Properties and Photochemistry of a Halorhodopsin from the Haloalkalophile, *Natronobacterium pharaonis*

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Pharaonis halorhodopsin is a light-driven transport system for chloride, similarly to the previously described halorhodopsin, but we find that it transports nitrate as effectively as chloride. We studied the photoreactions of the purified, detergent-solubilized pharaonis pigment with a gated multichannel analyzer. At a physiological salt concentration (4 M NaCl), the absorption spectra and rate constants of rise and decay for intermediates of the photocycle were similar to those for halorhodopsin. In buffer containing nitrate, halorhodopsin exhibits a second, truncated photocycle; this difference in the photoreaction of the pigment occurs when an anion is bound in such a way as to preclude transport. As expected from the lack of anion specificity in the transport, the photocycle of pharaonis halorhodopsin was nearly unaffected by replacement of chloride with nitrate. All presumed buried positively charged residues, which might play a role in anion binding, are conserved in the two pigments. At the extracellular end of the presumed helix C, however, an arginine residue is found in halorhodopsin, but not in pharaonis halorhodopsin, and an arginine-rich segment between the presumed helices A and B in halorhodopsin is replaced by a less positively charged sequence in pharaonis halorhodopsin (Lanyi, J. K., Duschl, A., Hatfield, G. W., May, K., and Oesterhelt, D. (1990) *J. Biol. Chem.* 265, 1253–1260). One or both of these alterations may explain the difference in the anion selectivity of the two proteins.

Bacterial rhodopsins are widespread in halophilic archaeobacteria. The first proteins of this type, the proton pump bacteriorhodopsin (Stoeckenius et al., 1978; Stoeckenius and Bogomolni, 1982; Lanyi, 1984), and the chloride pump halorhodopsin (Lanyi, 1986; Hegemann et al., 1987; Oesterhelt and Tittor, 1989), as well as two rhodopsins with sensory functions (Spudich and Bogomolni, 1988), were discovered in the neutrophilic *Halobacterium halobium* (Lanyi, 1984). Consistent with this, we found that the primary structure of the halorhodopsin-like protein in the membranes of *Natronobacterium pharaonis*, termed pharaonis halorhodopsin, showed only 65% identity with halorhodopsin, and the divergence at the DNA level was even greater (Lanyi et al., 1990).

In the present study, we have investigated the light-dependent chloride pump of *N. pharaonis*, the only protein of this type besides halorhodopsin so far discovered. As for halorhodopsin, regulation of the internal salt concentration and possibly energy supply in times of oxygen starvation are the likely functions of this pigment. A comparison of pharaonis halorhodopsin with halorhodopsin should provide information about common and divergent structural and chromophoric features which play roles in two different light-driven chloride transport systems, as well as on evolution and mechanism of bacterial rhodopsins in general.

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The appearance of two parallel photocycles in nitrate has clearly to do with the way this anion is bound, and we have suggested ways to account for this and other observations (Lanyi, 1990). Thus, halorhodopsin will discriminate between chloride and nitrate, although not as much as expected from previous reports (Schobert and Lanyi, 1982). In keeping with the fact that N. pharaonis contains much less pigment than H. halobium (cf. below), its light-dependent transport activity was considerably lower.

Throughout the purification procedure, the pharaonis halorhodopsin was assayed by flash spectroscopy with a single wavelength instrument for measuring transient absorption changes, described by others (Kamo et al., 1979). Right-side-out envelope vesicles were produced (Lanyi and MacDonald, 1979) from H. halobium strains OD2W and L-33. These were exhaustively dialyzed against unbuffered 1.5 M Na2SO4, in the dark at room temperature, followed by the transport assays without and with added chloride or nitrate. Transport was followed by measuring the initial rate of pH changes upon illumination with yellow light, in the presence of uncoupler, as described before (Schobert and Lanyi, 1982). When the anion dependence of the transport was tested, the KCl inside the combination pH electrode (Ross, model 81-03, Orion Corp., Boston, MA) was replaced with 2.5 M Li2SO4 so as to prevent chloride contamination of the envelope suspensions from leakage through the electrode junction. Under buffering conditions transport was determined with a TPP+ electrode, as described by others (Kamo et al., 1979).

