Kinetic isotope effects reveal an ice-like and a liquid-phase-type intramolecular proton transfer in bacteriorhodopsin

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Abstract The mechanism of the intramolecular proton transfer in the membrane protein bacteriorhodopsin (bR) is studied. The kinetic isotope effects after H/D exchange were determined for the individual photocycle reactions and used as an indicator. Significant differences in the kinetic isotope effects are observed between the intramolecular proton transfer on the release and the uptake pathways. The results suggest a fast intramolecular proton transfer mechanism in the proton release pathway, which is similar to the one proposed for ice, where the rate limiting step is the proton movement within the H bond. However, the reactions in the intramolecular proton uptake pathway occur in a mechanism similar to the one suggested for liquid water, where the rate limiting step is given by a rotational rearrangement of H bonded network groups. We propose that the experimental evidence for a proton wire mechanism given here for bacteriorhodopsin is of general relevance also for other proton transporting proteins.

Key words: Kinetic isotope effect; Bacteriorhodopsin; Hydrogen bonded network; Proton movement

1. Introduction

A suitable system for the investigation of proton transfer reactions in biological systems is the retinal protein bacteriorhodopsin. After light excitation of the light adapted BR 570 ground state the protein undergoes a photocycle with the intermediates J, K, L, M, N, and O which is accompanied by vectorial proton transfer (for recent overview see [1]). Until now the following reactions have been identified (see also Fig. 3): in the BR to J transition the retinal chromophore isomerizes from its all-*trans* into the 13-*cis* configuration. With this transition the pK of the central proton binding site, the protonated Schiff base which connects retinal to K216 of the protein, is drastically reduced. In the L to M transition the Schiff base proton is transferred to its counterion, the internal proton acceptor D85 [2,3]. Further groups that are not yet identified must be involved in the almost simultaneous proton release to the external medium. Reprotonation of the Schiff base occurs during the M to N transition from D96 [3,4]. The proton passes the 12 Å distance between D96 and the Schiff base via an intramolecular hydrogen bonded network [5]. This hydrogen bonded network can be monitored by absorption changes of a broad continuum absorption in the infrared spectral range that is indicative of large proton polarizibility caused by collective proton fluctuation [5].

D96 is reprotonated during the N to O transition from the cytoplasmic side [6], involving further not yet identified proton carriers. Additionally, D85 deprotonates in the O to BR recovery to another group which is also unidentified [6]. Molecular dynamics simulations and different experimental observations suggest the existence of several bound water molecules that may contribute to specific proton-translocating hydrogen bonded networks in the proton release and proton uptake pathways [7–10].

Here we report on a qualitative difference between the intramolecular proton conduction in the proton release and the proton uptake pathways. Even though in general the details of the high proton mobility on the atomic level are poorly understood and experimental results seem contradictory, a main characteristic of unidirectional proton transfer in a hydrogen bonded network is the occurrence of two alternating steps: (i) the fast proton displacement within an individual H bond from the donor to the acceptor and (ii) a slower rearrangement of the H bond to the initial state which involves rotational movements of the respective donor and acceptor groups [11–15]. These steps are also referred to as the migration of an ionic and a bonding defect ('hop and turn mechanism'). In ice the H_2O molecules are highly ordered, forming a hexagonal lattice, where each of the two H donor and acceptor sites are hydrogen bonded to H acceptor and donor sites of adjacent H_2O molecules. The rate limiting step for proton transfer in ice was described as the proton displacement within the H bond [11,12]. In contrast, the rate limiting step in liquid water was described to be the rotational movement. The different rate limiting steps result in different kinetic isotope effects (KIE) upon H/D exchange. If the rotational rearrangement is rate limiting, a KIE of $\sqrt{2}$ is observed, reflecting the mass ratio between proton and deuteron. If the proton displacement within the H bond is rate limiting much larger KIEs are reported [11,12,15].

In order to determine the nature of H-bonded networks in the proton release and uptake pathways and the mechanism of proton transfer related to them, we analyzed the KIEs on the proton pumping photocycle.

