Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Photochemistry and Photobiology B: Biology 82 (2006) 16–20
Journal of Photochemistry and Photobiology B:Biology
www.elsevier.com/locate/jphotobiol

The influence of the halide ions on the photochemical reaction cycle of pharaonis halorhodopsin

Klára Magyari [a], Viorica Simon [a], György Váró [b,*]

[a] *Department of Physics, University "Babes-Bolyai" Cluj-Napoca, Romania*
[b] *Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, P.O. Box 521, Szeged H-6701, Hungary*

Received 20 June 2005; received in revised form 4 August 2005; accepted 8 August 2005
Available online 10 October 2005

Abstract

Sodium salt of chloride, bromide and iodide were used to elucidate the effect of the size of the anion on the binding to pharaonis halorhodopsin and its transport during the photocycle of this retinal protein. Spectroscopic titration revealed an apparent strong binding constant of 2 mM for chloride, 0.23 mM for bromide and 5 mM for iodide. In the case of iodide a second, week binding constant of about 10 M could be estimated. This second binding constant was similar to that observed earlier for nitrate. By changing the halide ions, only the transitions in the second half of the photocycle were affected, which contained intermediates N, O, and HR′. The O to HR′ transition becomes faster with increasing ion volume, meaning that the ion uptake is accelerated. This effect shows a direct correlation with the ion radius. With increasing ion concentration the N–O–HR′ equilibrium changed in such a way that the accumulated O tended to decrease. This tendency was overruled in iodide, by the appearance of the second binding constant. The increasing iodide concentration, up to 100 mM decreases the accumulation of the intermediate O, due to kinetic reasons, but at higher ion concentration the amount of O increases, although its decay becomes faster. This effect correlates with the appearance of the second iodide bound to the protein.

Keywords: Retinal protein; Chloride transport; Photocycle; Absorption kinetics

1. Introduction

Pharaonis halorhodopsin (pHR) is a light-driven chloride ion pumping retinal protein, found in the cell membrane of *Natronobacterium pharaonis* [1,2]. Its amino acid sequence was established to be very similar to the salinarum halorhodopsin and bacteriorhodopsin found in *Halobacterium salinarum* [3], showing a 66% and 25% homology, respectively. The photoactive molecule in the pHR is a retinal bound to lysine 256 in helix G via a protonated Schiff-base. Under all conditions, 85% of the retinal in the protein is in all-*trans*, 15-*anti* and 15% in 13-*cis*, 15-*syn* configuration [4,5]. Although the retinal composition of the sample is not homogeneous, there is no evidence that the 13-*cis* containing pHR is photoactive or takes part in the transport process, resulting a homogeneous photocycle [4].

The structure of salinarum halorhodopsin is known with a 1.8 Å resolution [6]. Based on the high homology between the two chloride ion pumps and their similar photocycle, it can be assumed that the pHR has similar structure. In the structure, the binding site of the ion was determined and it shows that a cluster of three water molecule is in the binding pocket [6], suggesting that ions of different size could be accommodated.

Spectral titration in function of different sodium salts show that the pHR binds different anions, inducing a blue shift in the spectrum [7]. If the titration was effectuated at a constant sodium concentration by exchanging the non-bonding sulfate to the titrated anion, the binding constant

* Corresponding author. Tel.: +36 62 599620; fax: +36 62 433133.
E-mail address: varo@nucleus.szbk.u-szeged.hu (G. Váró).

1011-1344/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jphotobiol.2005.08.005

of the pHR at pH 6 was found to be 2 mM with a 0.75 binding order for chloride [4], 10 mM for azide [8], and 11 mM with a 0.9 order for nitrate [9]. In the case of nitrate a second, low-affinity binding constant of about 7 M was observed [9].

In sodium sulfate the pHR has a photocycle with only one red shifted, K like intermediate. Electric signal measurements reveal that during the photocycle there are internal charge motions in the protein, but there is no net charge transfer across the membrane [4]. In 100 mM chloride the photocycle contains a succession of intermediates (noted by K, L, N, O and HR′). Each intermediate has a well-determined absorption spectrum in visible. Their kinetics could be described by a model containing sequential, first order equilibrium reactions [4]. At 1 M chloride concentration the intermediate O can not be observed, for kinetic reasons. It was shown that the ion release and uptake steps are that of N–O and O–HR′ transitions, respectively [10].

