Blue Halorhodopsin from *Natronobacterium pharaonis*: Wavelength Regulation by Anions†

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ABSTRACT: Halorhodopsin, the chloride pump from *Natronobacterium pharaonis* (pharaonis hR), was isolated under conditions of low ionic strength. The quotient between the optical densities of pharaonis hR in 4 M NaCl at 280 and 577 nm amounts to 1.1, indicating a high purity of the protein and integrity of the chromophore. Gel filtration chromatography of the purified pharaonis hR at neutral pH and in the absence of inorganic salts leads to a shift of the absorption maximum to 600 nm (pharaonis hR^{blue}). The purple color can be regained by the addition of anions such as chloride, iodide, azide, nitrate, and also fluoride. The absorption maxima are dependent on the nature of the anion and the pH. At pH 7, sulfate does not influence the 600-nm absorption, while at pH 4.5 the absorption maximum is shifted to 581 nm. The blue form of halorhodopsin (pharaonis hR^{blue}) was titrated with different sodium salts, such as chloride, azide, and nitrate. The half-maximal binding is in the millimolar range, with $Br < Cl < NO_3 < N_3 < BrO_3$. Deprotonation of the Schiff base can be accomplished by treatment of pharaonis hR or pharaonis hR^{blue} with base. The apparent pK of the Schiff base in pharaonis hR^{blue} was determined to be 8.5. The pK shifted to 8.0 in the presence of 150 mM Na_2SO_4, whereas the pK of pharaonis hR in 150 mM NaCl is 9.6. In the photocycle of the chloride- and nitrate-containing pharaonis hR, a species similar to hR_{520} could be detected. Pharaonis hR reconstituted with azide shows photochemical reactions similar to the photocycle of bacteriorhodopsin. Absorbance changes at 410 nm are indicative of a deprotonated Schiff base, as it has also been described for halorhodopsin from *Halobacterium salinarium*. The light activation of pharaonis hR^{blue} also leads to photoreactions with only one intermediate, characterized by an apparent maximum around 520 nm.

Two different classes of retinal proteins have been described in the archaebacterium *Halobacterium salinarium*. The sensory rhodopsins are photoreceptors regulating the light-dependent behavior of the archaebacterium. The two members of the other group, bacteriorhodopsin (bR[1]) and halorhodopsin (hR), are light-driven ion pumps specific for protons and chloride, respectively. Halorhodopsin, sequenced first by Blanck and Oesterhelt (1987), has now been found in a variety of other halophilic archaebacteria (Otomo et al., 1992; Soppa et al., 1993). It has also been isolated from *Natronobacterium pharaonis*, a haloalkaliphilic archaebacterium (Duschl et al., 1990; Lanyi et al., 1990a), and was thus named pharaonis halorhodopsin (pharaonis hR).

The functional and physiological properties of the halobacterial system have been intensively investigated by several groups [for a review, see Lanyi (1990)]. The relevant functional attributes of hR include the light-activated photocycle characterized by the following sequence of intermediates: $hR_{578} \rightarrow hR_{600} \rightarrow hR_{520} \rightarrow hR_{640} \rightarrow hR_{578}$. During this reaction cycle, chloride is transported electrogenically from outside the cell to its interior. However, the ion specificity is not strict, since bromide and nitrate are also transported to some extent. The photocycle is dependent on the ionic strength and the presence of the anion. For example, chloride at low concentrations reveals an intermediate absorbing at 640 nm, whereas azide catalyzes the deprotonation of the Schiff base (Hegemann et al., 1985; Lanyi, 1986).

