Light-Driven Chloride Ion Transport by Halorhodopsin from *Natronobacterium pharaonis*. 1. The Photochemical Cycle†

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ABSTRACT: The photochemical cycle of the light-driven chloride pump, halorhodopsin from *N. pharaonis*, is described by transient optical multichannel and single-wavelength spectroscopy in the visible, and in the infrared. Titration of a blue-shift of the absorption maximum upon addition of chloride describes a binding site with a K_D of 1 mM. The reaction sequence after the all-*trans* to 13-*cis* photoisomerization of the retinal in this chloride binding form is itself dependent on chloride. At 2 M chloride it is described by the scheme: $HR \xrightarrow{h\nu} K \leftrightarrow L \leftrightarrow N \rightarrow HR$ that relaxes in a few milliseconds, and is very similar to the photocycle of bacteriorhodopsin under conditions where the retinal Schiff base cannot deprotonate. At lower chloride concentrations, *e.g.*, 0.1 M, however, a red-shifted state termed O appears between N and HR, in equilibrium with N. The absorption spectra of K, L, N, and O are very similar to their counterparts in the bacteriorhodopsin photocycle. As in their equivalents in bacteriorhodopsin, in the N state the retinal is still 13-*cis*, but it is reisomerized in the O state to all-*trans*.

Halorhodopsin is a light-driven chloride ion pump in the cytoplasmic membranes of halobacteria (Schobert & Lanyi, 1982). Similarly to bacteriorhodopsin in the same organisms (Mathies et al., 1991; Oesterhelt et al., 1992; Ebrey, 1993; Lanyi, 1993), photoisomerization of the all-*trans* retinal to 13-*cis* initiates its transport cycle (the "photocycle"), but instead of a proton a chloride ion is translocated, and in the reverse direction, *i.e.*, into the cytoplasm rather than outward (Oesterhelt & Tittor, 1989; Lanyi, 1990). It has been argued that there must be an analogy between proton transport in bacteriorhodopsin and chloride ion transport in halorhodopsin (Oesterhelt & Tittor, 1989; Oesterhelt et al., 1992). Comparisons of bacteriorhodopsins and halorhodopsins from various species show sequence identities of 25% to 30% between the two kinds of proteins (Blanck & Oesterhelt, 1987; Lanyi et al., 1990a; Otomo et al., 1992; Soppa et al., 1993). The extent of identity is greater in the putative seven transmembrane helical segments of the proteins, and the residues in what is identified in bacteriorhodopsin as the retinal binding pocket (Henderson et al., 1990) are particularly highly conserved. The notable exceptions to this are the two aspartates, D85 and D96, in bacteriorhodopsin, that are components of the proton translocation pathway. In all halorhodopsins sequenced to date, the corresponding residues are threonine and alanine, respectively. D85 is the more important residue of the two, as the acceptor of the Schiff base proton in the first proton transfer reaction of the bacteriorhodopsin photocycle. Indeed, the Schiff base and D85 have been suggested to comprise the active site (Kataoka et al., 1994).

We have recently found that the D85T (but not the D85N) residue replacement converted bacteriorhodopsin from a proton pump into a chloride ion pump (Sasaki et al., 1995). This demonstrated that the ion specificity of the transport resides in the side-chain of residue 85. When an aspartate, it participates with the Schiff base in a proton donor−acceptor pair, but when a threonine, it will evidently hydrogen-bond, at some stage of the transport process and probably together with the Schiff base, to a chloride ion. The D85T mutant of bacteriorhodopsin has low binding affinity for chloride, however. We believe that many of the residues specific to each protein, other than residue 85, have the function to optimize the conduction of either protons or chloride ions to and from the active site, while many of the conserved residues provide the means to accomplish the translocation. Thus, we suspect that the transport of a cation and an anion in the two ion pumps are variants of a *single* mechanism. This would be a surprising and far-reaching conclusion, but cannot be evaluated before the chloride transport mechanism in halorhodopsin is understood. In this paper, and the accompanying one (Váró et al., 1995a), we report our attempts to begin a description of the photocycle and chloride translocation in halorhodopsin from *Natronobacterium pharaonis*.

What are the clues supplied to halorhodopsin by the more thoroughly investigated bacteriorhodopsin photocycle? While

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some controversy still exists over the photocycle of the latter protein (as reviewed in Lanyi, 1993), many investigators feel that its basic features are understood. A change in the geometry of the Schiff base, D85, and bound water (Brown et al., 1994; Kandori et al., 1995), and distortions of the retinal chain (Fahmy et al., 1991; Maeda et al., 1994; Yamazaki et al., 1995) upon photoisomerization to 13-*cis*, that become evident after the K → L reaction,[1] cause the transfer of a proton from the retinylidine group to the anionic D85 in the L → M_1 reaction. This is followed by release of a proton to the extracellular surface. Reprotonation of the Schiff base from D96 is ensured by the "reprotonation switch" that changes the proton transfer pathway toward the cytoplasmic side in the M_1 → M_2 reaction (Váró & Lanyi, 1991a; Zimányi et al., 1992a; Kataoka et al., 1994). The switch seems to be associated with a large-scale protein conformational change, revealed directly by diffraction (Dencher et al., 1989; Koch et al., 1991; Nakasako et al., 1991; Subramaniam et al., 1993; Han et al., 1994; Kataoka et al., 1994). It was detected indirectly by the effect of hydrostatic pressure on the $M_1 \leftrightharpoons M_2$ equilibrium (Váró & Lanyi, 1995), which suggested a volume increase consistent with the structural change inferred from electron diffraction of the M state. Proton transfer from D96 restores the protonated Schiff base in the M → N reaction, and during the lifetime of N, the now anionic D96 is reprotonated from the cytoplasmic surface. Reisomerization of the retinal to all-*trans* occurs during the N → O reaction (Bousché et al., 1992; Souvignier & Gerwert, 1992; Smith et al., 1983). This is followed by internal proton transfer which restores the anionic state of D85 and thus the initial BR.

It is not immediately clear how such a mechanism would transport chloride in halorhodopsin. The suggestion has been made (Oesterhelt et al., 1986) that instead of deprotonation of the Schiff base, chloride might bind to the positively charged protonated retinylidine nitrogen and be translocated upon its displacement as the retinal undergoes *trans* → *cis* reisomerization in the photocycle. This model has not been put to a test because the chloride transfer steps in the halorhodopsin photocycle had not been identified.

