Characterization of the Azide-Dependent Bacteriorhodopsin-Like Photocycle of Salinarum Halorhodopsin

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ABSTRACT The photocycle of salinarum halorhodopsin was investigated in the presence of azide. The azide binds to the halorhodopsin with 150 mM binding constant in the absence of chloride and with 250 mM binding constant in the presence of 1 M chloride. We demonstrate that the azide-binding site is different from that of chloride, and the influence of chloride on the binding constant is indirect. The analysis of the absorption kinetic signals indicates the existence of two parallel photocycles. One belongs to the 13-*cis* retinal containing protein and contains a single red shifted intermediate. The other photocycle, of the all-*trans* retinal containing halorhodopsin, resembles the cycle of bacteriorhodopsin and contains a long-living M intermediate. With time-resolved spectroscopy, the spectra of intermediates were determined. Intermediates L, N, and O were not detected. The multiexponential rise and decay of the M intermediate could be explained by the introduction of the "spectrally silent" intermediates M_1, M_2, and HR′, HR, respectively. The electric signal measurements revealed the existence of a component equivalent with a proton motion toward the extracellular side of the membrane, which appears during the M_1 to M_2 transition. The differences between the azide-dependent photocycle of salinarum halorhodopsin and pharaonis halorhodopsin are discussed.

INTRODUCTION

Salinarum halorhodopsin (sHR), a small retinal protein in the cell membrane of *Halobacterium salinarum* (Matsuno-Yagi and Mukohata, 1977; Lindley and MacDonald, 1979), is a light driven chloride ion pump. Its structure, at 1.8 Å resolution (Kolbe et al., 2000) bears great similarity to the proton pump, bacteriorhodopsin (BR) (Lanyi et al., 1990; Váró, 2000). The protein binds a retinal to its lysine residue (K242) through a protonated Schiff base. The protonated Schiff base and the amino acid surrounding of the retinal determines the absorption maximum of the protein (λ_{max} = 573 nm) (Schobert and Lanyi, 1982; Oesterhelt and Tittor, 1989; Lanyi, 1990). It shows 65% sequence identity with the other light-driven chloride pump, found in *Natronobacterium pharaonis*, the pharaonis halorhodopsin (pHR) (Lanyi et al., 1990). In both halorhodopsins photon absorption causes isomerization of the retinal from all-*trans* 15-*anti* to 13-*cis*, 15-*syn*, which triggers a sequence of thermal reactions. The photocycle intermediates are defined by changes in the visible and infrared spectra, and their sequence is accompanied by the translocation of a chloride ion from the extracellular space into the cytoplasm.

The lack of the aspartate residue equivalent to the proton acceptor D85 in bacteriorhodopsin (T111 in sHR) is consistent with the fact that in physiological conditions the retinal Schiff base does not deprotonate in the sHR photocycle. There have been numerous attempts to describe the photocycle (for reweave, see Lanyi, 1986b; Oesterhelt et al., 1992; Váró, 2000). The study of the photocycle of sHR had to take into account the fact that unlike BR, the sample after light adaptation contains ~25% 13-*cis* retinal with a different photocycle from the all-*trans* (Váró et al., 1995c; Zimányi and Lanyi, 1997). In the presence of chloride the 13-*cis* photocycle was rather simple, containing two intermediates. The first, slightly red-shifted one is followed by an intermediate with almost the same spectrum as sHR. The all-*trans* photocycle had several intermediates (named K, L_1, L_2, and N) in sequence. In absence of chloride ion a third photocycle was observed, having only a single red shifted intermediate (Váró et al., 1995c). In pHR the situation is simpler. Although the two isomers of the retinal are also present, the 13-*cis* containing protein does not show photoactivity (Scharf and Engelhard, 1994; Váró et al., 1995a,b). The chloride transporting photocycle of pHR has five intermediates in sequence (K, L, N, O, HR′), whereas in sulfate, similarly to the sHR, there is only a red-shifted intermediate.

Electric signals have been measured both on sHR (Bamberg et al., 1984; Dér et al., 1985b) and pHR (Kalaidzidis et al., 1998; Ludmann et al., 2000; Kulcsár et al., 2000). These signals characterize the charge motions inside the protein and the ion translocation process across the membrane. The relative electrogenicity of the pHR photocycle intermediates (Ludmann et al., 2000) show the change of the electric dipole magnitude of intermediate relative to the ground state of the protein (Trissl, 1990; Gergely et al., 1993).

Besides chloride, some other anions are also transported by halorhodopsin. Although pHR transports nitrate with the same efficiency as chloride, sHR transports it with approx-

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imately one-third efficiency (Duschl et al., 1990). Another anion, azide, is used in the study of bacteriorhodopsin mutants as a substitute for proton donor or acceptor when they are altered in the protein (Tittor et al., 1989; Zimányi and Lanyi, 1993; Tittor et al., 1994). In chloride-free solution, azide confers on pHR a proton transporting photocycle by replacing the missing proton donor and acceptor (Váró et al., 1996). Kinetic analysis suggested that this photocycle is very similar to that of bacteriorhodopsin (Kulcsár et al., 2000). In contrast, several groups concluded that in sHR the azide induces a side reaction in the chloride transporting photocycle, leading to an M-like intermediate with deprotonated Schiff base (Hegemann et al., 1985; Oesterhelt et al., 1985; Lanyi, 1986a).