The binding of anions to hR has been studied extensively. Ogurusu et al. (1984) concluded from the dependency of the absorption spectrum of hR on the concentration of chloride that hR has a single chloride binding site. From further spectroscopic analysis of the binding of different anions to hR in Na_2SO_4, which itself does not bind to the protein, it has been postulated that anions occupy two different sites in hR (Schobert et al., 1986; Lanyi et al., 1990b). One of these sites binds only polyatomic anions (site I) such as perchlorate, whereas the other (site II) is specific for anions like chloride. Nitrate is able to bind to both sites. These two sites might participate in the uptake and release of the chloride during transport (Lanyi et al., 1990b). New results from FTIR spectroscopy presented by Walter and Braiman (1994) indicated that only one anion binding site exists. The discrepancies between this view and the proposal of Lanyi and co-workers (Lanyi et al., 1990b) were explained by the assumption of a one-site, two-state model (Walter & Braiman, 1994).

Recent studies using resonance Raman spectroscopy supported the view that the chloride in hR is close to the Schiff base (Maeda et al., 1985; Pande et al., 1989; Diller et al., 1991), and it was concluded that the positive Schiff base cation is weakly hydrogen-bonded to a complex counterion including chloride, Asp238, and Arg108 (Ames et al., 1992). A similarity between the hR and bR counterion complexes is quite evident in the observation of an acid purple form of bR (Fischer & Oesterhelt 1979; Dér et al., 1991) and in studies of anion binding to bR mutants in which Asp85 is neutralized (Marti et al., 1992).

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[1] Abbreviations: hR, halorhodopsin; bR, bacteriorhodopsin; DM, *N*-dodecyl β-D-maltoside; SDS, sodium dodecyl sulfate; CD, circular dichroism.

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The analysis of the binding sites and their functional role has been complicated by the instability of the pigment to conditions of low ionic strength. In this article, the isolation of the analogous pigment pharaonis hR from *N. pharaonis* (Duschl et al., 1990; Lanyi et al., 1990a) under low-salt conditions is described. This preparation allows the removal of bound anions, which shifts the absorption maximum to 600 nm. The spectrum of this blue pharaonis hR (pharaonis hR^{blue}) bears a close resemblance to that of bR at low pH (acid bR^{purple}) (Fischer & Oesterhelt, 1979; Mowery et al., 1979) and that of the cation-depleted blue membrane of bR (bR^{blue}) (Kimura et al., 1984). Similar to acid bR^{purple} (Fischer & Oesterhelt, 1979; Mowery et al., 1979; Dér et al., 1991), the purple form of pharaonis hR is regained by adding certain anions. This observation provides a tool to study the anion binding site(s) of pharaonis hR.

MATERIALS AND METHODS

All chemicals used were of analytical grade.

Strains and Cell Culture. Natronobacterium pharaonis strain SP1(28) was grown under low oxygen tension (1 L cell culture in 2-L Erlenmeyer flasks shaken at 130 rounds/min and under continuous illumination) for 120 h at 40 °C on a synthetic growth medium. Growth medium contained the following salts: 3.4 M NaCl, 175 mM Na_2CO_3, 100 mM sodium acetate, 10 mM trisodium citrate, 27 mM KCl, 4 mM $MgSO_4$, 2 mM Na_2HPO_4, 5 μM $FeSO_4$, 4 μM $CuSO_4$, 4 μM $MnCl_2$, 3 μM $CaCl_2$, and 3 μM $ZnSO_4$; vitamins: 15 μM thiamine, 15 μM folic acid, 2 μM D-(+)-biotin; 0.8 mM Gly; and L-amino acids: 48 mM Glu, 7.6 mM Leu, 2.9 mM Cys, 0.8 mM Ile, 0.6 mM Met, and 0.4 mM each of Ala, Arg, Asp, Pro, Ser, Thr, Tyr, and Val. The medium was titrated with 25% HCl to a pH of 9.3. The cells were harvested by centrifugation and washed three times with 4 M NaCl.

Purification of Pharaonis hR. All steps were carried out at room temperature. All buffers used during column chromatography contained 80 μM phenylmethanesulfonyl chloride (PMSF) as a protease inhibitor.