The photocycle of halorhodopsin from *Halobacterium salinarium* was reported (Tsuda et al., 1982; Taylor et al., 1983; Hegemann et al., 1985; Oesterhelt et al., 1985; Lanyi & Vodyanoy, 1986; Tittor et al., 1987; Zimányi et al., 1989) to contain intermediates analogous to the bacteriorhodopsin photoproducts but without the M state that contains a deprotonated Schiff base. We reported recently (Váró et al., 1995b) that the red-shifted intermediate, which appeared to arise in the millisecond time-range and had been placed earlier in the reaction sequence, originates in fact from the photocycle of the 13-*cis* state at high chloride concentrations, and from the non−chloride-binding form at low chloride concentrations. The photocycle of the chloride-binding form with all-*trans* retinal, which corresponds to the transport cycle, was therefore described by the scheme:

$$HR \xrightarrow{h\nu} K \leftrightharpoons L \leftrightharpoons N \rightarrow HR$$

much like the photocycle of bacteriorhodopsin when D85 is protonated at low pH (Mowery et al., 1979; Váró & Lanyi, 1989) or D85 is replaced by asparagine (Otto et al., 1990; Thorgeirsson et al., 1991; Kataoka et al., 1994). However, according to a recent report (Scharf & Engelhard, 1994), the photocycle of another halorhodopsin, from *Natronobacterium pharaonis*, with 65% sequence identity with the chloride pump in *H. salinarium* (Lanyi et al., 1990a), contains so much of the red-shifted intermediate that one would have to suspect that it might be an authentic photoproduct of the chloride-binding all-*trans* protein. Because of this, and because of the fact that binding of chloride shifts the absorption maximum toward shorter wavelengths (Scharf & Engelhard, 1994) rather than in the other direction as in *H. salinarium* halorhodopsin (Lanyi et al., 1990b), we suspected that there might be differences between the two halorhodopsins. In this report we describe the properties and the photoreactions of the *N. pharaonis* protein, and recount further results relevant to chloride transport in the paper that follows (Váró et al., 1995a).

MATERIALS AND METHODS

Halorhodopsin-containing membranes were prepared from *Halobacterium salinarium* (formerly *halobium*) strain L33 which was transformed with an independently replicating vector to be described elsewhere, with the *bop* promoter, the *Natronobacterium pharaonis hop* structural gene, and the novobiocin resistance gene for selection. The amount of *H. salinarium* halorhodopsin expressed in these cells is negligible compared to the greatly enhanced amount of *N. pharaonis* halorhodopsin. After inoculating from a novobiocin-containing (1 μg/mL) culture, growth at 40 °C in medium lacking novobiocin with shaking for 3−4 days was followed by centrifugation, washing of the cells in 25% NaCl, and dialysis against 40 volumes of water in the presence of DNase I. The lysed cells were centrifuged at 30 000 rpm for 1 h (T35 rotor in a Beckman L5-75B ultracentrifuge) and washed with 0.1 or 0.2 M NaCl, and the resulting membrane fragments collected in a discontinuous sucrose gradient containing 0.1 or 0.2 M NaCl, as described for purple membranes (Oesterhelt & Stoeckenius, 1974). As in the earlier preparations of *H. salinarium* halorhodopsin-containing membranes (Váró et al., 1995b), because unphysiologically low salt concentration was used to disrupt the halophilic cells the preparation contained small membrane fragments rather than closed vesicles. The absorbance ratio at 280 and 580 nm was about 4, indicating that about half of the protein in these membranes was halorhodopsin. A small extent of contamination with cytochrome that absorbed at about 415 nm (see legends to Figures 1, 4, 5, and 7) was tolerated. The membrane fragments were stored with sucrose at −70 °C, and centrifuged, washed, and resuspended in the buffer of choice before use, similarly to purple membranes. All spectroscopy in the visible was with these membranes encased in polyacrylamide gels, as before (Váró et al., 1995b).

Flash excitation with a frequency-doubled Nd-Yag laser and measurements of absorption changes with an intensified diode array were as previously described (Zimányi et al., 1989; Váró & Lanyi, 1991b; Zimányi & Lanyi, 1993). The spectra are averages from 400 to 600 repetitions. The buffer was always 50 mM MES, pH 6.0, in addition to the other salts present as specified. The temperature was regulated at 22 °C throughout. Singular value decomposition (SVD)

[1] Abbreviations: K, L, N, and O are intermediates of the photocycle of halorhodopsin, in analogy of the intermediates of bacteriorhodopsin that include also M; MES, 2-[2-morpholino] ethanesulfonic acid.

treatment (Golub & Kahan, 1992) was used to decide the number of spectral components, as well as to remove some of the noise in the time-resolved difference spectra. The spectra are shown after this noise-filtering. The spectral analyses that utilize the multidimensional fitting programs SEARCH (for finding the spectra of intermediates) and RATE (for fitting various kinetic models) were described earlier (Zimányi & Lanyi, 1993; Váró et al., 1995b).

Chloride transport was measured in cell envelope vesicles (Lanyi & MacDonald, 1979) by following the light-dependent pH changes that originate from passive proton uptake in response to the negative-inside membrane potential created (Lanyi & Oesterhelt, 1982; Schobert & Lanyi, 1982; Duschl et al., 1990). We avoided chloride contamination from the glass electrode (Ross Combination semimicro electrode #810300, Orion Research Inc. Boston, MA) by filling it with 1.5 M Na_2SO_4 instead of the usual KCl.

Before retinal extractions, bacteriorhodopsin, and halorhodopsin from *H. salinarium* and *N. pharaonis*, were dark-adapted by keeping them in the dark in 1 M NaCl, 20 mM phosphate, pH 6, for 2 days. When light adapted, it was by illumination at 4 °C with yellow light (filter Y52) from a 1-kW halogen-tungsten lamp for several minutes until no further change in absorption occurred. (However, halorhodopsin from *N. pharaonis* did not show spectral shift upon the illumination.) Extraction of retinal was carried out before and immediately after the illuminations, by a method described earlier (Scherrer et al., 1989). A total of 100 μL of membrane suspension was mixed rapidly with 250 μL of ice-cold ethanol on a vortex mixer. After 2 min on ice with intermittent mixing, 250 μL of ice-cold hexane was added and the sample was mixed continuously for 2 min. The emulsion was centrifuged with a hand-rotating centrifuge, and the retinal isomers in the hexane (upper) phase were immediately separated by HPLC (Shimadzu, LC-7A). A Zorbax-Silcolumn (4.6 × 150 mm; Shimadzu) was used for the separations of the retinal isomers, with 8% diethyl ether in hexane as solvent, at a flow rate of 1.5 mL/min, and monitored at 365 nm with a UV-VIS spectrophotometric detector (Shimadzu, SPD-7AV).