**RESULTS**

**Transport Properties of Pharaonis Halorhodopsin**—Light-driven transport was determined by following passive proton uptake, induced by the electrogenic transport of chloride (Schobert and Lanyi, 1982). Right-side-out envelope vesicles from both N. pharaonis SP1 and H. halobium OD2W showed broad pH optima for the initial rate of H+ import between pH 5.5 and 9 upon illumination (Fig. 1), in presence of uncoupler. This confirms an earlier report on the relative pH independence of chloride transport by halorhodopsin (Lanyi and Schobert, 1983). In keeping with the fact that N. pharaonis contains much less pigment than H. halobium (cf. below), its light-dependent transport activity was considerably lower. Fig. 1 shows that transport activity declined at about the same high pH (>9) in the two systems. The decline of transport activity at high pH is not due to insufficient uncoupler activity, because stepwise additions of uncoupler, up to 10

![FIG. 1. pH dependence of chloride transport by envelope vesicles from H. halobium (containing halorhodopsin) and N. pharaonis (containing pharaonis halorhodopsin). The rate of light-driven chloride uptake was determined by following proton influx in 4 M NaCl, in the presence of 2 μM SF 8447, as described under "Materials and Methods." Protein concentration was 2 mg/mL.](http://www.jbc.org/)

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1 The abbreviations used are: FPLC, fast protein liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; TPP+, tetraphenyl phosphonium cation; SDS, sodium dodecyl sulfate; pharaonis photointermediates are designated as PH2 with subscripts according to their analogy to the halorhodopsin intermediates.

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**Photochemistry of Pharaonis Halorhodopsin**

Zimányi and Lanyi, 1989b). The procedure of two parallel photocycles in nitrate has clearly to do with the way this anion is bound, and we have suggested ways to account for this and other observations (Lanyi, 1990). Thus, halorhodopsin will discriminate between chloride and nitrate, although not as much as expected from previous reports (Schobert and Lanyi, 1982). Right-side-out envelope vesicles were produced (Lanyi and MacDonald, 1979) from H. pharaonis strain SP1 (from W. D. Grant, University of Leicester, United Kingdom), and H. halobium strain OD2W and L-33 were grown in a complex medium and harvested as reported previously (Lanyi and MacDonald, 1979). The growth medium for N. pharaonis is described elsewhere (Lanyi et al., 1990). Yeast extract for this medium was from Difco, and casamino acids were from Sigma.

Pharaonis halorhodopsin was purified by the procedure described below.

**TWEEN WASH**—Membranes from 80 liters of cell culture, obtained as described previously for H. halobium (Duschl et al., 1988) and suspended in about 300 ml of 4 M NaCl solution, were mixed with an equal volume of 0.1 M NaCl, 25 mM Tris·HCl, pH 8, 6% (w/v) Tween 20 (Sigma) and stirred for 30 min at 4 °C. The mixture was centrifuged for 1 h at 140,000 g at 4°C, the supernatant discarded, and the pellets washed with 400 ml of 4 M NaCl, 25 mM Tris, pH 8.0. Finally the membranes were homogenized in an equal volume of the same buffer.

**Cholate Solubilization**—The Tween-washed membranes were mixed with sodium cholate (Serva Fine Biochemicals) from a 20% (w/v) solution, to bring the cholate concentration to 3% (w/v). After stirring for 90 min at room temperature in the dark, the solution was centrifuged as above.

**Phenyl-Sepharose Chromatography**—The supernatant was loaded on a phenyl-Sepharose (Pharmacia LKB Biotechnology Inc.) column, 15 × 2.5 cm, previously equilibrated with 0.5% cholate, 4 M NaCl, 5% Tris, pH 8.0 (buffer A). The column was then washed with 2 liters of buffer A and eluted with 0.5% Lubrol PX (Sigma), 4 M NaCl, 25 mM Tris, pH 8.0 (buffer B). Fractions containing pharaonis halorhodopsin were combined for the next step.