In nitrate the photocycle is very similar to that observed in chloride, but the transported ion is much larger [7]. The electric signal measurements show that a charge is transported across the membrane [9]. At several molar nitrate concentration the O intermediate does not disappear form the photocycle, which was concluded to be the effect of the second, low-affinity binding of the ion [9]. Beside of these changes in the photocycle another effect was also observed, the apparent release and rebinding of a small fraction of the excited retinal, inside the retinal pocked, during the photocycle [9]. By using cell envelope vesicles of pHR, it was shown that both chloride and nitrate are transported with the same efficiency [2].

When the bathing solution of the sample contains azide, instead of chloride, the transported ion was estimated to be a proton [8]. The photocycle looks similar to that of the proton transporting bacteriorhodopsin [7,11] and by the use of cell envelope vesicles was shown that not the azide ion is translocated through the membrane [8]. Based on kinetic isotope effect measurement, recently was proposed that the transported ion, instead of proton could be a negative hydroxyl ion [12]. In proton transporter bacteriorhodopsin and proteorhodopsin the water to deuterium oxide exchange caused a marked change in the photocycle. The deuteron exchange in pHR did not affect the chloride and nitrate photocycle of it, and surprisingly the azide photocycle also remained unaffected [12].

In bacteriorhodopsin at low pH, by using the method of exchanging HCl to HBr it was possible to show that the transported ion was the halide [13]. There are only preliminary information about the effect of different anions on the photocycle of pHR [7]. Based on these the effect of the halide ions (chloride, bromide, and iodide) was investigated, to better understand the process of ion transport by pharaonis halorhodopsin across the membrane.

2. Materials and methods

pHR was produced in *Halobacterium salinarum* strain L33, in which the *Natronobacterium pharaonis hop* gene was introduced [4]. The measurements were performed on membranes encased in polyacrylamide gel [14]. All salt solutions were mixed by using 1 M Na_2SO_4 and 2 M of NaCl, NaBr or NaI in such a way that although the halide concentration varied, the total Na concentration was constant at 2 M. The solutions contained 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid) at pH 6.

During the experiment the sample was in a 4×10 mm cuvette incubated in a temperature controlled sample holder. The photocycle was initiated by a laser flash from a frequency-doubled Nd-YAG laser (Surelite I-10, Continuum, Santa Clara, Ca). The measuring light was provided by a 55 W halogen lamp with heat filter. Measurements were effectuated at four wavelengths (500, 590, 620 and 640 nm) selected by a monochromator placed between the sample and the photomultiplier. By these the change of the measuring wavelength did not affect the light intensity going through the sample. The up to one second long signals were recorded with a transient recorder card (NI-DAQ PCI-5102, National Instruments, Austin, TX) with a 50 ns resolution. Each measurement was the average of 100 signals. At the end of the measurement the linear time base was converted to logarithmic by averaging in the time interval between logarithmically equidistant points. The signals were fitted to photocycle model by using RATE program [15].

3. Results and discussion

Although there were preliminary information about the binding of different halide ions to pHR [7] the exact binding constants were determined by spectral titration with NaCl, NaBr and NaI. In all three cases, the spectra shifted toward blue with increasing anion concentration as shown for bromide titration (Fig. 1(a)). The relative changes calculated at two wavelengths 560 and 640 nm were plotted and titration curves were fitted to the points (Fig. 1(b)). For chloride the binding constant and order were as determined earlier (not shown) [4]. Similar to that observed in nitrate [9], in the case of iodide a second, low-affinity binding constant could be estimated, although its exact value was not determined, due to limited number of points at the high halide concentrations. The binding constants and reaction orders are listed in Table 1. The strength of the second binding constant seems to correlate to the radius of the ions.

Comparing the absorption kinetic signals measured in 100 mM halide concentration (Fig. 2) reveals that the fast part of the photocycle does not depend on the anion. The signals measured at 500 and 590 nm look unchanged in the whole time domain. In the ms time domain, the positive amplitude of the absorption signals measured over 600 nm decreases by changing chloride to bromide or iodide. To emphasize the changes the signals measured at 640 nm were normed at the 100 μs time point (Fig. 3). With the increase of the size of the anion the positive amplitude, located at around 10 ms, decreases.