For the FTIR measurements the membranes containing halorhodopsin were suspended in 20 mM phosphate, pH 7.0, containing 10 mM, 100 mM, or 1 M NaCl, and centrifuged at 90 000 rpm for 10 min in rotor TL100.3 of the Beckman Model TL-100 ultracentrifuge. The pellet was placed on a BaF_2 window of 18-mm diameter, and pressed with another window with a 12.5-μm thickness Teflon O-ring between them. The sample holder containing this sandwich was inserted into an Oxford DN1704 cryostat equipped with an Oxford ITC4 temperature controller. The temperature was maintained at 273 K for the measurements reported, but the spectra obtained at 265 or 293 K were essentially the same. Infrared spectra were collected in a BioRad FTS-60A/896 spectrometer. The spectra shown were obtained by averaging 128 interferograms at 2 cm^{-1} resolution. The sample window was tilted 45° relative to the probe light, and the quasi-continuous excitation light from an excimer-dye laser (540 nm, 17 ns pulse width, 50 Hz) was directed onto the sample at 90° to the probe light. The spectra were recorded both before and during the illumination, and the difference spectrum of the photoproducts *vs.* the unphotolyzed state was obtained by subtraction. The measurements were repeated 10−16 times and averaged to obtain the final spectra.

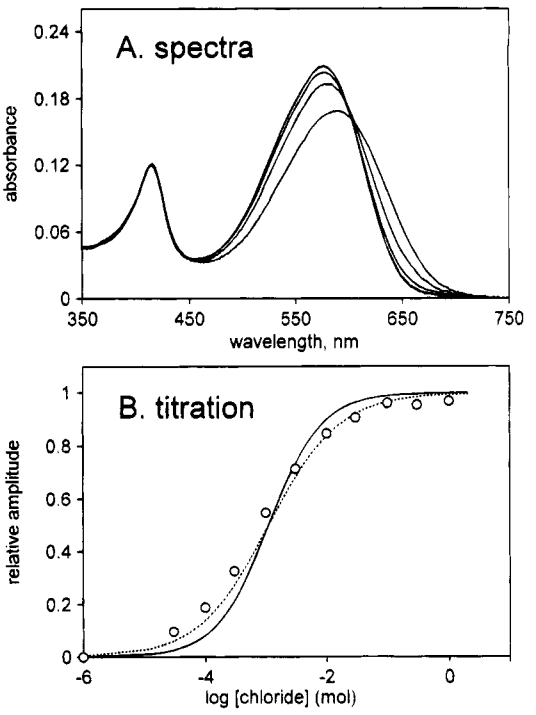

FIGURE 1: Chloride-dependent changes in the spectrum of the *N. pharaonis* halorhodopsin chromophore. (A) Absorption spectra in mixtures of 2 M NaCl and 1 M Na_2SO_4 (total concentration of Na^+ kept at 2 M). Chloride concentrations in the direction of increasing absorbance near 570 nm: zero, 3 mM, 100 mM, 2 M. The chloride-independent peak at 420 nm is due to cytochrome contamination of the membranes. (B) Amplitudes of the difference spectra with and without chloride vs. the logarithm of chloride concentration. Solid line: best fit of a titration curve with first-order binding of chloride. Dotted line: best fit with 0.75-order binding.

RESULTS

Spectroscopic Titration with Halides. The absorption maximum of *N. pharaonis* halorhodopsin shifts toward the blue in the presence of chloride (Scharf & Engelhard, 1994). Figure 1A shows that this shift is largely accounted for by a simple transformation of one spectral species (maximum at 590 nm) to another (maximum at 577 nm) upon binding of the chloride. This is different from the spectral changes in *H. salinarium* halorhodopsin, where titration of the same kind of membrane preparation revealed a small red-shift instead of a large blue-shift upon adding chloride, and a more obvious amplitude increase that was not clearly related to the shift (Váró et al., 1995b). The apparent binding constant in Figure 1B is 1 mM, as found before (Scharf & Engelhard, 1994). The amplitudes associated with the spectral shift are somewhat better described by a titration curve with a reaction order of 0.75 chloride ions (dotted line) than with 1.0 (solid line), in the same way as pH dependencies of proton-involved processes at the surfaces of bacteriorhodopsin are often represented by lower than first-order binding of protons (Holz et al., 1989; Miller & Oesterhelt, 1990; Marti et al., 1991, 1992; Cao et al., 1991, 1995). Essentially the same titration curve was obtained from the amplitude of the spectral changes that originate from the chloride-dependent photocycle (Váró et al., 1995a), suggesting that the chloride at the binding site for the spectral shift determines also the photocycle. The spectral changes in *H. salinarium* halorhodopsin had yielded an apparent binding constant of 20 mM for chloride, and for reasons that were not clear the

Table 1: All-*trans* and 13-*cis* Retinal Isomeric Contents of the Halorhodopsins from *N. Pharaonis* and *H. salinarium*, and Bacteriorhodopsin, in the Dark-Adapted and Light-Adapted States[a]

		all-*trans*	13-*cis*
Halorhodopsin (*N. pharaonis*)	light-adapted	77	23
	dark-adapted	83	17
Halorhodopsin (*H. salinarium*)	light-adapted	83	17
	dark-adapted	48	52
Bacteriorhodopsin	light-adapted	98	2
	dark-adapted	39	61

[a] The data for bacteriorhodopsin are given as controls. Previous measurements have given the all-*trans* contents in light-adapted samples as 100%, and in dark-adapted samples as 40% (Scherrer et al., 1989).

apparent binding constant from the amplitude of the chloride dependence of the photocycle was several fold higher (Váró et al., 1995b).

Absence of Light/Dark Adaptation. The isomeric contents of retinal shift toward increased all-*trans* at the expense of 13-*cis* upon sustained illumination of both bacteriorhodopsin (Sperling et al., 1977; Harbison et al., 1984; Scherrer et al., 1989) and halorhodopsin from *H. salinarium* (Maeda et al., 1985; Lanyi, 1986; Váró et al., 1995b), and the original thermal equilibrium is reestablished upon incubation for hours in the dark. We find that such a light/dark adaptation does not occur in halorhodopsin from *Natronobacterium pharaonis*. Neither have we seen a change in the absorption maximum upon illumination or in the difference spectra upon photoexcitation after storing for several days in the dark (not shown), nor do we observe a change in the isomeric composition of the retinal (Table 1). Likewise, absorption changes at single wavelengths measured in dark-adapted or continuously light-adapted samples were essentially identical (not shown). The samples contain mostly all-*trans* retinal with or without previous illumination, and the rest is mostly 13-*cis* retinal, in about 20% amount. Rapid dark-adaptation as a cause of these observations is excluded by the fact that the spectrum is virtually the same whether measured during illumination, with an optical multichannel analyzer, or while dark-adapted, with a conventional spectrophotometer (not shown).