In the present study we demonstrate that azide binds to sHR at a site different from the chloride-binding site. The binding constant is influenced by the presence of chloride ion but not with competitive kinetics. In a solution with high azide concentration and no chloride present two parallel photocycles could be identified. Most probably one belongs to the 13-*cis* retinal containing protein, having only a red shifted intermediate. The other photocycle associated to the all-*trans* retinal containing protein has an M-like intermediate.

MATERIALS AND METHODS

Halorhodopsin-containing membranes were prepared from *Halobacterium salinarum* strain L33, transformed with an independently replicating vector with the *bop* promoter and *hop* structural gene, resulting in a greatly enhanced production of sHR. The preparation of sHR membrane suspension was described earlier (Oesterhelt and Stoeckenius, 1974; Váró et al., 1995c).

The measuring and analyzing techniques were the same as described (Kulcsár et al., 2000). In short, all spectroscopic and absorption kinetic measurements were performed with sHR containing membranes encased in polyacrylamide gels. Electric signal measurements were carried out on oriented gel samples. All bathing solution contained 10 mM MES (2-[*N*-morpoilno]ethanesulfonic acid) buffer and the pH was set to 7. The temperature of the sample was 20°C if not otherwise mentioned.

Continuous illumination was performed with a 250-W halogen lamp with heat filter and a 500-nm-long pass filter. Laser excitation was with a frequency-doubled Nd-YAG laser (Surelite 10, 2 = 532 nm, Continuum, Santa Clara, CA). Time-resolved difference spectra were measured with a gated optical multichannel analyzer (Zimányi et al., 1989) and the intermediate spectra calculated with the standard procedure (Gergely et al., 1997). Absorption kinetic signals were recorded at five wavelengths with a transient recorder card (National Instruments, NI-DAQ PCI-5102) and the signals fitted with RATE and EYRING program as described (Kulcsár et al., 2000).

RESULTS

The replacement of NaCl or Na_2SO_4 with NaN_3 produced a shift of the spectrum toward the blue (Fig. 1). The spectral

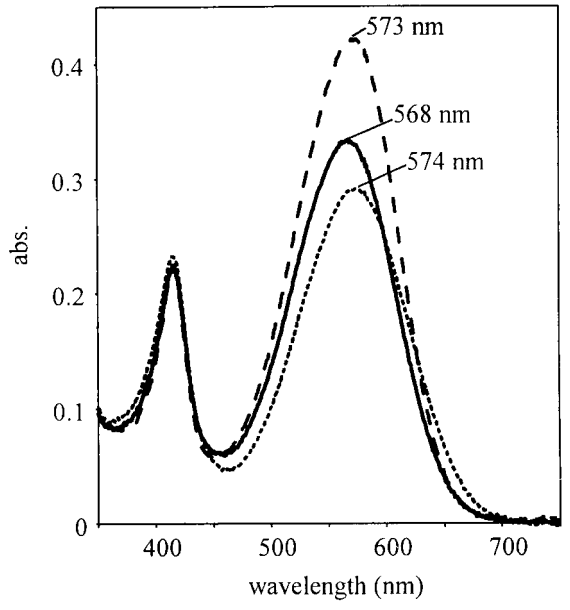

FIGURE 1 Absorption spectrum of salinarum halorhodopsin in 2 M NaCl (*broken line*), 1 M Na_2SO_4 (*dotted line*), and 2 M NaN_3 (*continuous line*). The peak at 415 nm is produced by a membrane bound cytochrome. The solutions had 10 mM MES, pH 7, 20°C.

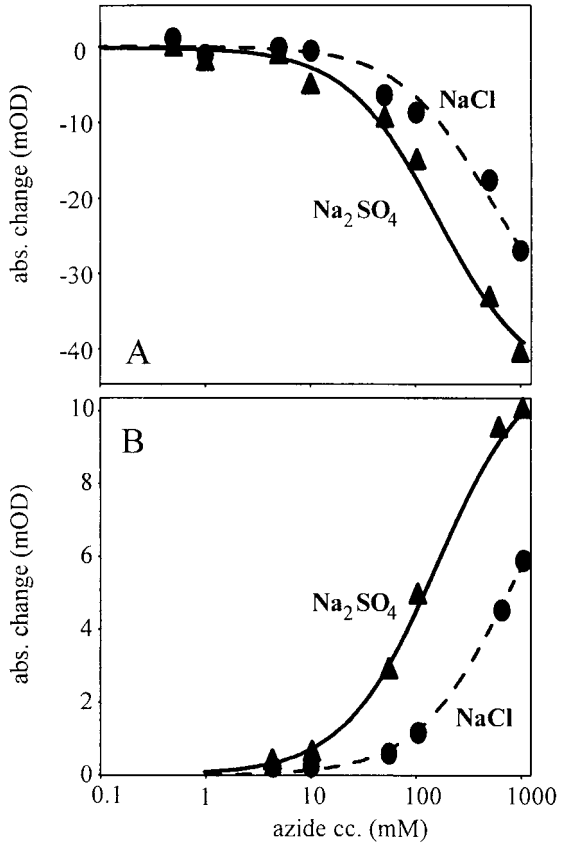

FIGURE 2 Binding of azide to halorhodopsin. Titration with NaN_3 was performed in NaCl (●) and Na_2SO_4 (▲). The fit revealed a binding constant of 1 M (*dotted line*) and 150 mM (*continuous line*). (*A*) Spectroscopic titration based on the absorption change of the spectrum measured at 630 nm. (*B*) Titration of the amplitude of the absorption kinetic signal measured at 410 nm.