**Hydroxylapatite Chromatography**—An HR 16/10 FPLC column (Pharmacia LKB Fine Biochemicals) was packed with hydroxylapatite (Bio-Gel HT; Bio-Rad), suspended in 0.5% Lubrol PX, 4 M NaCl, 25 mM Tris, pH 7.2, 1 mM NaH2PO4 (buffer C). The column was equilibrated with 100 ml of buffer B and the protein was loaded. After washing with 300 ml each of buffer B and buffer C, respectively, the pharaonis halorhodopsin was eluted with 200 ml of 0.5% Lubrol PX, 4 M NaCl, 25 mM Tris·HCl, pH 8.0, 5 mM NaH2PO4 (buffer D). The column was run at 150 ml/h, with the aid of a Pharmacia model P-500 pump. For the preparation of buffer D, porcine-free Lubrol PX (Surfact-Amps PX; Pierce Chemical Co.) was used. Occasionally, after elution with buffer D all visible material was eluted from the column with buffer E, which contained 0.5% NaH2PO4. Neither the material obtained by this final elution, nor that eluted during the wash procedure, contained any detectable amount of pharaonis halorhodopsin. Purple colored fractions were combined and the buffer was replaced, in an Amicon-concentrator or by dialysis overnight, with buffer B. The final purification was by a second hydroxylapatite chromatography, performed as the first one.

Through the purification procedure, the pharaonis halorhodopsin was assayed by flash spectroscopy with a single wavelength instrument for measuring transient absorption changes, described before (Duschl et al., 1988). In these assays absorption changes at 570 or 633 nm were followed, within 10 ms after a laser flash at a wavelength of 579 nm. For each measurement 512 flashes were averaged. Absorption spectra were recorded on a Shimadzu UV-250 spectrophotometer connected to an IBM-XT computer.

Transients spectra in the photocycle of pharaonis halorhodopsin were obtained with a Princeton Instruments (Princeton, NJ) optical spectrophotometric multichannel analyzer (OSMA). The instrument and criteria for the analysis of the data are described elsewhere (Zimányi et al., 1989, 1994).

Right-side-out envelope vesicles were produced (Lanyi and MacDonald, 1979) from N. pharaonis, and H. halobium strains OD2W and L-33. These were exhaustively dialyzed against unbuffered 1.5 M Na2SO4, in the dark at room temperature, followed by the transport assays without and with added chloride or nitrate. Transport was followed by measuring the initial rate of pH changes upon illumination with yellow light, in the presence of uncoupler, as described before (Schobert and Lanyi, 1982). When the anion dependence of the transport was tested, the KCl inside the combination pH electrode (Ross, model 81-03, Orion Corp., Boston, MA) was replaced with 2.5 M Li2SO4 so as to prevent chloride contamination of the envelope suspensions from leakage through the electrode junction. Under buffering conditions transport was determined with a TPP+ electrode, as described by others (Kamo et al., 1979).
times that used otherwise, yielded no greater transport. The same conclusion could be reached from data without uncoupler (not shown). The result is surprising because *N. pharaonis* had been isolated from a highly alkaline environment, and the optimal pH for its growth is well above that of *H. halobium* (Tindall et al., 1980; Soliman and Truper, 1982). It seems likely that the decline of transport at high pH was caused by the light-dependent protonation of the Schiff base of the two halorhodopsins, which is known to occur under these conditions for *H. halobium* halorhodopsin at least (Lanyi and Schobert, 1983; Hegemann et al., 1985), and generates a semi-stable and inactive blue-shifted pigment.