Transport of Chloride. Figure 2 shows the initial rate of transport for halorhodopsins from *H. salinarium* and *N. pharaonis*, assayed by measuring passive proton uptake in cell envelope vesicles in the presence of an uncoupler (Lanyi & Oesterhelt, 1982; Schobert & Lanyi, 1982; Duschl & Wagner, 1986), as a function of chloride concentration. As expected from the equilibrium binding constant in Figure 1B, the half-maximal chloride concentration for transport in *N. pharaonis* halorhodopsin is 1 mM. It is 50 mM for transport by *H. salinarium* halorhodopsin, a value more consistent with the half-maximal chloride concentration required for its chloride-dependent photocycle, than with the chloride binding equilibrium for its spectral shift (Váró et al., 1995b). We note in Figure 2 that, as reported earlier (Duschl et al., 1990), there must be significant transport activity for *N. pharaonis* halorhodopsin even in the absence of chloride. The line for this titration curve is drawn with a limiting value of 10% activity at zero chloride concentration, consistent with the measured rate in 1.5 M Na_2SO_4. Since, unlike *H. salinarium* halorhodopsin, this protein will transport nitrate as well as chloride (Duschl et al., 1990), we assume that the transport in the absence of chloride

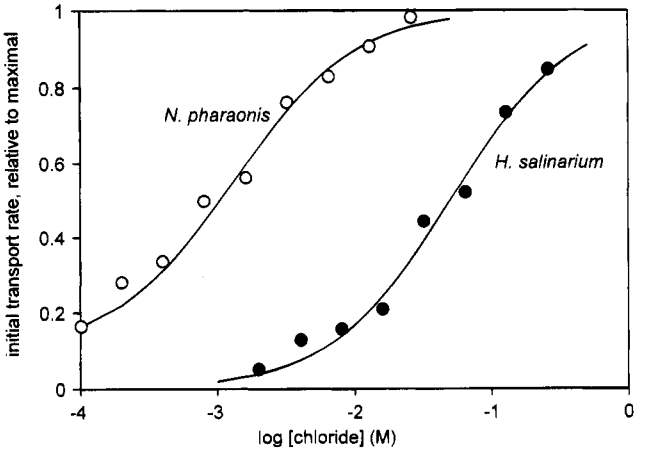

FIGURE 2: Chloride dependence of the initial light-dependent transport rate in halobacterial cell envelope vesicles containing either *N. pharaonis* (open circles) or *H. salinarium* (closed circles) halorhodopsin. The electrogenic uptake of chloride was followed by measuring light-dependent pH increase due to the passive uptake of protons. Conditions: mixtures of 3 M NaCl and 1.5 M Na_2SO_4, pH 6.7, 5 μM CCCP, 23 °C.

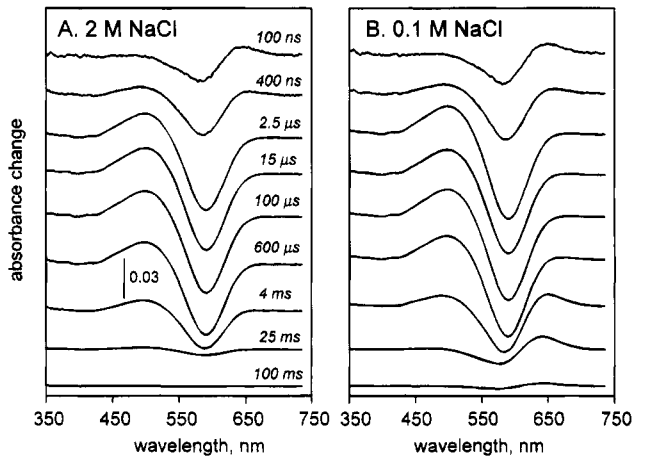

FIGURE 3: Difference spectra after pulse photoexcitation of *N. pharaonis* halorhodopsin in 2 M NaCl (A), and 0.1 M NaCl + 0.95 M Na_2SO_4 (B). The delay times between the laser pulse and the measurements of the spectrum are given in A.

represents a low but definite affinity also for sulfate as transport substrate.

Transient Spectroscopy at Two Selected Chloride Concentrations. As one would expect for a chloride transport system, halorhodopsin from *H. salinarium* exhibited different photochemical cycles in the presence and absence of chloride (Váró et al., 1995b). However, the photocycle of the 13-*cis* form of the chromophore contributed a long-lived red-shifted spectral species that had to be subtracted from the measured changes to obtain the photocycle of the all-*trans* form relevant for transport. Time-resolved difference spectra were measured for *N. pharaonis* halorhodopsin in 2 M NaCl and in 0.1 M NaCl plus 0.95 M Na_2SO_4. Figures 3A and B show a series of difference spectra at various delay times under these conditions. Remarkably, the spectra at saturating, *i.e.*, 2 M, chloride concentration (Figure 3A) lack the spectral features identified in the other protein as the photoproduct of the 13-*cis* form under these conditions. In fact, the spectra are similar to the spectra for *H. salinarium* halorhodopsin *after* subtraction of the contribution of the 13-*cis* cycle (compare with figure 3A in Váró et al., 1995b). It appears that although the protein contains some 13-*cis* retinal (cf.

above), it has virtually no detectable photoproducts on the time-scale of these measurements.

Even 0.1 M NaCl is well above saturation in the chloride binding equilibrium (Figure 1B), and the spectra under these conditions (Figure 3B) will have originated also entirely from the photoreaction of the chloride binding form. Up to 100 μs after photoexcitation the spectra are very similar to those at the higher chloride concentration, but those that follow have a distinctly different shape (Figure 3B). Consistent with earlier results (Scharf & Engelhard, 1994), absorbance increases considerably between 600 and 700 nm in the millisecond time domain. Unlike in *H. salinarium* halorhodopsin (Váró et al., 1995b), this large increase of absorbance is due not to artifact from the presence of two photocycles but to the formation of a red-shifted species in a single cycle. That it is observed at 0.1 M chloride but not at 2 M contrasts with the observations with the other protein, where the photocycle associated with chloride showed an amplitude increase with increasing chloride concentration, but not changes in the kinetics or the kind of intermediates that accumulated.