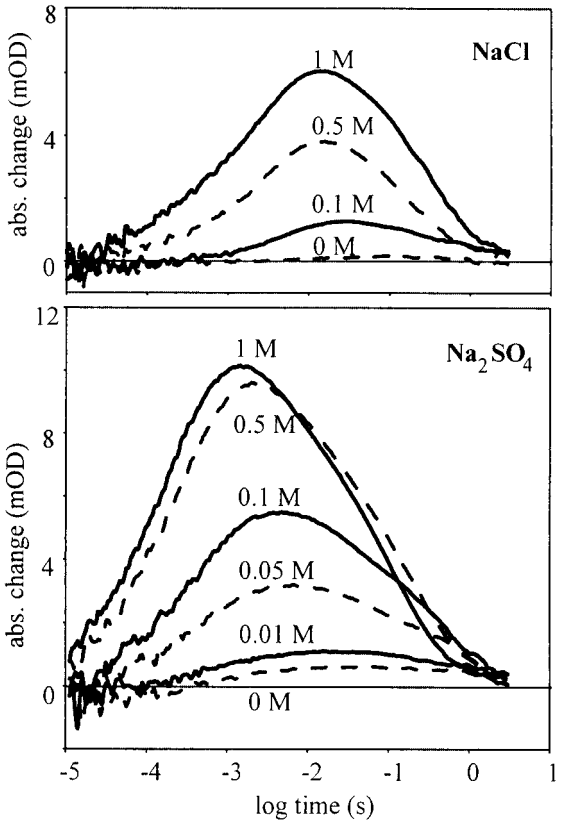

FIGURE 3 Flash-induced absorption changes in the presence of different azide concentrations measured at 410 nm. The appearance of the deprotonated Schiff base is detected. Keeping the Na concentration constant, 2 M NaCl or 1 M Na_2SO_4 was mixed with 2 M NaN_3 to get the desired azide concentration. All solutions had 10 mM MES, pH 7, 20°C.

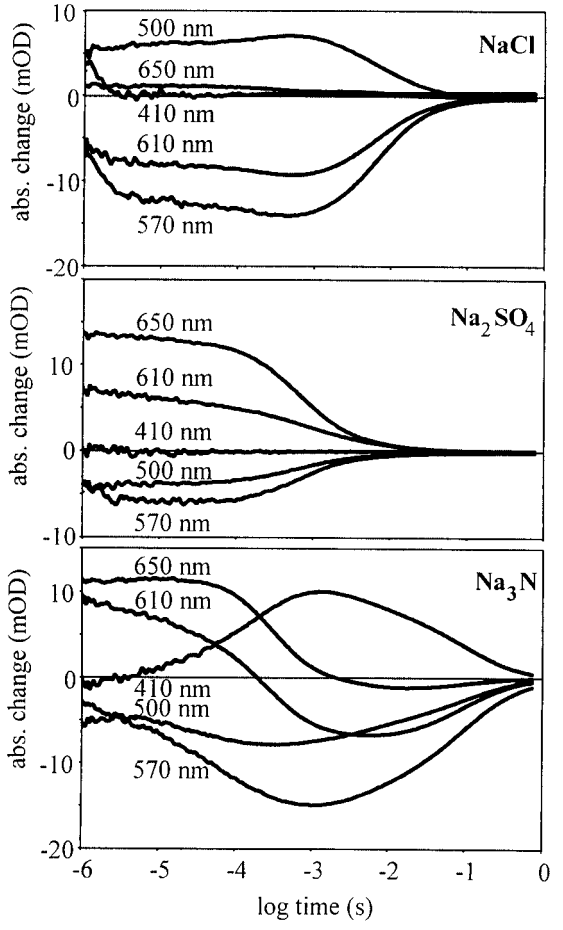

FIGURE 4 Absorption kinetic signals measured at five wavelengths with halorhodopsin in 2 M NaCl, 1 M Na_2SO_4, and 2 M NaN_3. All solutions had 10 mM MES, pH 7, 20°C.

changes were used to titrat azide binding, as done previously for chloride replacement with sulfate (Váró et al., 1995c). The replacement of 2 M NaCl with azide resulted a binding constant of ~1 M. When 1 M Na_2SO_4 was gradually replaced with 2 M NaN_3, keeping the sodium concentration constant, a binding constant of 150 mM was observed (Fig. 2 *A*). A third titration was performed at a constant 1 M NaCl concentration, gradually replacing 0.5 M Na_2SO_4 with 1 M NaN_3, which yielded a binding constant of 250 nM (data not shown).