**Anion Dependence of Electrogenic Transport by Envelope Vesicles**—It was reported previously that in envelope vesicles from *H. halobium* strain L-33 the passive proton movements, which occurred in response to the electrical potential caused by chloride transport, were not seen when chloride was replaced with nitrate (Schobert and Lanyi, 1982; Hazemoto et al., 1984). Upon reexamining this question, we find that this is not so. Fig. 2 (upper panel) shows the initial rate of proton influx during the illumination of *H. halobium* OD2W envelope vesicles, as a function of added chloride and nitrate concentration. Although half-maximal transport was observed at about 40 mM chloride, as before (Schobert and Lanyi, 1982; Hazemoto et al., 1984), nitrate elicited about 30% of the proton influx observed with chloride. Half-maximal transport with nitrate was at about 100 mM. A number of control experiments (repeated illuminations, addition of sulfate instead of chloride or nitrate, etc.) indicated that the transport in the presence of nitrate was not an artifact. Addition of nitrate (0–200 mM) to an assay mixture containing 50 mM chloride caused partial inhibition of the transport (not shown). Envelope vesicles from *H. halobium* L-33, the strain used in the earlier reports, gave essentially the same results as strain OD2W (not shown). Fig. 2 (lower panel) shows proton influx rates of *N. pharaonis* envelope vesicles. In contrast to the *H. halobium* envelopes, which show a 3-fold specificity in favor of chloride, *N. pharaonis* vesicles did not greatly discriminate between these anions. Half-maximal transport in this system was at 25 mM chloride and at 15 mM nitrate, and the maximal rates with the two anions were comparable. With these vesicles a very small extent of alkalinization (2 nmol of H+/min) was observed even without chloride or nitrate (not shown), which suggests that sulfate, present in the assays at 1.5 M concentration, may be transported also.

Since the natural habitat of *N. pharaonis* contains <0.3 mM nitrate, but high concentrations of HCO₃⁻ and CO₃²⁻ (Imhoff et al., 1979; Tindall et al., 1980), we examined the possibility that the latter anions might be also transported by pharaonis halorhodopsin. In these assays, otherwise carried out as above, the transport was followed with a TPP⁺ electrode which detects a negative inside electrical potential by following the redistribution of the membrane-permeant cation TPP⁺, upon illumination (Kamo et al., 1979). In the presence of chloride, envelope vesicles from both *H. halobium* OD2W and *N. pharaonis* showed large light-dependent uptake of TPP⁺. Bicarbonate (100 mM) or carbonate (100 mM), at pH 8.2 and 9.8, respectively, elicited no detectable light-dependent 1¹H⁺⁺ uptake, however, in *N. pharaonis* vesicles (the pHₐ of HCO₃⁻/ CO₃²⁻ is 9.6 under these conditions). Thus, the physiological substrate of the pigment is chloride.

**Purification of Pharaonis Halorhodopsin**—Because pharaonis halorhodopsin is less tolerant of detergents than halorhodopsin, and the pharaonis halorhodopsin content of SP1 cells is only 10% or less of the halorhodopsin content of *H. halobium* OD2W, purification of the pharaonis pigment was more difficult. Thus, the procedure we recently developed for halorhodopsin isolation (Duschl et al., 1988) had to be modified. As expected for an integral membrane protein, and similarly to halorhodopsin, pharaonis halorhodopsin was not solubilized by Tween 20. Tween-washed membrane from *N. pharaonis* was therefore a good starting material for the purification; it was also better suited for our initial flash spectroscopy in identifying the presence of pigment than untreated membranes, where the high carotenoid content made such measurements difficult.

Pharaonis halorhodopsin, solubilized in cholate, was bound to phenyl-Sepharose under similar conditions as halorhodopsin (Duschl et al., 1988). However, unlike halorhodopsin, it could not be successfully eluted by octyl glucoside, since after application of a 0.5% octyl glucoside buffer an instant change of color from purple to yellow indicated destruction of the pigment. Lubrol PX-containing buffer eluted the pigment from the column without bleaching. Binding of pharaonis halorhodopsin to hydroxyapatite could be achieved either by including 1 mM Mg²⁺ in the buffer, or by lowering the pH from 8.0 to 7.2. The pigment was eluted by phosphate-containing buffer (5 mM), as expected for an acidic protein. Indeed, according to the gene sequence (Lanyi et al., 1990), in pharaonis halorhodopsin the ratio of acidic to basic amino acid residues is 19:10.