Reconstruction of the Spectra of the Intermediates and Their Kinetics in the Presence of Chloride. Time-resolved spectra, as in Figure 3 but with many more time-points, were used first to calculate the spectra of the intermediates of the photocycles then calculate their time-dependent concentrations, and finally to test the fits of various kinetic schemes. For such data, the first and most important fact to decide is the number of spectrally and kinetically distinguishable intermediates. The three highest-ranking basis spectra in SVD analysis (Golub & Kahan, 1992) of 31 difference spectra at different delay times in 2 M chloride had weights of 1.74, 0.17, and 0.029. The pairwise products of the correlations of the weights and the time-dependencies were 0.95, 0.78, and 0.16, respectively. Thus, this set of spectra contains two significant spectrally distinct components. As in the case of *H. salinarium* halorhodopsin (Váró et al., 1995b), however, analysis with only two spectra (K and L) yielded the unrealistic result that the concentration of the K intermediate first decreased in the cycle and then increased. Thus, we introduced a third component, N. This consideration produced the scheme K $\leftrightharpoons$ L $\leftrightharpoons$ N $\rightarrow$ HR as the simplest alternative. Other than for two L substates, this is the photocycle that we suggested for the other halorhodopsin. For this scheme, a search for the component spectra with shapes that obey simple criteria (Zimányi & Lanyi, 1993) yielded the spectra shown in Figure 4A, and decomposition of the data into these spectra produced the time-courses for their concentration shown in Figure 4B. Both spectra and kinetics are similar to those for the other halorhodopsin, except that the L $\leftrightharpoons$ N interconversion is about 10 times slower (compare with figures 4A and B in Váró et al., 1995b).

The same analysis gave different results for spectra measured in 0.1 M chloride. This was as expected, given the appearance of a red-shifted state at the lower but not the higher chloride concentration (compare the spectra at 600 μs and later in Figures 3A and B). The four highest-ranking SVD basis spectra had weights of 1.90, 0.30, 0.07, and 0.02, and the products of the correlations of the weights and the time-dependences were 0.95, 0.87, 0.79, and 0.14. Thus, these data contain three significant spectral components. Since in 100 mM chloride there cannot be any contribution

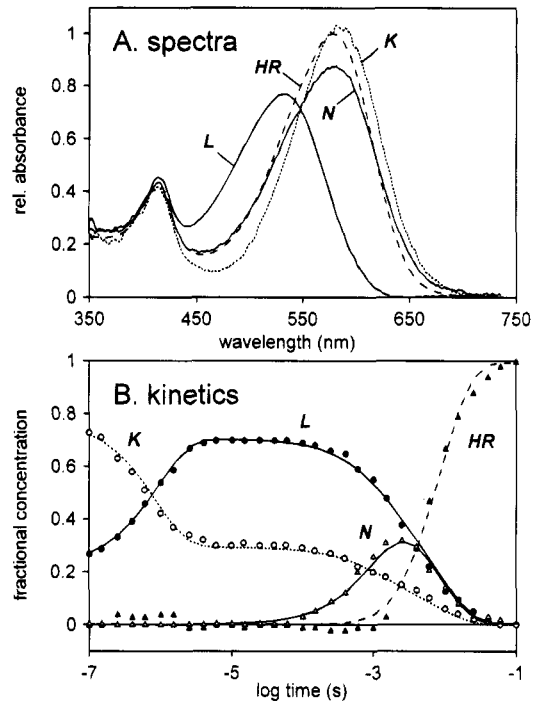

FIGURE 4: Calculated spectra of the photointermediates (A) and their kinetics (B) in 2 M NaCl. The spectrum with dashed line in A is that of the unphotolyzed chromophore. The peak at 420 nm is due to cytochrome contamination of the membranes. The symbols in B are the calculated time-dependent concentrations of K, L, N, and HR. The lines are the best fits of the model, K $\leftrightharpoons$ L $\leftrightharpoons$ N $\rightarrow$ HR (see text), with rate constants in s^{-1}: $k_{KL} = 8.3 \times 10^5$; $k_{LK} = 3.5 \times 10^5$; $k_{LN} = 610$; $k_{NL} = 270$; $k_{NHR} = 280$.

from the photocycle of the chloride-free form, the spectra at 0.1 M and 2 M chloride must both originate from the chloride-binding form. The appearance of the third spectral component at the lower chloride concentration indicates that this photocycle is itself dependent on chloride. While this dependence will be described in detail in the accompanying paper (Váró et al., 1995a), here we attempt to describe the consequences of lowering the chloride concentration on the kinetic model. The spectral changes until about 100 μs are unchanged (compare Figures 3A and B). Thus, we retain the photocycle in 2 M chloride up to this time, *i.e.*, K $\leftrightharpoons$ L $\leftrightharpoons$ N, with the additional intermediate (named O in analogy with the red-shifted state at the end of the bacteriorhodopsin photocycle) placed tentatively between N and HR. This analysis yielded the spectra shown in Figure 5A, and the time-dependence of the concentrations shown in Figure 5B. The spectra of K, L, and N were indeed nearly identical to those at 2 M chloride (compare with Figure 4A). The spectrum for the halorhodopsin O state has about the same relationship (red-shift and amplitude change) to the spectrum of the unphotolyzed state as in bacteriorhodopsin (Lozier & Niederberger, 1977; Váró & Lanyi, 1991b). This is consistent with the fact that, as will be shown below, the retinal in this O state is already reisomerized to all-*trans*, as in bacteriorhodopsin.

Deciding in favor of a suitable kinetic scheme for the N to HR segment of the photocycle was influenced by the decay kinetics of the O state. Although the kinetics from the difference spectra in Figure 5B were too noisy for such analysis, single wavelength measurements at 640 and 590 nm indicated that the decay of O and the recovery of HR at $\leq$0.1 M chloride were clearly biphasic (Váró et al., 1995a).

