# THE KINETICS OF THE PHOTOCHEMICAL REACTION CYCLE OF DEUTERATED BACTERIORHODOPSIN AND PHARAONIS HALORHODOPSIN

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*Abstract*. Kinetic isotope effects in the photochemical reaction cycle of bacteriorhodopsin and pharaonis halorhodopsin were determined in $H_2O$ and $D_2O$ at normal pH, to get insight into the proton dependent steps of the transport process. All the steps of the bacteriorhodopsin photocycle at normal pH exhibited a strong isotope effect. In the case of halorhodopsin in both the chloride and nitrate transporting conditions the photocycle was not strongly affected by the deuterium exchange. In the case of chloride, a slight slow down of the photocycle could be observed. On the opposite, in the nitrate transport conditions a reverse effect is present.

*Key words*: bacteriorhodopsin, halorhodopsin, deuterium, photocycle, absorption kinetics.

## INTRODUCTION

Bacteriorhodopsin and halorhodopsin are retinal proteins belonging to the family of seven-helical transmembrane proteins. Upon light excitation, they go through a reaction cycle ("photocycle") during which translocate an ion (proton, chloride or nitrate ion) across the cell membrane [2, 22, 27].

The most studied retinal protein is the bacteriorhodopsin (BR), found in *Halobacterium salinarum*. It uses the light energy to transport proton from the cytoplasmic to the extracellular side of the membrane [6, 16, 18]. The spectral and kinetic properties of the BR intermediates and the structural changes during the proton transport are well understood [8, 18, 21]. In short: after absorbing light by the retinal, bound to lysine 216, a charge separation along the retinal chain occurs in the fs time domain [9], followed by an all-*trans* to 13-*cis* isomerization of it in

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several ps (J to K transition). The isomerization is followed by a local protein relaxation around the retinal in less than 10 μs (K to L transition). The Schiff-base of the retinal deprotonates by transferring a proton to the acceptor Asp-85 (L to $M_1$ transition occurring in about 100 μs) and a proton is released from the release group on the surface of the membrane to the external medium. The Schiff-base accessibility changes from the extracellular to the cytoplasmic side ($M_1$ to $M_2$ transition occurring also in less than 100 μs) and it reprotonates from the proton donor Asp-96 ($M_2$ to N transition occurring in the ms time domain). The retinal reisomerizes to its original all-*trans* form, a proton is taken up from the cytoplasmic side and the proton from the acceptor is transferred to the proton release group (N to O to BR transitions occurring also in the ms time domain) returning the protein to its original state [19, 24]. For almost all steps the crystallographic structure of the BR is already known [13, 15, 18, 25, 26].

The salinarum halorhodopsin (sHR) was discovered also in *Halobacterium salinarum*. This membrane protein transports chloride ion inside the cell [27]. A similar chloride ion pump, the pharaonis halorhodopsin (pHR) was identified in the *Natronobacterium pharaonis* [5, 17]. The spectra of intermediates and the photocycle kinetics of both HR-s show similarities to that of BR, only no M intermediate could be identified [29]. Although the crystallographic structure of the unexcited sHR is known [11], there is no information about the structure of the intermediates and the steps of ion translocation. It was shown that beside chloride the pHR transports nitrate [1, 5] or proton [14, 31]. In the proton transporting photocycle of pHR, observed in the presence of azide, instead of chloride, an M like intermediate could be observed [14].

Recently a gene of a new proton pumping retinal protein, the proteorhodopsin (PR) was identified in the γ-proteobacterium, a marine bacterioplankton. The protein was functionally expressed in *Escherichia coli* [2, 3].

In the following study we use as a reference the BR and try to draw conclusions about the ion transporting function of the pHR, by measuring the effect of deuteration on the absorption kinetics after laser excitation. The effects shade light in one side on the transported ion, while on the others side the involvement of the exchangeable protons and the change in the hydrogen bonds inside the protein.

## MATERIALS AND METHODS

BR was prepared form *Halobacterium salinarum* strain L33 with the bop gene introduced. pHR was produced using the same strain, in which the *Natronobacterium pharaonis hop* gene was introduced [30]. The measurements were performed on membrane encased in polyacrylamide gel [23, 30] on samples

having optical density around 0.5 at 570 nm. To ensure a good $H_2O$ to $D_2O$ exchange and the same measuring conditions, before the measurements all the samples were dried on $P_2O_5$ and re-soaked in the desired salt solution. The desired concentration of salt solution was freshly prepared from dried salt (NaCl or $NaNO_3$ as required) and $H_2O$ or $D_2O$. The pH was adjusted with HCl, DCl, NaOH and NaOD respectively. The pD of the heavy water solution was taken 0.4 units higher to the measured pH [4]. In the similar samples, compared in water and heavy water, the pH and pD respectively were adjusted to be equal, resulting in equal proton/deuteron concentration. As no buffer was used, the pH (pD) of the solution was checked before and after the measurement and only those signals were taken into account where the pH (pD) change was less than 0.2 units. There was no observable spectral difference between the matching pair of samples prepared in this way.

The excitation of the sample was performed with a frequency-doubled Nd-YAG laser (Surelite I-10, Continuum, Santa Clara, CA). For the absorption kinetic signals the measuring light was provided by a 55 W halogen lamp with a heat filter. Measurements were effectuated at four or five different wavelengths. The wavelength selection was made by a monochromator placed between the sample and the photomultiplier. The signals were recorded in a time interval between 1 μs and 10 s, using a transient recorder card (NI-DAQ PCI-5102, National Instruments, Austin, TX). The signals were fitted to photocycle models by using RATE program [21].