Fig. 3A shows the absorption spectrum of purified, Lubrol-solubilized pharaonis halorhodopsin. The absorption maximum in the visible region is 577 nm, or about 3 nm red shifted from that for halorhodopsin in the presence of Lubrol.
This process is the thermal conversion of HRKL into HRi, which results in a major band, which corresponds to monomeric pharaonis halorhodopsin, running at an apparent molecular mass of 25.2 kDa, a minor band at 50 kDa is a dimer of this protein, since it is somewhat behind the position of halorhodopsin. The electrophoresis was in 15% acrylamide gel, which was silver-stained.

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(e.g. Schobert et al., 1986). The 280/570 nm absorbance ratio is 1.4, indicating a high degree of purity by the criterion often used for bacterial rhodopsins. Densitometric tracing of a silver-stained SDS-acrylamide gel is given in Fig. 3B. The major band, which corresponds to monomeric pharaonis halorhodopsin, ran at an apparent molecular mass of 25.2 kDa, i.e. somewhat behind the position of halorhodopsin. The minor band at 50 kDa is a dimer of this protein, since immunoblots indicated that antibodies raised against a synthetic peptide derived from the COOH-terminal sequence of pharaonis halorhodopsin reacted with this band also (Lanyi et al., 1989). The electrophoresis was in 15% acrylamide gel, which was silver-stained.

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Because the spectral transition if Fig. 4A is already underway between 0.55 and 1.74 μs, estimation of the kinetics of the transition required extrapolation to zero time after the flash. This was done with an equation based on first-order reaction kinetics, as described earlier (Zimányi et al., 1989):

\[
\frac{1}{C} = \frac{\exp(-kt_1) - \exp(-kt_2)}{\exp(-kt_3) - \exp(-kt_4)}
\]

where \(C\) is the scaling factor used to bring the difference spectra into superimposition, \(t_1\) and \(t_2\) are the times at which the first and last spectra were taken, and \(k\) is the rate constant of the transition. Fig. 4B shows \(1/C\) for the experimental points, and lines connecting calculated points from Equation 1 for a \(t_0\) of 1.5 μs. Thus, the half-time of the PHRKL → PHRL transition is 1.5 μs. The HRKL → HR^t transition under the same conditions has a half-time of 0.87 μs (Zimányi and Lanyi, 1989b).

From the above calculation we produced a spectrum extrapolated to zero time, analogously to the treatment of similar data for halorhodopsin (Zimányi et al., 1989), which agreed well with the experimentally obtained 60 ns difference spectrum (not shown). Thus, the first spectral information we could obtain in these experiments relates to the PHRKL species, and any contribution by an earlier intermediate (which might be designated as PHR^t) to the spectra was negligible.

No further observable spectroscopic changes occurred until several hundred μs after the flash. In the millisecond time range a difference spectrum, indicative of a blue-shift and amplitude decrease, of rather small magnitudes, developed (Fig. 5). This feature was not seen in halorhodopsin (Zimányi et al., 1989). At still later times, between 4.34 and 60 ms, recovery of the parent pigment was observed (Fig. 6A). The good isosbestic point in this time domain, (through which a 1.35-ms spectrum, also included, does not pass), argues that once the spectral change in Fig. 5 is completed the recovery of the pigment is by a simple process. The latter is represented by a single exponential (Fig. 6B), which has a half-time of 42 ms. The recovery of halorhodopsin under the same conditions has a half-time of 95 ms.