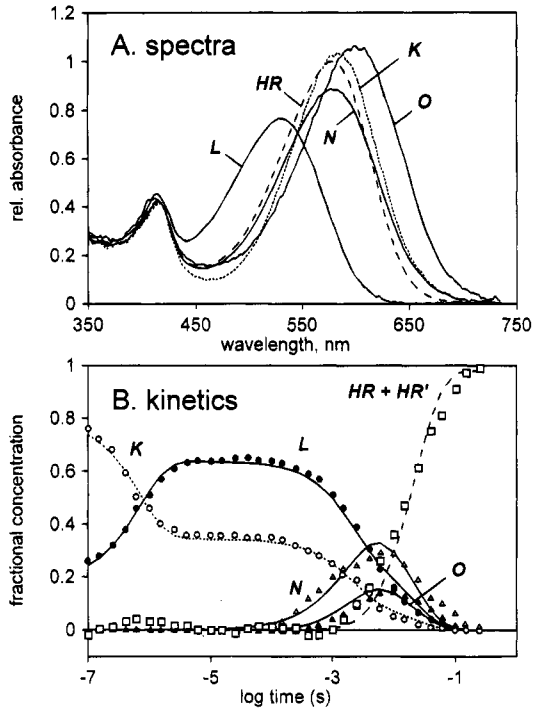

FIGURE 5: Calculated spectra of the photointermediates (A) and their kinetics (B) in 0.1 M NaCl + 0.95 M Na_2SO_4. The spectrum with dashed line in A is that of the unphotolyzed chromophore. The peak at 420 nm is due to cytochrome contamination of the membranes. The symbols in B are the calculated time-dependent concentrations of K, L, N, O, HR′, and HR. The lines are the best fits of the model, K ⇔ L ⇔ N ⇔ O ⇔ HR′ → HR (see text), with rate constants in s^{-1}: $k_{KL} = 6.7 \times 10^5$; $k_{LK} = 5 \times 10^5$; $k_{LN} = 500$; $k_{NL} = 140$; $k_{NO} = 2100$; $k_{ON} = 3300$; $k_{OHR'} = 330$; $k_{HR'O} = 55$; $k_{HRHR'} = 35$.

Various models were tried to account for this. Fitting a scheme where the decay of N to HR is via two pathways, one directly and the other through O, did not yield two exponentials because the best fit eliminated the O → HR pathway and the fit was poor (not shown). On the other hand, the scheme containing K ⇔ L ⇔ N and two O states, each produced from N and each decaying to HR, fit the data well, as did the K ⇔ L ⇔ O ⇔ N → HR and the K ⇔ L ⇔ N ⇔ O ⇔ HR′ → HR sequences (not shown). In the last scheme HR′ is a state whose spectrum is undistinguishable, within error, from that of unphotolyzed HR, and its presence is revealed only by the additional time-constant in the decay of the O intermediate. From a kinetic point of view all of these models were valid, and deciding among them could not be on the basis of the goodness of the fits. However, not all kinetic models are reasonable for a chloride transport system. We considered a model physically valid if the chloride dependence of the measured kinetics originated from two chloride-dependent steps only, one in which chloride is taken up and another which is the reverse of chloride release during the transport cycle. This is the case for the pH dependence of reactions in the bacteriorhodopsin photocycle, where the two proton-dependent steps so identified are associated with the uptake and release of the transported proton (Zimányi et al., 1992b, 1993). When the fits were tested at various chloride concentrations as described in the accompanying paper (Váró et al., 1995a), too many of the calculated rate constants were chloride dependent, and not in any consistent way, in all of these schemes except the last. In that model we could clearly identify a chloride

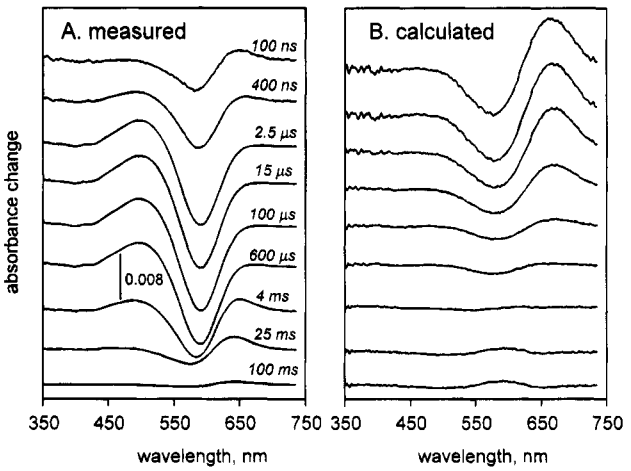

FIGURE 6: Difference spectra after pulse photoexcitation of *N. pharaonis* halorhodopsin in 1 M Na_2SO_4. (A) Measured spectra; (B) calculated spectra for the anion-free form of the protein (see text). The delay times between the laser pulse and the measurements of the spectrum are given in A.

uptake and a release step. We suggest, therefore, that the photocycle contains the K ⇔ L ⇔ N ⇔ O ⇔ HR′ → HR sequence. The lines in Figure 5B show the time courses of K, L, N, and O in this scheme. The fits of the concentrations of N and O to their predicted decay kinetics are consistent with the model although only roughly and with a great deal of noise, because, unlike in Figure 4B, both N and O are present and under these conditions their spectra and therefore their amplitudes are not well defined.

Reconstruction of the Spectra of the Intermediates and Their Kinetics in the Absence of Chloride. Figure 6A shows difference spectra measured in 1 M Na_2SO_4, *i.e.*, in the absence of chloride. In view of the small but measurable transport activity under these conditions, and detailed examination of the difference spectra at chloride concentrations down to zero (Váró et al., 1995a), we consider these spectra as the sums of the photocycles of the form without bound anion and the form that contains sulfate bound in the same manner as chloride. We extrapolated the rates of the chloride-dependent steps (Váró et al., 1995a) in the cycle to zero chloride, and after some trial and error subtracted 14% of the photocycle of the chloride-binding form. Figure 6B shows the spectra remaining after the subtraction. They resemble remarkably the measured spectra for *H. salinarium* halorhodopsin in 1 M Na_2SO_4, where no such subtraction was needed (compare with Figure 1C in Váró et al., 1995b).

The spectra after such subtraction should contain, to the extent that the weights used in the subtraction are correct, the spectral changes due to the photocycle of the anion-free form only. These spectra could be described with a single spectral component (Figure 7A) that decays with more than one time constant, up to three or four (Figure 7B). Given the numerous assumptions used in arriving at the points plotted, a precise fitting seems pointless. A two-component fit (line in Figure 7B) would give 17 μs and 1.6 ms. This would be similar to the photocycle of the chloride-free form of *H. salinarium* halorhodopsin, where a single spectral component much like in Figure 7A sufficed (compare with Figure 6A in Váró et al., 1995b) and the kinetics were best described by two decay time-constants of about 300 μs and 3.8 ms.