Increasing azide concentration, starting out from 2 M NaCl or 1 M Na_2SO_4, produced an amplitude increase in the laser flash induced absorption kinetic signal measured at 410 nm (Fig. 3). This absorption change is characteristic to the appearance of the deprotonated Schiff base and is very similar to that characteristic to intermediate M in the bacteriorhodopsin photocycle. The amplitude of the 410-nm kinetic signal in function of azide concentration titrated with the same binding constants as the spectral changes (Fig. 2 *B*). In the presence of chloride the rise of the absorption change is slower, but in both chloride and sulfate the increasing amplitude was accompanied by acceleration of the rise of the signal. There was much less effect of azide on the decay of the signal. The effects of the three different salts upon the absorption kinetic traces measured at five wavelengths are shown in Fig. 4. The M-like absorption signal appears only in azide. It is absent both in chloride transporting photocycle (in the case of 2 M NaCl) or in that of nontransporting one when the solution contains only sulfate.

To determine the spectra of intermediate of the sHR photocycle, in the presence of azide, transient kinetic spectroscopic measurements were performed. The measured difference spectra were submitted to singular value decomposition analysis as described earlier (Golub and Kahan, 1992; Gergely et al., 1997). Based on the weight factors of the basis spectra and the autocorrelation of the spectra and their time dependent amplitude, the first four components could be considered different from noise, suggesting the existence of four independent spectral components. Two basis spectra were very strong (1.75 and 1.0 weight; 0.95 and 0.93 correlation), and the other two were much weaker (0.049 and 0.043 weight; 0.43 and 0.49 correlation). All of the

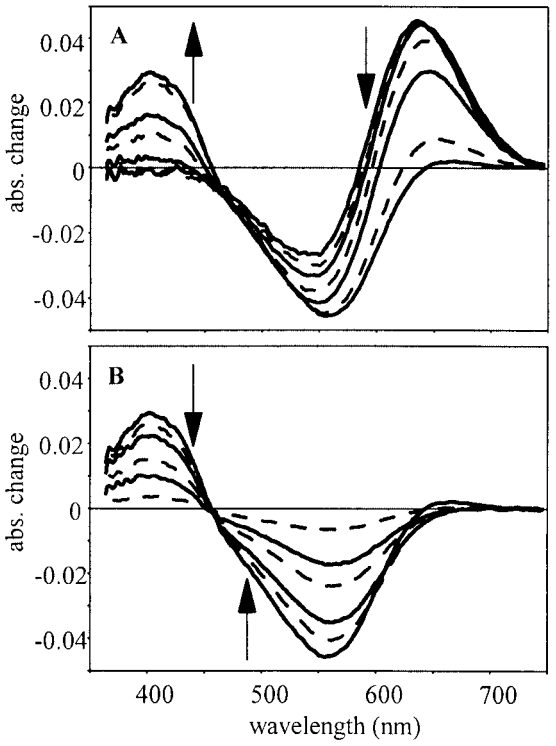

FIGURE 5 Singular value decomposition filtered time-resolved difference spectra measured on salinarum halorhodopsin in the time interval of: (*A*) 1 μs to 1 ms and (*B*) 1 ms to 400 ms. Measuring conditions consisted of 2 M NaN_3, 10 mM MES, pH 7, 20°C.

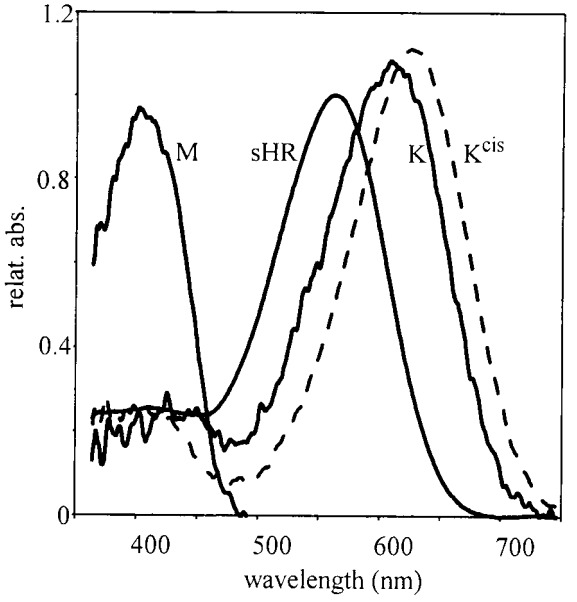

FIGURE 6 Spectra of intermediates calculated from the difference spectra shown in Fig. 5.

following components had even lower weight and one order of magnitude lower correlation. Noise-filtered difference spectra were reconstructed from the four singular value decomposition components (Fig. 5). The difference spectra taken at the shortest delay time after the photoexcitation show a red shifted component, which shifts even more toward red and decays to the blue absorbing component (Fig. 5 *A*). The existence of more than two intermediates in the early times is indicated by the fact that there is no isosbestic point observed during this transformation. Later the blue absorbing M-like component decays with an isosbestic point at ~450 nm, showing that only one intermediate is present, or all the components return to sHR in equilibrium with each other. The search for the intermediate spectra was performed as described (Gergely et al., 1997; Kulcsár et al., 2000). The early part of the photocycle showed the existence of three intermediates (Fig. 6) and in the second part of the photocycle no other intermediate could be detected. The existence of two red shifted intermediates suggested the possibility of two parallel photocycles.