Absolute spectra of the pharaonis halorhodopsin intermediates were calculated from the difference spectra according to criteria described before (Zimányi et al., 1989). As shown in Fig. 7, these are analogous to the halorhodopsin intermediates and consist of PHR^tL (from a 60-ns spectrum), PHRL (from a 54-μs spectrum), and PHR^t (from a 4.34-μs spectrum). The absorption maxima of these species are at 578, 525, and 519 nm, respectively. No red-shifted intermediate
the good agreement of the spectra of PHRKL and PHRL with buffer the spectra are somewhat different from those in chlor-
as A, conditions as in Fig. 4. A, flash-induced difference spectra, at the aonis halorhodopsin photocycle between 1.35 and 60 ms.

B, semilogarithmic plot of the total photocycling fraction in the time range of A, which indicates the \( t_1/2 \) for the recovery of the parent pigment.

(i.e. PHR\(_0\)) is seen, similarly to the case of halorhodopsin, where this kind of species also does not accumulate appreciably at very high chloride concentrations (Zimányi and Lanyi, 1989b).

Comparison of the Pharaonis Halorhodopsin Photocycles in Chloride- and Nitrate-containing Buffers—Halorhodopsin exhibits different photocycles in the presence of chloride and nitrate (Lanyi and Vodyanoy, 1986; Titter et al., 1987; Zimányi and Lanyi, 1989b). Flash-induced difference spectra for pharaonis halorhodopsin were measured, at increasing delay times after the flash, in buffer containing 0.4 M Na\(_2\)SO\(_4\) plus either 100 mM NaCl or 100 mM NaNO\(_3\). Fig. 8 contains data in the sub-microsecond time scale. In the presence of chloride the difference spectra greatly resemble those reported, in similar experiments, for halorhodopsin (Zimányi and Lanyi, 1989a, 1989b, 1989c), as regards the positions of the maxima and the isosbestic point. This is as expected from the good agreement of the spectra of PHRKL and PHR\(_1\) with the analogous halorhodopsin intermediates (Fig. 7). In nitrate buffer the spectra are somewhat different from those in chloride: in the shorter time-delay spectra a positive absorption change in the red region suggests that under these conditions the first intermediate is red-shifted from PHRKL. Such a difference between chloride and nitrate was seen also for halorhodopsin, but the intensity of the additional band was much larger (Zimányi et al., 1989; Ziminyi and Lanyi, 1989b).

At longer delay times the difference spectra in chloride and nitrate buffers were more similar to one another (Fig. 9). The striking difference from halorhodopsin is that neither set of spectra contain any indication of an HR\(_0\)-like, red-shifted intermediate, which in the case of the other pigment dominates the spectra in nitrate (Zimányi and Lanyi, 1989b). The recovery of the parent pharaonis halorhodopsin occurred with a \( t_1/2 \) of 9.2 ms in chloride buffer, and 4.1 ms in nitrate buffer. Bivin and Stoeckenius (1986) reported \( t_1/2 \) of 2 ms in the presence of chloride, and 110 ms in the absence of both chloride or nitrate, for this pigment in the membranes of N. pharaonis. The discrepancy with chloride may be that our data are for the detergent-solubilized pigment, and at 3 °C rather than at room temperature; we have no results in the absence of both chloride and nitrate, as used by Bivin and Stoeckenius (1986). For halorhodopsin the corresponding half-times in 100 mM chloride and 100 mM nitrate are 30 and 4.9 ms.

If an appropriately scaled spectrum of pharaonis halorhodopsin is added to the difference spectra in Figs. 8 and 9, absolute spectra for the mixture of intermediates at the delay times used will be obtained. Fig. 10 shows such spectra at selected delay times in chloride and nitrate buffers. The absorption spectra for the parent species, also included in Fig. 10, have maxima at 577 and 571 nm, respectively. As discussed
Fig. 10. Spectra for the intermediates produced in the pharaonis photocycle at 60 ns, 15 μs, and 3.8 ms. These delay times were selected so as to show individual species, according to our analysis, rather than mixtures. The spectra in chloride (upper panel) and in nitrate (lower panel) containing buffers were calculated from difference spectra, such as shown in Figs. 8 and 9. Pharaonis halorhodopsin (-), absorption maximum at 577 nm in chloride and 571 nm in nitrate; 60 ns (---), absorption maximum 578 nm in chloride and 580 nm in nitrate; 15 μs (---), absorption maximum 525 nm in chloride and 529 nm in nitrate; 3.8 ms (-----), 521 nm in chloride and 528 nm in nitrate.

