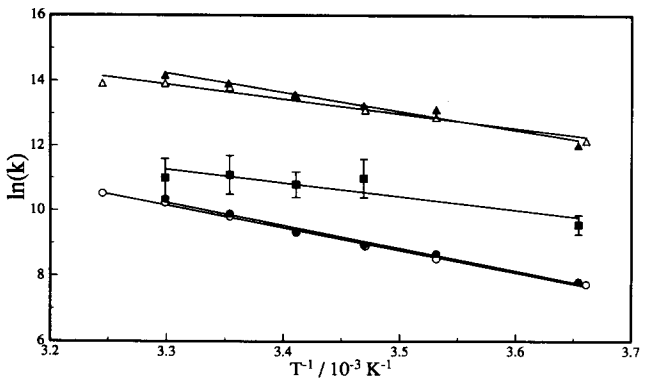

Fig. 3. Temperature dependence of rate constants of the BR photocycle measured with absorption flash photolysis (open symbols, triangle: $\lambda_{obs} = 590$ nm, circle: $\lambda_{obs} = 412$ nm) and beam deflection (filled symbols); see text for additional rate constant of the beam deflection signal (filled box).

ance parameters; a comparison would be therefore erroneous. Should the retinal chromophore be involved in the 20 μs process, a high absorbance change would be expected at the observation wavelengths, and the amplitude corresponding to this intermediate would be large. The monoexponential behaviour of the 1.4 and 80 μs decays at the respective observation wavelengths indicate that the 20 μs transient exhibits a negligible absorbance at those wavelengths. A monoexponential rise of M with $\tau \approx 80$ μs (20°C, pH 4–8) is documented by other authors, e.g., by Eisfeld et al. [21], while a slow component of some hundred μs in addition to the 80 μs decay at 550 nm is not in contradiction to our results. Although other authors detected a fast component ($\tau < 6$ μs or < 10 μs respectively, at room temperature) of approx. 10% amplitude in additon to a rise time constant of about 80 μs for the M state [14,22], these processes are too fast in comparision with our 20 μs component in PBD. A component of some μs time constant and 10% amplitude or more in the absorbance change signal at 412 nm as well as at 550 nm can be excluded from our data. The reason for these differences between our results and those of other authors is unclear at the moment. It is important to remark, however, that our absorption measurements were performed under similar BR concentration and photon densities as those used for the PBD measurements. Concentration effects or fluence effects cannot be excluded as the reason for the differences between our data and those from other authors.

By monitoring the kinetics of the BR photocycle by two detection methods, time resolved absorption spectroscopy and PBD, we identified an additional process between the intermediates L and M, i.e., at the moment when the proton transfer is initiated. The similarity of the lifetimes and temperature-dependent behaviour of the shortest- and longest-lived components support the validity of the PBD approach. Thus, the 20 μs component reflects an additional process occurring in the system. On the molecular level, this process may reflect a protein conformational change necessary for the deprotonation of the Schiff base by approaching the proton acceptor. On the other hand, the new process may represent a conformational motion of the protein which activates the proton channel to the transport of the arriving proton. The conformational change of the protein now found between L and M is in agreement with the different structure of the protein in the M state compared to the BR ground state as observed by different diffraction techniques [23–25]. The additional strength of the PBD method is its capability to time-resolve protein movements in photoreceptors with μs resolution. The disadvantage, on the contrary, is its lack of specificity.

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