Different sequential and parallel models were fitted to the absorption kinetic signals. Models containing less than five intermediates resulted in very bad fits. Models containing five intermediates gave good fits, and any extra intermediate introduced only slight improvement. Three models resulted in rather good fits:

1) model with sequential reactions:

$$K \Leftrightarrow K^{cis} \Leftrightarrow M_1 \Rightarrow M_2 \Leftrightarrow sHR' \Rightarrow sHR$$

2) model with branching after the first intermediate:

$$K \Leftrightarrow M_1 \Rightarrow M_2 \Leftrightarrow sHR' \Rightarrow sHR \text{ and}$$

$$K \Leftrightarrow K^{cis} \Rightarrow sHR$$

3) model with two parallel photocycles:

$$K \Leftrightarrow M_1 \Rightarrow M_2 \Leftrightarrow sHR' \Rightarrow sHR \text{ and}$$

$$K^{cis} \Rightarrow sHR$$

The three models had almost the same number of independent parameters and the same goodness of the fit. It is interesting to note that in all models considered the reaction between intermediates M_1 and M_2 become unidirectional, although the fit was started by allowing the reaction in both directions. The introduction of more than two M intermediates did not improve the fit. The fit of the model with two parallel photocycles to the absorption kinetic signals is shown on Fig. 7. When the fits of the three models were calculated for measurements performed at different temperature (Fig. 8), only the parallel model resulted linear Eyring plots (ln k versus $1/T$, in which k is the rate constant of the transition and T is the temperature measured in K) for all of the rate constants (data not shown). Based on these obser-

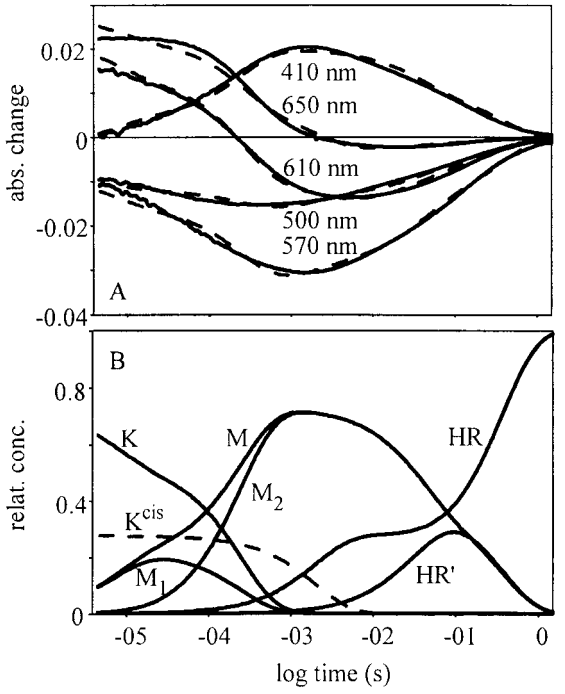

FIGURE 7 (*A*) Fit of the photocycle model (*broken line*) to the absorption kinetic signals (*continuous line*). The error of the fit was less than ±10%. (*B*) Time evaluation of the concentration of the intermediates.

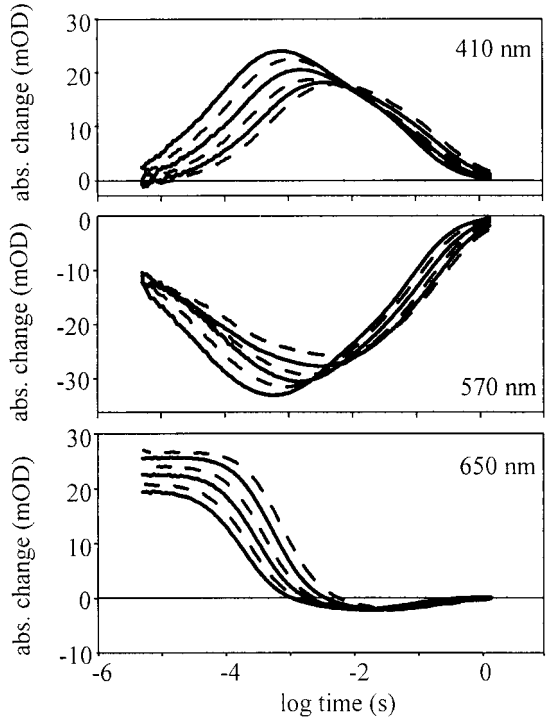

FIGURE 8 Absorption kinetic signals measured on salinarum halorhodopsin at different temperatures. From left to right the temperatures are 30°C, 25°C, 20°C, 15°C, 10°C, and 5°C. The other measuring conditions were the same as in Fig. 5.

vations the third model with two parallel photocycles was considered valid. The thermodynamic parameters calculated from the Eyring plots allow drawing the energetic picture of the photocycle. The photocycle containing K^{cis} has a single unidirectional transition with a free energy barrier of ~50 kJ/mol. The other photocycle shows an entropy driven transition between the K and M_1 intermediates, whereas an enthalpy driven transition between M_2 and HR′ (Fig. 9). There is no information about the energy absorbed at intermediate K. As the transition between M_1 an M_2 is unidirectional (denoted by the broken line on Fig. 9), there is no information about the energy level corresponding to that of M_2. Both K and M_2 were put arbitrarily to the same energy level.