above, at 60 ns the spectrum is attributable to the first observable (i.e. PHRkl) intermediate. In chloride buffer this is virtually identical to HRKl (Zimányi et al., 1989), but Fig. 10 (lower panel) and Fig. 8B both suggest that in nitrate buffer it is somewhat red-shifted. At 15 μs the spectrum is attributable to the second (i.e. PHRl) intermediate. In chloride buffer its absorption maximum is at 526 nm, as in 4 M NaCl (Fig. 6), but in nitrate buffer it is at 529 nm. In chloride-containing buffer the 3.8 ms spectrum reveals a third intermediate (i.e. PHRl), seen also in 4 M NaCl (Fig. 7), with a blue-shifted absorption maximum at 521 nm. An analogous second L-like intermediate, with lower amplitude but no blue-shift, seems to exist in the presence of nitrate as well (Fig. 10, lower panel). Most importantly, at no time is there an O-like intermediate, which in the case of halorhodopsin absorbs at 640 nm (Tittor et al., 1987; Zimányi and Lanyi, 1989a, 1989b; Zimányi et al., 1989).

DISCUSSION

Transport of chloride by halorhodopsin-containing envelope vesicles is probably limited at high pH by the light-dependent slow deprotonation of the Schiff base of the pigment, generating a species which absorbs in the blue (Ogrurus et al., 1981; Lanyi and Schobert, 1983). We had expected that pharaonis halorhodopsin would have an extended pH range on the alkaline side, since N. pharaonis was isolated from an extremely alkaline saline lake, with a pH above 10.5 (Tindall et al., 1980). Our finding of similar pH dependence of the two systems (Fig. 1) is contrary to this. Together with the observation that N. pharaonis grows best at pH 8.5–9.5 under laboratory conditions (Tindall et al., 1980; Soliman and Triper, 1982), this suggests that either (a) the species is best adapted to conditions other than its normal habitat, or (b) the pH in the upper part of the trona crust of the alkaline salt lakes, where the organism proliferates (Tindall et al., 1980), is lower than in the body of the lake.

Pharaonis halorhodopsin could be purified by a procedure similar to one described for halorhodopsin (Duschl et al., 1988). The most important difference encountered was the bleaching of the pharaonis pigment when bound to phenyl-Sepharose and exposed to octyl glycoside. This cannot have been an effect of the detergent alone, because treatment of Tween-washed membranes with octyl glycoside did not destroy the pigment. On silver-stained SDS-acrylamide gels only few and weak bands were visible besides the major band of pharaonis halorhodopsin at 25.2 kDa and its dimer at 50 kDa (Fig. 3B). As judged by the absolute spectrum also, the preparation was virtually pure (Fig. 3A): the A280/A570 absorbance ratio of 1.4 was lower than the values (1.7–1.85) reported for halorhodopsin (Steiner and Oesterhelt, 1983; Taylor et al., 1983; Duschl et al., 1988). Even if this reflects less absorption at 280 nm, because pharaonis halorhodopsin contains only 8 tryptophans, as opposed to 10 in halorhodopsin, the preparations are comparable in purity.