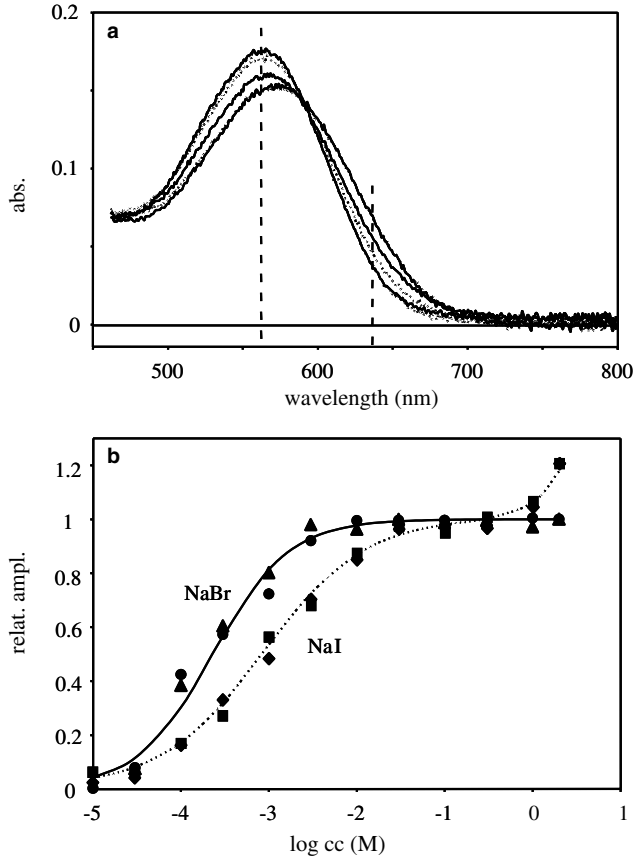

Fig. 1. Halide dependent changes in the spectrum of pharaonis halorhodopsin. (a) The absorption spectra increases and shifts toward blue by increasing bromide concentration. (b) The relative change calculated at 560 nm (◆, ▲) and 640 nm (■, ●) yields titration curves for each halide ion, from which the binding constants and reaction order, were calculated (see Table 1).

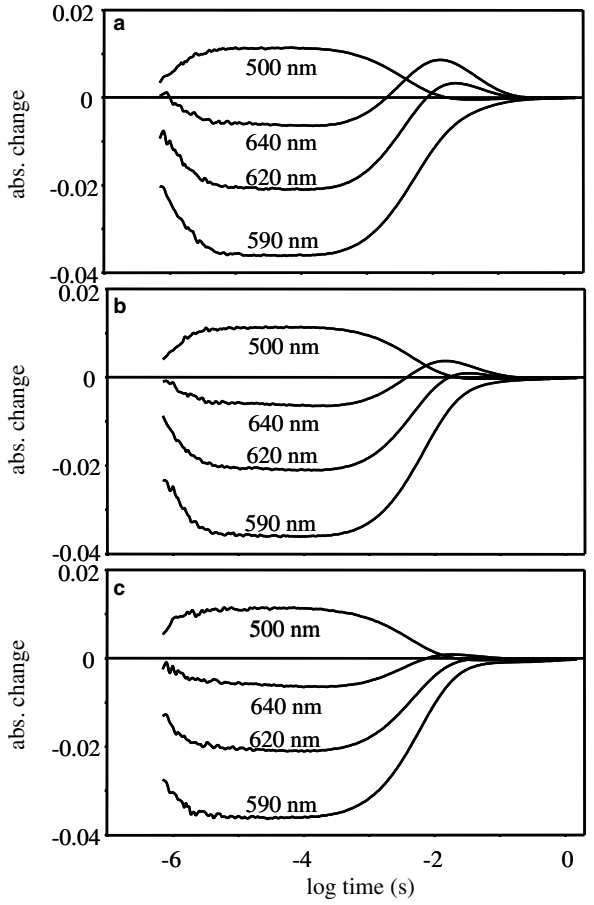

Fig. 2. Absorption kinetic signals measured at four wavelengths on pharaonis halorhodopsin in 100 mM sodium chloride (a), sodium bromide (b) and sodium iodide (c). The solution contained 950 mM sodium sulfate, 50 mM MES at pH 6.

The set of signals shown in Fig. 2 could be fitted with the previously published sequential equilibrium reaction model [4]

$$K \Longleftrightarrow L \Longleftrightarrow N \Longleftrightarrow O \Longleftrightarrow HR' \Rightarrow HR$$

The RATE program characterized the goodness of the fit with calculating χ^2 (the sum of the square of the residuum of the fit). In all three cases, this value was very low and varied in a narrow range, between 0.00018 and 0.00022, showing that the fits were very good and they can be compared. The result of the fit to the signals containing chloride (Fig. 4) was very similar, to that published earlier [4]. The absorption changes in the μs time domain correspond to the appearance of the equilibrium between the K and L intermediates. The following part up to 0.1 ms is dominated by this equilibrium, which is anion independent. The decay part of the K–L equilibrium, gives rise to the N, O and HR′ intermediates, which steps are already anion dependent. By changing the chloride to bromide and iodide the maximum concentration of intermediate N decreases, the intermediate O disappears totally, while the concentration of intermediate HR′ increases (Fig. 5).