## RESULTS AND DISCUSSIONS

The isotope effects exhibited by different intermediates of BR photocycle at neutral pH showed a strong dependence on the proton involvement in the respective step of the photocycle. Thus, for the deuterated sample, in the μs time domain only the K to L transition appears and a lot of L intermediate accumulates as compared to the sample in normal water where the intermediate M appears already at this step of the photocycle (Fig. 1). The K to L transition is a local rearrangement of the amino acid side chains. This transition does not require large scale H bond rearrangement and/or proton motion [10, 20] and shows, therefore, a moderate isotope effect as previously found [7, 10, 12]. The K to L transition becomes slower about 1.3 times during the proton-deuteron exchange. All the following transitions are strongly affected by this perturbation, in agreement with the earlier observations [4, 7, 12], suggesting the involvement of the proton in all the steps of the photocycle. The largest change is in the L to M transition, which is related to the Schiff-base deprotonation and the proton release step. This step becomes one order of magnitude slower by changing the proton to deuteron. Crystallographic studies revealed that during this step H-bond restructuration occurs [4, 15, 26]. The M to N transition, the reprotonation of the Schiff-base is isotope dependent at both pH-s, becoming about 2 times slower in $D_2O$.

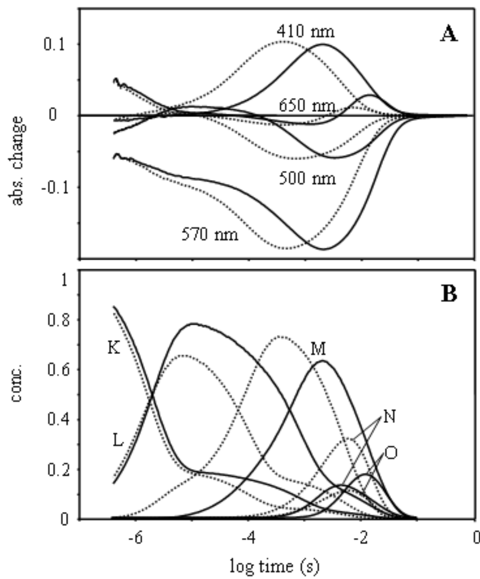


Fig. 1. Absorption kinetic signals measured on BR in $H_2O$ (broken line) or $D_2O$ (continuous line) at pH (pD) 7, panel A, and the time-dependent concentrations in the fitted photocycle model, panel B.

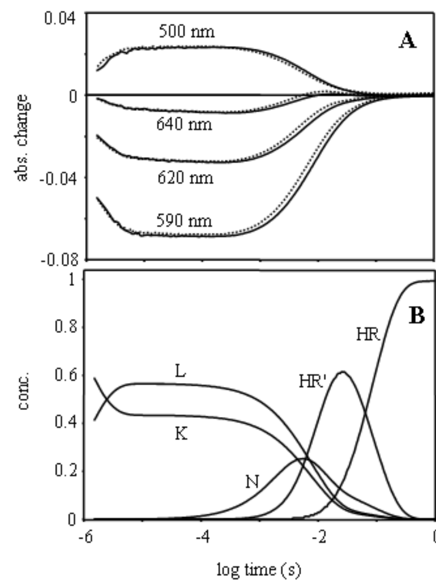


Fig. 2. Absorption kinetic signals measured at four wavelengths on pharaonis halorhodopsin. Measuring conditions: 1 M NaCl dissolved in $H_2O$ (broken line) or $D_2O$ (continuous line) at pH (pD) 6, panel A, and the time-dependent concentrations in the fitted photocycle model, in panel B.

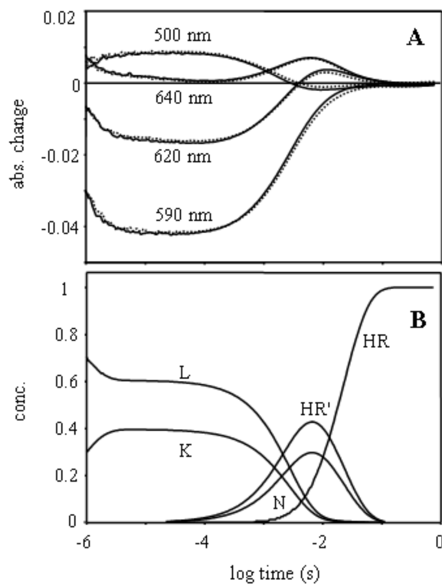


Fig. 3. Absorption kinetic signals measured at four wavelengths on pharaonis halorhodopsin. Measuring conditions: 200 mM $NaNO_3$ dissolved in $H_2O$ (broken line) or $D_2O$ (continuous line) at pH (pD) 6, panel A, and time-dependent concentrations in the fitted photocycle model, in panel B.

The effect of the deuteron exchange was studied on pHR in two different ion transporting conditions [28]. It is interesting to note that no considerable isotope effect could be observed in the case of any transported ion. Therefore, it made no sense to calculate the concentrations of the intermediates for the deuterated samples, as they should look nearly the same as in the case of the normal sample. In the case of the chloride, a slight slow down of the photocycle can be observed (Fig. 2, NaCl), but looking to the nitrate transport on several traces a reversed effect is manifested (Fig. 3, $NaNO_3$).

## CONCLUSIONS

Based on these in the case of pHR a general conclusion can be drawn that, in contrast to the strong isotope effect found in BR, during the ion transport no major proton motions and/or H-bond rearrangements happen in the protein, or in that part of the protein which is accessible to proton deuteron exchange.

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