2. Materials and methods

For H/D exchange the purple membranes (1.5 mg bR/ml) suspended in 150 mM phosphate buffer and 500 mM KCl were freeze dried and resuspended in D_2O. This procedure was repeated twice and afterwards the pH/pD was measured. Absorbance changes of the samples in H_2O and D_2O were recorded at pH/D 8 and T = 288 K in the visible spectral range and analyzed by global fitting. For the details of sample preparation, data acquisition and the global fit procedure see [16].

3. Results

The applied conditions of the measurements achieve kinetic separation of the photocycle intermediates without significant

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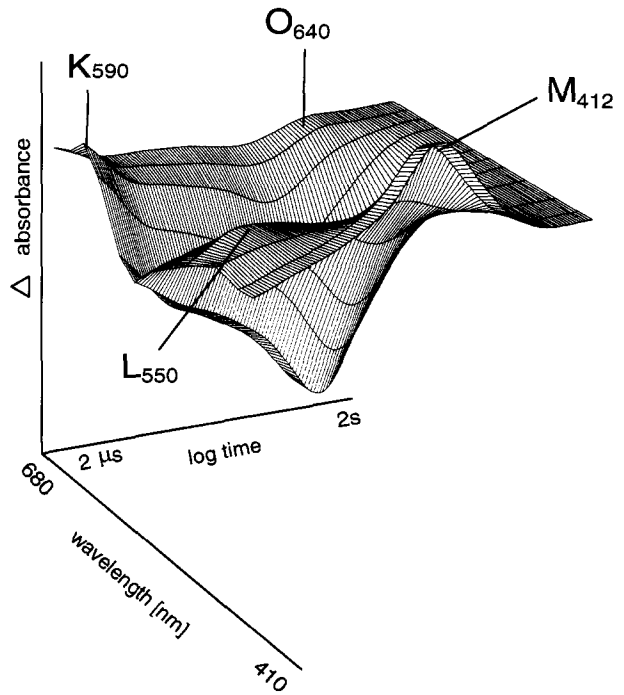

Fig. 1. 3D graph showing the occurrence of the bR photocycle intermediates K_{590}, L_{550}, M_{412} and O_{640} at pD 8 in D_2O, T = 288 K. The N_{550} intermediate cannot be seen in this graph due to the strong absorption of the BR_{570} initial state. No significant back reactions and therefore no mixing of the intermediates occurs.

back reactions and therefore no significant mixing of the intermediates occurs. The obtained apparent rate constants under these specific conditions describe the individual photocycle reactions and come close to intrinsic rate constants. This can be checked by comparison of the respective amplitude spectra. In Fig. 1 a three-dimensional plot of the fitted absorbance changes between 410 and 680 nm is given. The amplitude spectra, representing the spectral dependence of the rate constants, are given in Fig. 2 for H_2O (A) and for D_2O (B). The k_1 amplitude spectra (nomenclature according to [6]) describe the K to L transition as seen by the disappearing absorption (positive amplitude) around 610 nm for the K intermediate and the appearing absorption (negative amplitude) between 540 and 400 nm for the L intermediate. A biphasic L to M transition is described by the rate constants k_2 and k_5 with dominating contributions of k_2. The L to M transition is indicated by the disappearing absorption around 550 nm and appearing absorption around 410 nm for M. The spectral dependence of k_1, k_2 and k_5 is almost identical in H_2O and in D_2O. The M decay is described by three rate constants (k_3, k_4, and k_6). The rate constant k_4 describes the M to N transition as indicated by a disappearing absorption at 410 nm and an appearing absorption around 570 nm with a shoulder at 530 nm. The spectrum of the deuterated sample shows, in addition to these spectral changes, a shoulder at 640 nm due to a small contribution of the O intermediate. Nevertheless, the k_4 amplitude spectra taken in H_2O and in D_2O are still comparable. The k_7 amplitude spectrum describes the decay of N to BR. Due to the large overlap of the N and BR absorption this is not reflected as a clear positive amplitude around 530 nm as compared to the k_4 amplitude spectra but by an increase at 530 nm. The k_3 and k_6 amplitude spectra describe mostly an M to BR reaction. The nice agreement of the amplitude spectra obtained in H_2O and in D_2O suggests that the corresponding rate constants describe the same photocycle reactions. Therefore the rate constants can be directly compared to determine the KIE.