The observed concentration changes can be explained by the acceleration of the rise of HR′ with almost one order of magnitude, accompanied by a slight acceleration of its decay (Fig. 5(c)), what can be observed also on the decay of the absorption kinetic signals (Fig. 2). In the same time the rise of N and the N–O transition does not change considerably, resulting in a decreasing amount of N and O without observable change in their kinetics (Fig. 5(a) and (b)). The changes in the final decay of the equilibrated K and L intermediates are also minor (not shown).

Table 1
The molecular mass, ion radius and the binding parameters of anions, bound to pHR

Anion	m_w	R (nm)[a]	Binding constant	Binding order
Cl^-[b]	35	0.181	2 mM	0.75
Br^-	80	0.195	0.23 mM	1
I^-	127	0.216	5 mM	0.75
			15 M	1.7
NO_3^-[c]	62	0.264	11 mM	0.9
			7 M	1

[a] Ion radii from [16].
[b] Binding parameters from [4].
[c] Binding parameters from [9].

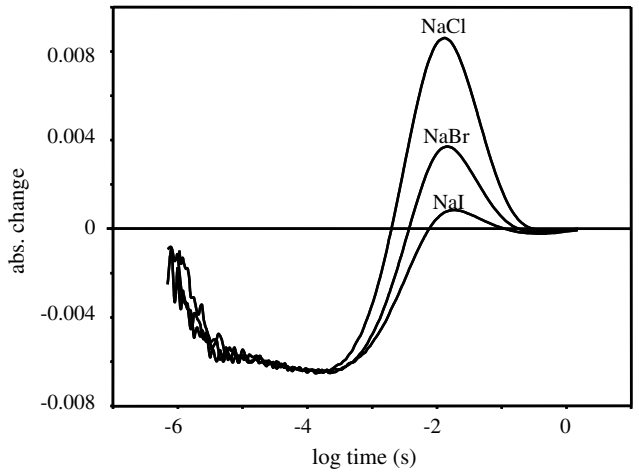

Fig. 3. The amplitude change of the absorption kinetic signal measured at 640 nm. The signals were normed at 100 μs. Measuring conditions were the same as in Fig. 2.

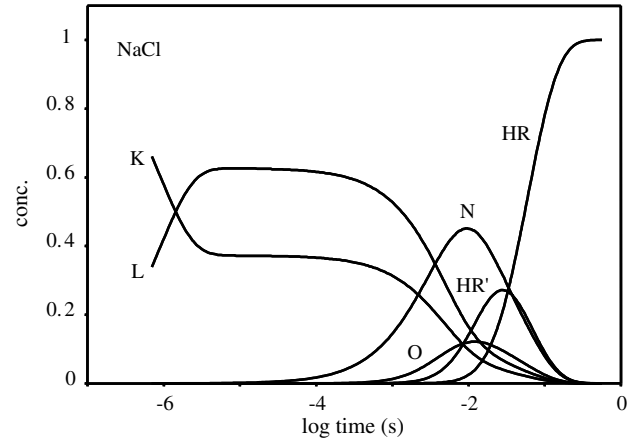

Fig. 4. The time dependent concentration changes of the photocycle intermediates, calculated from the absorption kinetic signals shown in Fig. 2(a).

From the literature is known that the chloride release step is the N–O transition, while the uptake happens at the O–HR′ transition [10]. From the acceleration of the rise of HR′ intermediate an unexpected observation can be concluded. By increasing the size of the ion the uptake of it is facilitated. This could be due to some steric enhancement, which is supported by the appearance of the second binding site, as the size of the ion increases. One possible explanation could be that as the size of the anion increases the negative charge is more delocalized. On the pHR containing negatively charged membrane the charges are localized, the interaction of a charge close to the entrance to the binding site with the approaching ion could diminish, increasing the probability of the ion to enter in the interior of the protein, where the binding site is located [6]. In the same time by penetrating in the protein the larger ion, changes the conformation of the protein giving space for a second binding site.