The charge motions during the photocycle were determined by electric signal measurements on oriented gel samples. Due to high conductivity of the solutions only current measurements could be performed. In the presence of 2 M NaCl the electric signal, after a fast negative component, had a positive one appearing in the submicrosecond time scale (Fig. 10, broken line). In 1 M Na_2SO_4 only a negative component could be identified (Fig. 10, dotted line). In 2 M azide after the negative component (Fig. 10, continuous line), a positive component could be identified also but only in the millisecond time domain (Fig. 10, inset). As this positive component was slow, it had very small amplitude. At the same high amplification the sulfate signal remained negative, whereas that corresponding to chloride had small positive amplitude (data not shown).

To compare our measurements to that effectuated by others under continuous illumination (Hegemann et al., 1985) similar measurements were performed (Fig. 11) in the presence of chloride and sulfate at different azide concentrations. In the presence of chloride similar signals were measured as reported previously (Hegemann et al., 1985). Without chloride ion present in the solution, after switching off the light, a steady-state M-like component was observed. Similar component but in much smaller amplitude could be identified also in the presence of chloride. With increasing azide concentration, the amplitude of the 410-nm signal first increased but at highest concentrations decreased again.

DISCUSSION

The aim of this study is to characterize the azide-dependent photocycle of sHR and compare it with the proton pumping photocycle of BR and pHR. The details of the function observed by spectroscopic and kinetic analysis, the energetic diagram or the electric signals, helps in a better understanding of the function of these multiple ion pumps.

If sodium chloride is exchanged with sodium sulfate, only the amplitude of the spectrum of sHR decreases accompanied by a slight broadening. The concentration de-

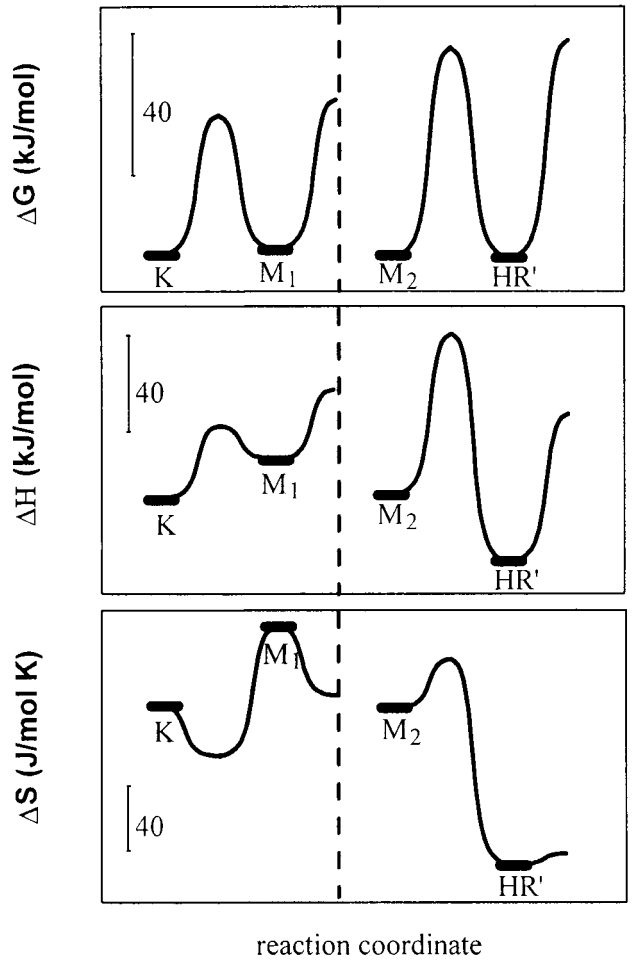

FIGURE 9 The free energy, enthalpy, and entropy diagram of the salinarum halorhodopsin all-*trans* azide photocycle. The M_1 to M_2 transition is unidirectional, leaving undetermined the position of the energy level corresponding to M_2, represented by the broken line between the two intermediates.

pendence of the amplitude change shows a 20 mM binding constant of the chloride ion (Váró et al., 1995c). The position of the chloride and all of the H bonds determining the binding site are known from the structure of sHR (Kolbe et al., 2000). When instead of chloride azide is used for titration a 150 mM binding constant is determined (Fig. 2). During this titration not only the amplitude of the spectrum increases, but also the position of the maximum is shifted from 574 to 568 nm (Fig. 1). In presence of 1 M NaCl, when the chloride-binding site is already saturated, the sulfate to azide replacement results an azide-binding constant of 250 mM. If instead of sulfate sodium chloride is replaced by azide the amplitude decreases, accompanied by the same shift of the spectrum but in this case with a weaker apparent binding constant of 1 M (Fig. 2 *A*). The spectral shift and the azide binding with only a small decrease in affinity even in the presence of a 20-times excess of chloride at its own binding site indicate that the position where azide binds is