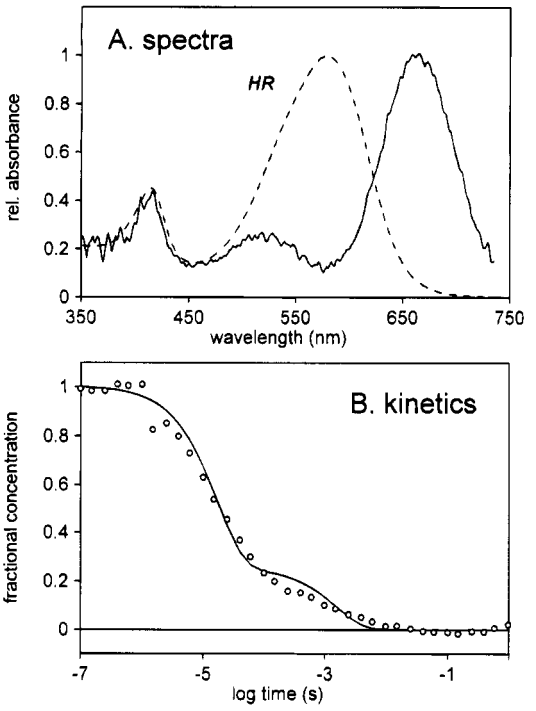

FIGURE 7: Calculated spectra of the single detected photointermediate (A) and its kinetics (B) of the anion-free form of *N. pharaonis* halorhodopsin, measured in 1 M Na_2SO_4. The contribution of the photocycle that occurs under these conditions, and resembles that of the chloride-binding form, was subtracted from the measured spectra as in Figure 6. The spectrum with dashed line in A is that of the unphotolyzed chromophore. The peak at 420 nm is due to cytochrome contamination of the membranes; the small peak near 500 nm is due to subtraction error. The symbols in B are the calculated time-dependent concentrations of the red-shifted photoproduct. The lines are the best fits of biexponential decay, with time-constants of 1.6 ms and 17.8 ms.

Infrared Spectra of the N and O Intermediates. To record the spectrum of the O intermediate by static FTIR spectroscopy, conditions under which the O state was stable were surveyed. However, it could not be stabilized even below 260 K. We measured its spectrum therefore in the photostationary state at 273 K. The difference spectra between illuminated and unilluminated halorhodopsin containing (a) 1 M, (b) 100 mM, and (c) 10 mM NaCl are shown in Figure 8. In 1 M NaCl the spectrum is attributable entirely to the L and/or N state *vs.* unphotolyzed halorhodopsin (cf. Figure 4B). We note that the frequencies of the C=C stretch modes are inversely proportional to the absorption maxima in the visible (Aton et al., 1977), and in bacteriorhodopsin this band is at 1530−1560 cm^{-1} for L and N (Smith et al., 1984; Diller et al., 1987; Fodor et al., 1988), and at 1509 cm^{-1} for O (Smith et al., 1983). In view of the presence of the 1552 cm^{-1} band in (a) of Figure 8 that thus corresponds to the C=C stretching mode of the chromophore in the L or N states, and the absence of the 1513 cm^{-1} band that would correspond to the O state, no accumulation of O is evident in this spectrum. When NaCl concentration is decreased to 10 mM, the spectrum (c) of the photostationary state contains mainly O by this criterion. Contamination with L or N is negligible because of the absence of 1552 cm^{-1} band. Therefore, we can attribute (a) in Figure 8 to an almost pure spectrum of the L or N state, and (c) to a nearly pure spectrum of O.

In the fingerprint region, the L *vs.* BR spectrum shows intense bands characteristic of 13-*cis* chromophore at 1192

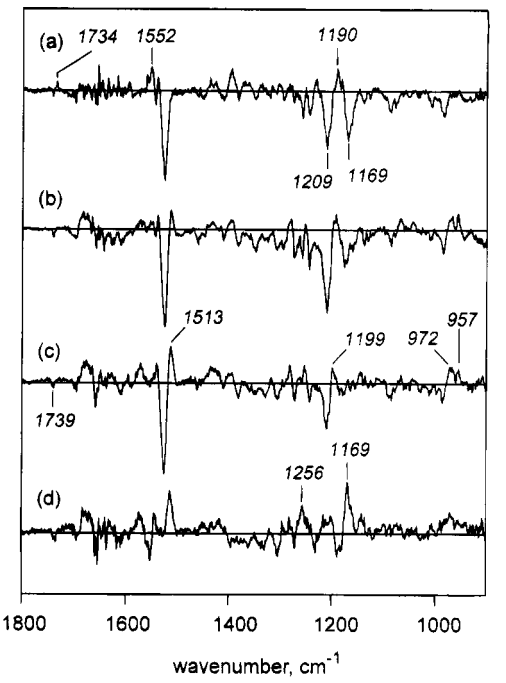

FIGURE 8: FTIR spectra of the photoproducts of *N. pharaonis* halorhodopsin at different chloride concentrations. (a) 1 M NaCl, (b) 100 mM NaCl, (c) 10 mM NaCl, (d) difference between (c) and (a). The full-scale absorbance for each trace is 0.001.

cm^{-1} on the positive side and of the depleted all-*trans* chromophore at 1202 and 1169 cm^{-1} on the negative side (Gerwert & Siebert, 1986). Based on the analogy of L and N states of halorhodopsin to that of the L state of bacteriorhodopsin, L and/or N in (a) of Figure 8 must have a chromophore in 13-*cis* configuration while HR has an all-*trans* chromophore. In contrast, the O *vs.* HR spectrum in (c) of Figure 8 shows much reduced negative band intensity at 1169 cm^{-1}, because of cancellation of the band by HR, demonstrating that O has an all-*trans* chromophore. This conclusion is further confirmed by the difference between the spectrum of L or N and that of O, shown in (d) of Figure 8, that contains an intense 1169 cm^{-1} band, and a positive band at 1256 cm^{-1}. The other characteristic band at 1209 cm^{-1} in HR must have been shifted to 1199 cm^{-1} in O, hence this band does not show complete cancellation in the O *vs.* HR spectrum. This was the case for the O *vs.* BR spectrum in Y185F (Bousché et al., 1992; Hessling et al., 1993). In these respects, the infrared spectrum of the O state of halorhodopsin is very similar to that of the O state of bacteriorhodopsin.