Table 1
Half-life times and isotope effects ($t_{1/2}$ D_2O/$t_{1/2}$ H_2O) for the measurements in H_2O and in D_2O

	$t_{1/2}$ H_2O [ms]	$t_{1/2}$ D_2O [ms]	$t_{1/2}$ D_2O/$t_{1/2}$ H_2O
(k_1) K→L	0.0013	0.0020	1.5
(k_5) M_1→M_2	0.02	0.03	1.5
(k_2) L→M	0.13	0.73	5.6
(k_3) M,N→BR	2.5	3.2	1.3
(k_4) M→N	6.7	11.0	1.7
(k_6) M→BR	27.5	46.0	1.7
(k_7) N→BR	99	151	1.5

The isotope effect of > 5 for k_2 describing the proton release reaction differs significantly from the isotope effects of all the other reactions. This difference reflects a change from an ice-like proton transfer without rate limiting molecular rotations inside the protein to a liquid-phase type, where such rotations are the rate limiting step.

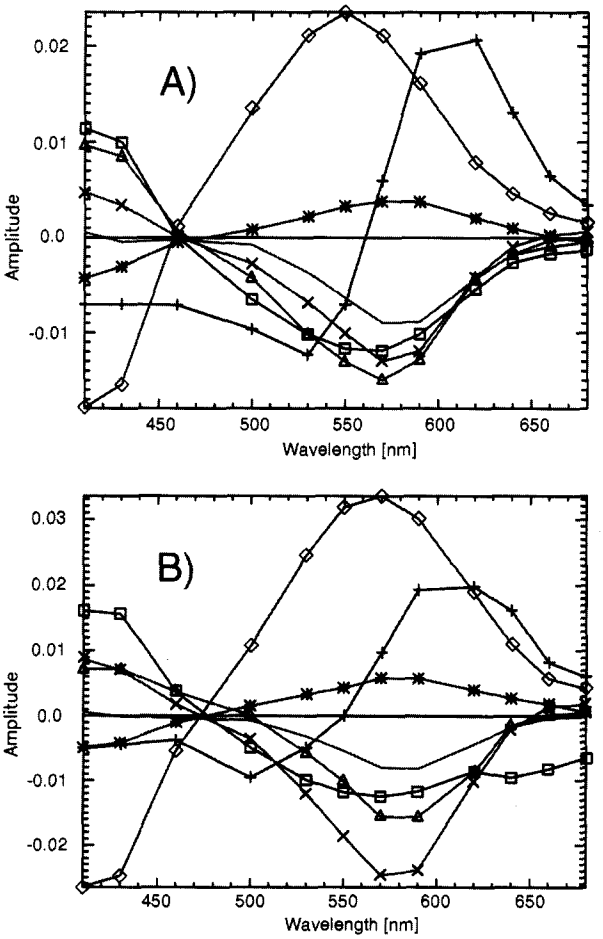

Fig. 2. Amplitude spectra describing the bacteriorhodopsin photocycle in the visible spectral range in H_2O (A) and in D_2O (B), pH/D = 8.0; T = 288 K. k_1: +, k_5: *, k_2: ◇, k_3: △, k_4: □, k_6: × and k_7: ·.