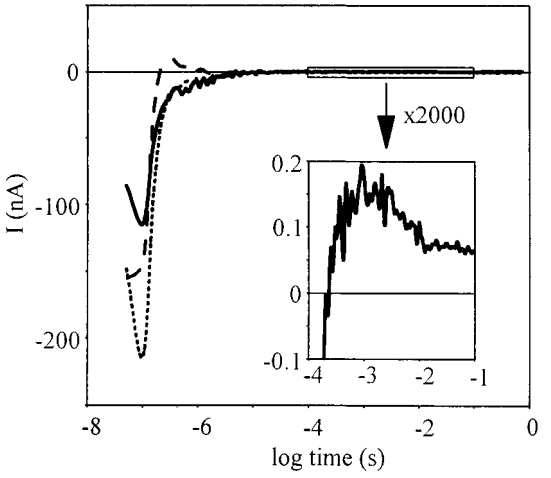

FIGURE 10 Light-induced electric signals of salinarum halorhodopsin measured on oriented gel samples in 2 M NaCl (*broken line*), 1 M $Na2SO_4$ (*dotted line*), and 2 M NaN_3 (*continuous line*). All solutions had 10 mM MES, pH 7, 20°C.

different from that of chloride. It appears that in sHR chloride does not compete with azide but only changes its binding properties. This is different from the case of pHR,

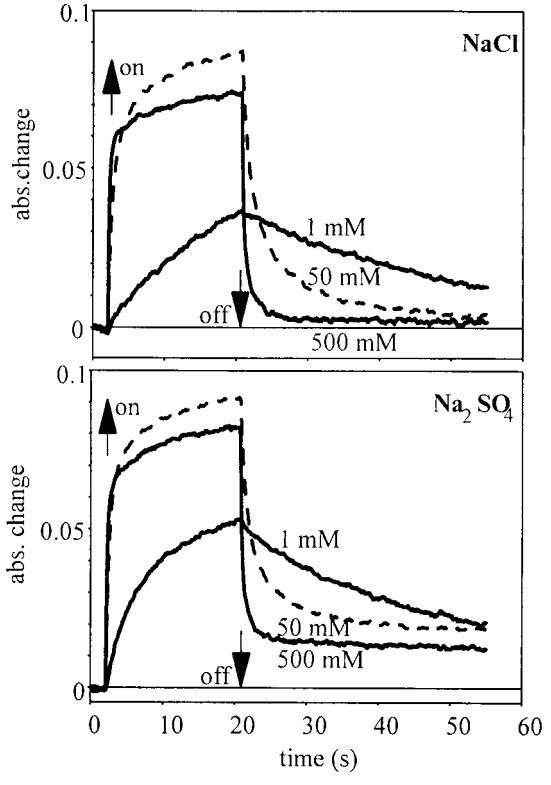

FIGURE 11 Absorption kinetic signals measured at 410 nm under continuous illumination of the sample. The arrows indicate where the light was switched on and off. Keeping the Na concentration constant, 2 M NaCl or 1 M Na_2SO_4 was mixed with 2 M NaN_3 to get the 1, 50, and 500 mM azide concentration. All solutions had 10 mM MES, pH 7, 20°C.

in which azide and chloride compete with each other in binding to the protein (Váró et al., 1996). The same binding constant were determined by the amplitude titration of the absorption kinetic signals measured at 410 nm, characteristic to the appearance of the deprotonated Schiff base. This indicates that the bound azide is required to detect the 410-nm signal and suggests that azide plays the role of the proton acceptor. The existence of the different binding site for chloride and azide explains the appearance of the M intermediate in high NaCl concentration in contrast to that observed earlier in pHR (Váró et al., 1996).

The photocycle of sHR is changed dramatically by replacement of chloride or sulfate with azide (Fig. 4). The chloride transporting photocycle is dominated by a positive absorption change measured at 500 nm, characteristic to intermediates L and N (Váró et al., 1995c). The nontransporting photocycle, measured in sulfate, has only a red shifted intermediate. When azide is added a more complex set of signals was measured at the same five wavelengths. The decay of red absorbing components is accompanied by the rise of a positive absorption change at 410 nm, indicating the appearance of an M-like intermediate, well known in the proton transporting photocycle of BR (Lanyi and Váró, 1995). An important difference from that photocycle is that no other intermediate, only the appearance of the HR-like component, can be observed in the decay part of the absorption kinetic signals. The same observation is corroborated by the time-dependent difference spectra measured with optical multichannel analyzer (Fig. 5). In the early difference spectra there is an absorption maximum ~650 nm, which decays, and no other peak in the red appears. Whereas the decay of the M-like component shows an isosbestic point at ~450 nm (Fig. 5 *B*) the time course of it is strongly multiexponential, suggesting the existence of the spectrally silent M- and HR-like intermediates.

The calculation of the intermediate spectra proved the existence of two red-shifted and one blue-shifted intermediate (Fig. 6). No other transient spectral component was found. The expected L-, N-, and O-like spectrum, based on the photocycle of BR (Lanyi and Váró, 1995) and that of proton transporting photocycle of pHR (Kulcsár et al., 2000), were missing from the sHR azide photocycle. The model fit to the absorption kinetic signals revealed that the most red-shifted intermediate belongs to a separate photocycle, probable to that of 13-*cis* retinal containing sHR. This is corroborated by the fact that the amplitude of K^{cis} is ~25% of the total amplitude, which is equal to the 13-*cis* content of the light adapted sHR (Zimányi and Lanyi, 1997). This photocycle is similar to that of 13-*cis* photocycle of BR with the difference that the two red-shifted intermediates were distinguished, decaying back to ground state (Gergely et al., 1994). In the presence of chloride the photocycle of the 13-*cis* retinal containing sHR also had two intermediates (Váró et al., 1995c) but with strongly overlapping spectra. Eventually a better data set of difference spectra could distinguish two spectra belonging to the 13-*cis* photocycle and these would lead to a better fit of the kinetic data at the early time points (Fig. 7).