The O *vs.* HR spectrum shows positive bands at 972 and 957 cm^{-1}. They are hydrogen-out-of-plane (HOOP) bands which can be enhanced when the chromophore is distorted. It appears therefore that O has an all-*trans* chromophore with a distorted conformation, like the O of bacteriorhodopsin (Smith et al., 1983; Bousché et al., 1992). In the region of the carboxylic C=O stretch vibrations, the spectrum of L or N shows a positive and a negative band at 1734 and 1739 cm^{-1}, respectively. In bacteriorhodopsin the 1739 cm^{-1} band was identified as the C=O stretch mode of the protonated D115 (Sasaki et al., 1994). This feature therefore indicates the perturbation of an aspartate, probably the protonated D156 which is the equivalent of D115 in bacteriorhodopsin. These bands were absent in the L *vs.* HR spectrum obtained at 170 K (our unpublished data), supporting the likelihood

that the spectrum in (a) of Figure 8 is not of the L but the N state, consistent with the kinetics (Figure 4B). However, the amide bands of the N state of bacteriorhodopsin (Rothschild et al., 1981; Marrero & Rothschild, 1987; Ormos, 1991; Braiman et al., 1991; Sasaki et al., 1992; Perkins et al., 1992) are not in this spectrum. A feature of interest in O is the negative band at 1734 cm^{-1}, indicating the possible deprotonation of D156.

DISCUSSION

With this report, there are now three different proposed photocycles for halorhodopsin containing all-*trans* retinal, in the presence of chloride. Much early work with halorhodopsin from *H. salinarium* had suggested the scheme

$$HR \xrightarrow{h\nu} K \rightarrow L \Leftrightarrow O \rightarrow HR' \rightarrow HR$$

where K, L and O were named in analogy with the more thoroughly described intermediates of the bacteriorhodopsin photocycle. The use of these designations was justified by the similarity of the vibrational difference spectra for K (Rothschild et al., 1988) and L (Fodor et al., 1987; Rothschild et al., 1988) to their equivalents in bacteriorhodopsin. However, the retinal was 13-*cis* in the presumed O state (Lanyi, 1984; Ames et al., 1992), unlike in bacteriorhodopsin where in the equivalent red-shifted intermediate it is already reisomerized to all-*trans* (Smith et al., 1983; Bousché et al., 1992; Souvignier & Gerwert, 1992). The reason is that this red-shifted photoproduct is not part of the all-*trans* photocycle (Váró et al., 1995b). A chloride dependence in what appeared as the O → L back-reaction implied release of chloride in the L → O reaction, presumably on the cytoplasmic side, and the O → HR′ step was thought to represent binding of chloride, presumably on the extracellular side, inferred from a chloride dependent shift of the absorption maximum of the unphotolyzed state. According to this scheme, therefore, once the L state is reached, the chloride bound on the extracellular side will have been translocated inside the protein to the cytoplasmic side, and this chloride is released as the O state is formed (Oesterhelt et al., 1986). The initial state is restored by chloride uptake at the extracellular surface at the last step, that completes the net transport.

We recently reported (Váró et al., 1995b) that the spectral features attributed to the intermediate referred to as the O state in the *H. salinarium* protein originate mostly or entirely from the photoreactions of the 13-cis state and the chloride-free form. When the contribution of these reactions to the spectral changes were subtracted, the photocycle scheme for the all-*trans* chromophore had to be revised. The photocycle of the chloride-binding form was described instead as

$$HR \xrightarrow{h\nu} K \Leftrightarrow L_1 \Leftrightarrow L_2 \Leftrightarrow N \rightarrow HR$$

where L_1 and L_2 had the same spectra but were distinguished on kinetic grounds. This scheme contained no chloride-dependent steps that would reveal where chloride is taken up and released. Rather, it resembled the photocycles of bacteriorhodopsin at low pH (Mowery et al., 1979; Váró & Lanyi, 1989) where D85 is permanently protonated, and the D85N (Otto et al., 1990; Thorgeirsson et al., 1991; Kataoka et al., 1994) and D212N (Needleman et al., 1991; Cao et al., 1993) mutants, where the Schiff base cannot deprotonate.

Finally, for halorhodopsin from *N. pharaonis*, we describe the photocycle here as

$$HR \xrightarrow{h\nu} K \Leftrightarrow L \Leftrightarrow N \Leftrightarrow O \Leftrightarrow HR' \rightarrow HR$$

While this sequence appears to resemble the earlier suggested scheme for *H. salinarium* halorhodopsin, there are important differences. First, the O state is confirmed here to be in the sequence, and its FTIR spectrum (Figure 8) indicates that in this state the retinal is already reisomerized to all-*trans*, while in the earlier proposed red-shifted state(s) the retinal was 13-*cis* (Lanyi, 1984; Ames et al., 1992). This finding confirms the validity of using the bacteriorhodopsin nomenclature. Second, the amplitude of the absorbance changes in the red, due to the O state of *N. pharaonis* halorhodopsin, *increases* as the chloride concentration is increased from zero to 0.1 M, rather than *decreases* as in the case of the other protein. In the following paper (Váró et al., 1995a) we show that the chloride-dependent steps in the simplest model we adopted for the kinetics are the O → N back-reaction (which implies chloride release in the N → O reaction) and the O → HR′ forward reaction (which implies chloride uptake in this step). Thus, the chloride translocation mechanism in this cycle is somewhat different from the one suggested by the earlier photocycle.

Is the more recent scheme for *H. salinarium* halorhodopsin, where an O state was not observed (Váró et al., 1995b), compatible with this reaction sequence? There are examples in the bacteriorhodopsin literature where conditions can be created in which the spectral changes due to an intermediate become progressively smaller and smaller until they are not observed in the photocycle (*e.g.*, the M state in Brown et al., 1994). This is the case even for *N. pharaonis* halorhodopsin. The amplitude of the absorbance changes due to the O state disappear, for kinetic reasons as the chloride concentration is increased to 2 M (Figure 3). They become very small also when 0.1 M NaCl is replaced with 0.1 M NaBr (not shown), and they are not observed after solubilization with Lubrol (Duschl et al., 1990). We assume that under these conditions the kinetics are poised against the accumulation of the O intermediate. Likewise, O might be produced but does not accumulate to a measurable extent in the photocycle of the *H. salinarium* protein.

We note that the photocycles of the chloride-free forms of the two halorhodopsins are very similar to each other, and resemble somewhat also the photocycle of 13-*cis* bacteriorhodopsin (Kalisky et al., 1977; Sperling et al., 1977; Hofrichter et al., 1989; Gergely et al., 1994). In the time-range studied, there is a red-shifted intermediate that decays in somewhat less than 1 ms, and with two apparent time-constants (compare Figure 7B with Figure 6B in Váró et al., 1995b).

It appears from these results that halorhodopsin from *N. pharaonis* is a more tractable system for studying the mechanism of chloride translocation than the protein from the other species. Importantly, its photocycle is very similar to that of the D85T mutant of bacteriorhodopsin which had changed from a proton pump to a chloride ion pump (Sasaki et al., 1995). This similarity will afford the means to assess the roles the different residues play in the cation and anion translocation processes in the two proteins.

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