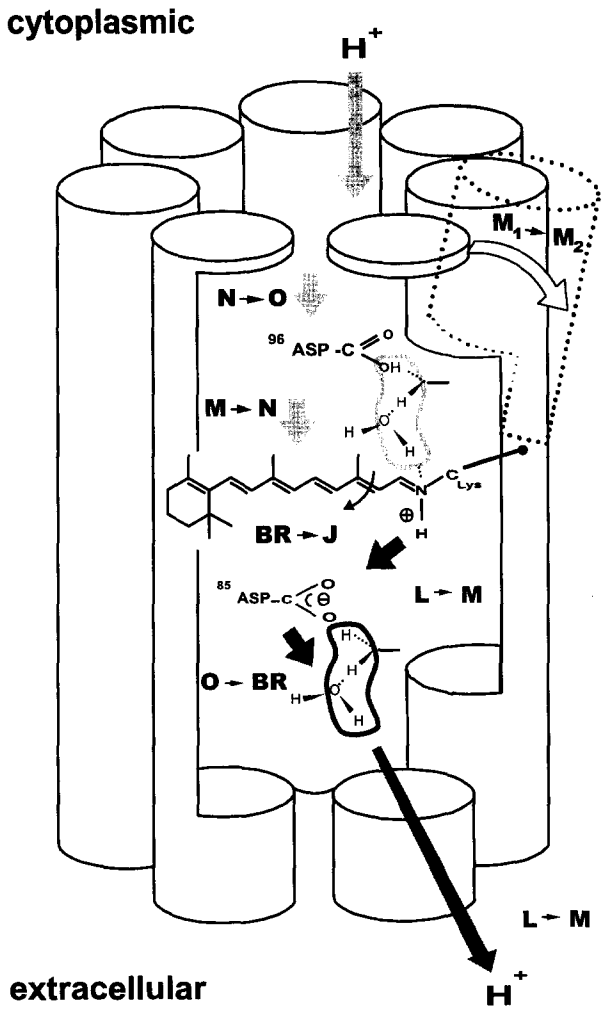

Fig. 3. After light induced all-*trans* to 13-*cis* retinal isomerization in the BR to J transition the Schiff base proton is transferred to Asp-85 in the L to M transition. Simultaneously a delocalized proton is released from the ice like H-bonded network to the external medium. In the M_1 to M_2 transition the central proton binding site, the Schiff base, is oriented from the proton release to the proton uptake side by a small protein backbone movement. The Schiff base is reprotonated from Asp-96 on the cytoplasmic side in the M to N transition. The proton is transferred from Asp-96 to the Schiff base in a water-like H-bonded network. Asp-85 is deprotonated in the 0 to BR transition.

4. Discussion

The kinetic isotope effects on the particular reactions are given in Table 1. Previously studied KIEs on the bR photocycle due to H/D exchange have been reported in the literature. However, they are difficult to compare because no amplitude spectra were presented in these investigations. Only by comparing the amplitude spectra can the corresponding rate constants be related to each other in H_2O and D_2O. Interestingly, except k_2, all rate constants show a kinetic isotope effect of $\sqrt{2}$ within the experimental error. This points to a proton transfer mechanism where the rate limiting step is given by the migration of a bonding defect. Only k_2, with a value larger than 5, shows a significant deviation in its kinetic isotope effect. Time-resolved step scan FTIR experiments show that the k_2 amplitude spectrum describes the proton transfer reactions in the L to M transition: the deprotonation of the Schiff base, the protonation of D85 and the proton release to the external medium (see Fig. 3) (R. Rammelsberg, B. Heßling and K. Gerwert, unpublished results). Double-flash time-resolved FTIR experiments show that the k_5 amplitude spectrum describes mainly the peptide bond movement in the M_1 to M_2 transition but no proton transfer reactions (B. Heßling, R. Rammelsberg and K. Gerwert, unpublished results) (Fig. 3). The strong isotope effect indicates a proton transfer without rate limiting rotational contributions and is thus similar to the one described for ice. In fact, several perturbations of the L to M transition have been reported that can now be explained with an ordered ice-like H-bonded structure: site-specific amino acid substitutions at position 82 between position 85 and the extracellular surface lead to uncoupling of Schiff base deprotonation and external proton release (Fig. 3). Mutagenesis of R82 leads to a retarded proton release and an accelerated M rise [17]. In addition, changes in the pH value of the external medium below a value of 6 also cause a delay of proton release after the proton uptake reaction [18]. The ice-like mechanism during the proton release reaction seems to support an almost irreversible step by increasing the entropy. The proton is expelled from a highly ordered protein environment into the less ordered aqueous bulk phase.

It is surprising that in a proton pump like bR all of the intramolecular proton uptake reactions occur in a mechanism similar to the one in the liquid phase. However, the change into liquid phase proton transfer in the M decay seems to be caused by the structure of the protein. There is a smaller number of possible H bond couples in the cytoplasmic proton uptake pathway as compared to the extracellular proton release pathway [19]. The extracellular proton release site is easily accessible to water whereas the proton uptake site between D96 and the Schiff base is very hydrophobic [19]. As liquid phase proton transfer is dominated by migration of a bonding defect to rearrange the network, a conformational change of the whole protein can be expected, which indeed occurs during the reprotonation step [4,20].

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