In the photocycle belonging to the all-*trans* retinal containing sHR intermediate K decays directly to M_1 with a time constant of ~30 μs. This is one order of magnitude slower than the decay of K in the bacteriorhodopsin photocycle (Lanyi and Váró, 1995; Ludmann et al., 1998). In the proton pumping photocycle of pHR, where a large amount of L is accumulated, the K to L decay is even faster (Kulcsár et al., 2000). Based on these it can be concluded that the L intermediate could be missing for kinetic reasons. If its decay is faster than that observed for K intermediate, L cannot accumulate during the photocycle. The same could be the case with intermediates N and O. By kinetic analyzes it cannot be decided whether these intermediates really are missing from the photocycle. The photocycle has an M_1 to M_2 unidirectional transition observed also in the BR photocycle at pH $>$ 6 (Ludmann et al., 1998). It is reasonable to assume, although not proven, that the M_1 to M_2 transition in the case of sHR has the same role as in the BR, the accessibility switch from extracellular to cytoplasmic conformation (Haupts et al., 1997; Brown et al., 1998; Lanyi, 1998).

The temperature dependence of the absorption kinetic measurements (Fig. 8) and their fit to the photocycle model revealed some of the energy relationships in the photocycle. Two transitions, K to M_1 and M_2 to HR′, were equilibrium reaction (Fig. 9). Although in the proton transporting photocycle of pHR, the K to L reaction was enthalpy driven, but in the presently studied sHR, the equivalent K to M_1 reaction is entropy driven. In the decay part both photocycles were enthalpy driven (Kulcsár et al., 2000). The unidirectional M_1 to M_2 reaction means a rather large free energy drop. It is interesting to note that in all of the cases, when the energetic picture of halorhodopsin was determined the HR′ state had entropically low energy. As the free energy levels were almost at the same level this low enthalpy is equivalent with an ordered protein structure, which later relaxes to the ground state (Váró et al., 1995a; Kulcsár et al., 2000).

The electric signal measurements provided new information about the photocycle. The electric signal measured in 2 M NaCl was rather similar to that measured earlier but in a lower salt concentration (Dér et al., 1985a, 1992). A fast negative component was followed by a positive one (Fig. 10, broken line). The negative component corresponds to a primary charge separation observed also in BR samples (Keszthelyi and Ormos, 1980; Groma et al., 1988). Although in pHR at high chloride concentration, the negative component was missing, the electrogenicity calculations proved that to intermediate K can also be attributed a negative component (Kulcsár et al., 2000). The positive component of the signal corresponds to the transporting step of the photocycle (Dér et al., 1992). In the case, when only

sulfate is present in the solution, the electric signal had only negative components (Fig. 10, dotted line). The recovery of the primary charge separation, which would result a small positive signal, could not be measured. When azide was added to the solution, after the fast negative charge separation, a characteristic positive signal was observed. This signal appeared in the same time domain as M_2 and could be followed up to 100 ms (Fig. 10, continuous line). The presence of the positive signal is a proof of a charge motion during this step of the photocycle. This charge motion has the same sign as that corresponding to chloride transporting step in HR or proton transporting step in BR. If it is accepted that the azide photocycle is related to proton transport (Bamberg et al., 1993, 1994; Váró et al., 1996), than this signal can be attributed to a motion from the cytoplasm to the extracellular direction.

From continuous illumination studies, similar to that presented in Fig. 11, it was concluded earlier that in the presence of azide the M intermediate is produced as a side reaction of the photocycle (Hegemann et al., 1985; Oesterhelt et al., 1985). Our flash experiments performed in the presence of chloride and sulfate indicate, however, that the absorption change measured at 410 nm cannot be explained as a side reaction of the chloride transporting or the sulfate photocycle. The M species produced by flash illumination and continuous illumination are different. The decay of the M-like intermediate is several orders of magnitude slower when measured after continuous illumination than the decay in the case of flash experiments (compare Figs. 3 and 11). After continuous illumination the rate of decay is dependent on azide concentration (Hegemann et al., 1985; Oesterhelt et al., 1985), but in the case of flash illumination it is nearly azide independent (Fig. 3). Thus, continuous illumination creates another kind of deprotonated Schiff base containing photoproduct unrelated to the azide photocycle we described here. A similar effect was observed in the case of BR exposed to very long illumination (Dancsházy et al., 1999; Dancsházy and Tokaji, 2000), although the production of the M-like intermediate in BR has a lower quantum efficiency and results in a stable product, which suggests that the basic effect producing it could be different. To understand the nature of this photoproduct, further studies are needed.

In this study the existence of a separate photocycle of sHR was proven, which can be related to proton transport across the membrane. It has the characteristic deprotonated Schiff base containing M intermediate. Its kinetics shows some similarities to the BR photocycle and that of the proton transporting photocycle of pHR.

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