The photocycle of detergent-solubilized pharaonis halorhodopsin at a high chloride concentration greatly resembles that of halorhodopsin. The differences are that (a) in the pharaonis pigment a second L-like intermediate, with a somewhat blue-shifted absorption maximum, follows the first one, and (b) the recovery of the parent pigment is twice as rapid. The significance of these (minor) differences between the two systems is not clear. When the pigments are compared with respect to their photoreactions in chloride and nitrate, however, a highly significant difference is seen: while halorhodopsin undergoes an HRl-containing photocycle in chloride, and this plus a second, truncated photocycle in the presence of nitrate (Zimányi and Lanyi, 1989b), the photoreactions of pharaonis halorhodopsin show no evidence for a second photocycle. Thus, in its photoreactions the pharaonis pigment does not discriminate between chloride and nitrate. This is in full accord with our finding that pharaonis halorhodopsin transports both of these anions equally well (Fig. 2, lower panel). The photocycle of pharaonis halorhodopsin in nitrate differs slightly from that in chloride (Fig. 10): (a) the KL intermediate in nitrate is somewhat red-shifted, although not as far as in the case of halorhodopsin in nitrate (Zimányi and Lanyi, 1989b), and (b) the L-intermediate in nitrate is somewhat red-shifted as well, while the second L-type intermediate is not blue-shifted.

The transport measurements with envelope vesicles from H. halobium show that, contrary to earlier reports (Schobert and Lanyi, 1982; Bamberg et al., 1984; Hazemoto et al., 1984), nitrate is transported by halorhodopsin, although with less effectiveness than chloride. As Fig. 2 (upper panel) indicates, half-maximal transport of nitrate is at a higher concentration than chloride, and at saturation less nitrate is transported than chloride. We had suggested some time ago (Schobert et al., 1983) that the appearance of the blue-shifted intermediate, which we now designate as HRl, is correlated with transport. The present findings clearly show what appeared to be a discrepancy between the transport specificity of halorhodopsin and its photoreactions: if a fraction of the pigment produces the HRl-containing photocycle in the presence of nitrate, it ought to transport nitrate to the extent that this photocycle is observed. We now find that this is indeed so. Thus, the HRl-containing photocycle can be unambiguously linked to transport.

The lack of an observed PHRo in the presence of chloride...
does not necessarily imply that this intermediate is not produced in the pharaonis halorhodopsin photocycle. Indeed, in the earlier report on membranes of *N. pharaonis* (Bivin and Stoeckenius, 1986), as well as in our experience with Tween-washed membranes (not shown), absorption rise in the red region suggests that PHRo will accumulate under some conditions, even if not in the detergent-solubilized pigment. This is understandable, since detailed kinetic analysis of HRo has shown that the appearance of this intermediate is very sensitive to variations in its rise and decay times (Ziminyi and Lanyi, 1989b). The existence of PHRo is suggested also by the dependence of the photocycle recovery rate on chloride concentration (t+ was 8.7 ms, and 42 ms at 0.1 M and 4 M chloride, respectively). For halorhodopsin this kind of dependence originates from the HRL → HRo + HR reaction scheme, because the rate of the HRo → HR transition is linearly dependent on chloride concentration (Ziminyi and Lanyi, 1989b). The existence of PHRo is suggested also by the dependence of the photocycle recovery rate on chloride concentration (t+ was 8.7 ms, and 42 ms at 0.1 M and 4 M chloride, respectively). For halorhodopsin this kind of dependence originates from the HRL → HRo + HR reaction scheme, because the rate of the HRo → HR transition is linearly dependent on chloride concentration (Ziminyi and Lanyi, 1989b).

Recently, we determined the primary structure of pharaonis halorhodopsin, and found that the sequence is about 65% identical with that of halorhodopsin (Lanyi et al., 1990). All arginine residues proposed to be buried inside the protein and involved in anion binding (Blanck and Oesterhelt, 1987; Lanyi et al., 1988), i.e. Arg-108, -161, and -200, are conserved in the two structures. However, Arg-103, which is thought to be located on the cytoplasmic interface of helix C (Schobert et al., 1988), is replaced by a valine residue in pharaonis halorhodopsin, and the charge of the A-B interhelical segment is changed from +4 to +1. Either of these differences might account for the observed lack of selectivity between chloride and nitrate in pharaonis halorhodopsin.

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Properties and photochemistry of a halorhodopsin from the haloalkalophile, *Natronobacterium pharaonis*.
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