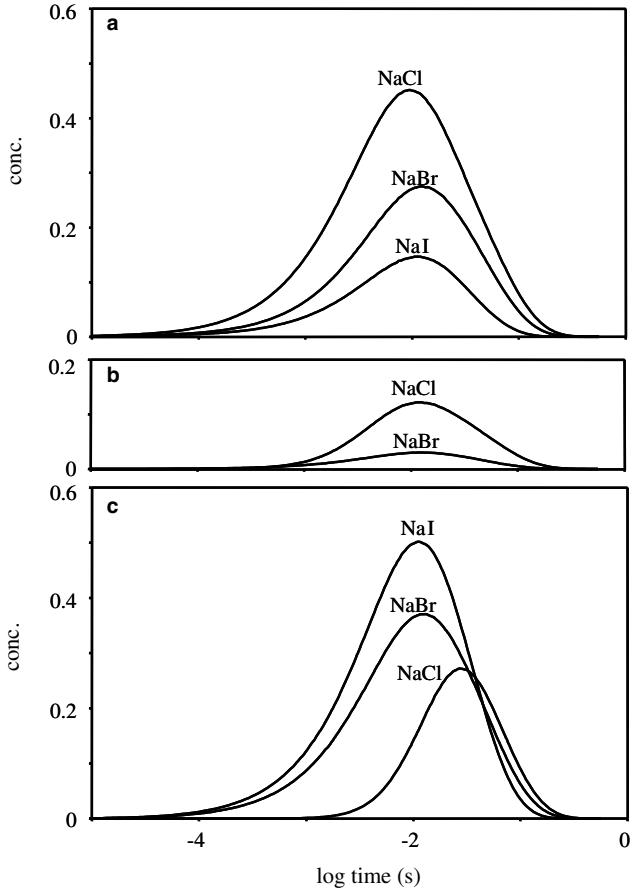

Fig. 5. Change of the concentration of the photocycle intermediates, by changing the halide ion: (a) intermediate N; (b) intermediate O; (c) intermediate HR′.

To understand the effect of the second binding site on the photocycle, the absorption kinetic signal at 640 nm was measured at different anion concentrations. In chloride (not shown) and bromide (Fig. 6(a)) the concentration dependence was similar to that measured earlier [10]. By increasing ion concentration the positive amplitude at around 10 ms decreased and disappeared at around 1 M. This led to the conclusion that at high ion concentration, due to kinetic reason, the intermediate O does not accumulate [10]. In the case of iodide the positive amplitude decreased up to 100 mM concentration and starts to increase, but with faster decay kinetics, as the concentration was further increased. In the same time a small negative component appeared at the end of the trace (Fig. 6(b)). The similar, but larger effect observed earlier in nitrate, was explained as the temporal break of the Schiff-base in some of the photoexcited pHR [9]. The accumulation of intermediate O at high iodide concentration seems to correlate with the binding of the second iodide to the protein.

The anomaly in spectral titration, the increase of the positive amplitude at around 10 ms and the appearance of the very slow negative component observed in the case of iodide and even better accentuated in nitrate, seems to correlate to the radius of the transported ion (see Table 1). These

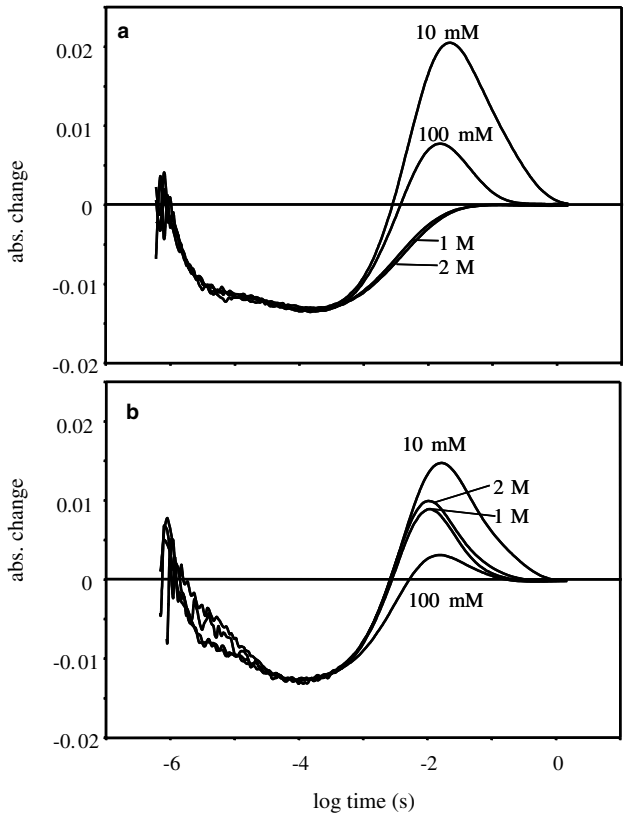

Fig. 6. Change of the amplitude of the absorption kinetic signal measured at 640 nm in function of increasing concentration of sodium bromide (a) and sodium iodide (b). The signals were normed at 100 μs. Measuring conditions were the same as in Fig. 2.

suggest that the size of the transported ion does not influence the efficiency of the transport [2], but introduces changes in the binding and the transport steps of the photocycle.

Acknowledgment

The National Science Research Fund of Hungary OTKA T048706 supported this work.

References

[1] D.B. Bivin, W. Stoeckenius, Photoactive retinal pigments in haloalkalophilic bacteria, J. Gen. Microbiol. 132 (1986) 2167–2177.
[2] A. Duschl, J.K. Lanyi, L. Zimányi, Properties and photochemistry of a halorhodopsin from the haloalkalophile, *Natronobacterium pharaonis*, J. Biol. Chem. 265 (1990) 1261–1267.
[3] J.K. Lanyi, A. Duschl, G.W. Hatfield, K.M. May, D. Oesterhelt, The primary structure of a halorhodopsin from *Natronobacterium pharaonis*: structural, functional and evolutionary implications for bacterial rhodopsins and halorhodopsins, J. Biol. Chem. 265 (1990) 1253–1260.
[4] G. Váró, L.S. Brown, N. Sasaki, H. Kandori, A. Maeda, R. Needleman, J.K. Lanyi, Light-driven chloride ion transport by halorhodopsin from *Natronobacterium pharaonis* 1. The photochemical cycle, Biochemistry 34 (1995) 14490–14499.
[5] L. Zimányi, J.K. Lanyi, Fourier transform Raman study of retinal isomeric composition and equilibration in halorhodopsin, J. Phys. Chem. B 101 (1997) 1930–1933.
[6] M. Kolbe, H. Besir, L.O. Essen, D. Oesterhelt, Structure of the light-driven chloride pump halorhodopsin at 1.8 Ĺ resolution, Science 288 (2000) 1390–1396.
[7] B. Scharf, M. Engelhard, Blue halorhodopsin from *Natronobacterium pharaonis*: wavelength regulation by anions, Biochemistry 33 (1994) 6387–6393.
[8] G. Váró, L.S. Brown, R. Needleman, J.K. Lanyi, Proton transport by halorhodopsin, Biochemistry 35 (1996) 6604–6611.
[9] Z. Bálint, M. Lakatos, C. Ganea, J.K. Lanyi, G. Váró, The nitrate transporting photochemical reaction cycle of the *Pharaonis halorhodopsin*, Biophys. J. 86 (2004) 1655–1663.
[10] G. Váró, R. Needleman, J.K. Lanyi, Light-driven chloride ion transport by Halorhodopsin from *Natronobacterium pharaonis*. 2. Chloride release and uptake, protein conformation change, and thermodynamics, Biochemistry 34 (1995) 14500–14507.
[11] A. Kulcsár, G.I. Groma, J.K. Lanyi, G. Váró, Characterization of the proton transporting photocycle of pharaonis halorhodopsin, Biophys. J. 79 (2000) 2705–2713.
[12] J. Szakács, M. Lakatos, C. Ganea, G. Váró, Kinetic isotope effect in the photochemical reaction cycle of ion transporting retinal proteins, J. Photochem. Photobiol. B 79 (2005) 145–150.
[13] A. Dér, S. Száraz, R. Tóth-Boconádi, Z. Tokaji, L. Keszthelyi, W. Stoeckenius, Alternative translocation of protons and halide ions by bacteriorhodopsin, Proc. Natl. Acad. Sci. USA 88 (1991) 4751–4755.
[14] P.C. Mowery, R.H. Lozier, Q. Chae, Y.W. Tseng, M. Taylor, W. Stoeckenius, Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin, Biochemistry 18 (1979) 4100–4107.
[15] K. Ludmann, C. Gergely, G. Váró, Kinetic and thermodynamic study of the bacteriorhodopsin photocycle over a wide pH range, Biophys. J. 75 (1998) 3110–3119.
[16] J. Israelachvili, Intermolecular and Surface Forces, Academic Press Ltd., New York, 1992.