Solubilization. Preparation of cell membranes and extraction with Triton X-100 were carried out as described in Scharf and Engelhard (1993). Triton-extracted membranes from 45 L of cell culture were suspended in 20 mM NaCl and 10 mM Tris (pH 8.0) to a protein concentration of about 9 mg/mL, mixed with 1.5 g of dodecyl maltoside dissolved in 25 mL of 20 mM NaCl and 10 mM Tris (pH 8.0), and stirred for 14 h in the dark. After centrifugation for 60 min at 360000*g*, the supernatant containing the solubilized pharaonis hR was collected.

Chromatography. The supernatant was applied to a DEAE-Sepharose CL-6B column (5.0 × 4.0 cm) that had been equilibrated with 0.1 M NaCl, 0.2% DM, and 10 mM Tris (pH 8.0). The column was washed thoroughly with 5 bed vol each of 0.1, 0.15, and 0.2 M NaCl, 0.2% DM, and 10 mM Tris (pH 8.0). Pharaonis hR was subsequently eluted (flow rate, 30 mL/h) by increasing the ionic strength of the buffer to 0.3 M NaCl.

The pharaonis hR fraction was diluted with 1 vol of 20 mM NaCl and 10 mM Tris (pH 8.0) and applied to a second DEAE-Sepharose CL-6B column (2.6 × 3 cm) equilibrated with 0.1 M NaCl, 0.2% DM, and 10 mM Tris (pH 8.0). After the column was washed with 2 bed vol of the equilibration buffer, pharaonis hR was eluted (flow rate, 20 mL/h) by 300 mL of a linear gradient of 0.15 to 0.4 M NaCl, 0.2% DM, and 10 mM Tris (pH 8.0). Fractions were collected and assayed spectrophotometrically for pharaonis hR and cytochrome content.

Fractions from the DEAE-Sepharose rechromatography were concentrated in Centriprep CP10 and Centricon C10 to a protein concentration of 5 mg/mL and then subjected to FPLC gel filtration in 0.5-mL portions (Superdex 200, HR16/60). The column was equilibrated and developed in 150 mM NaCl, 0.1% DM, and 10 mM Na_2HPO_4 (pH 8.0) at 1 mL/min. Peak fractions were collected manually and assayed spectrophotometrically.

Preparation of the Blue Species of Pharaonis hR. For buffer exchange, the purified pharaonis hR was passed over Sephadex G-25 (1.6 × 20 cm) in 10 mM sodium citrate/sodium phosphate with the appropiate pH at a flow rate of 0.4 mL/min in the dark.

pH Titration. Pharaonis hR^{blue} and pharaonis hR in 150 mM NaCl or 250 mM Na_2SO_4 were passed through a Sephadex G25 column that was equilibrated in 0.1% DM. These samples were diluted with 10 mM citrate/phosphate buffer at the appropriate pH with either no salt added or 150 mM NaCl and 250 mM Na_2SO_4 added, respectively. The pH was measured after recording the spectrum.

Preparation of the Hydroxide Form of Pharaonis hR. The purified pharaonis hR was passed over anion exchange resin AG 1-X8 (0.5 × 2 cm) in the hydroxide form at a gravity-controlled flow rate.

Analytical Procedures. Gel electrophoresis was performed essentially using the procedure of Laemmli (1970) in a 2-mm flat gel in a linear gradient from 10 to 17.5% acrylamide.

The protein content was determined after total hydrolysis of the samples in 6 N HCl with 0.1% phenol at 110 °C for 24 h with a Biotronik LC 7000 amino acid analyzer.

Spectroscopy. The visible spectra were recorded on a Perkin-Elmer Lambda 9 double-beam spectrophotometer. The circular dichroism (CD) spectra were recorded on a Jobin-Yvon M III dichrograph. The raw data were averaged and stored on a Nicolet 1074 (Nicolet, Madison, WI) transient recorder and transformed to ellipticity (θ) using a self-written program.

The photocycle kinetics were measured and analyzed as described by Müller et al. (1991).

RESULTS

Purification. The purification of halorhodopsin from *N. pharaonis* (pharaonis hR) was carried out from cells grown at low oxygen concentration. These conditions provided a 5-fold increase in the pharaonis hR content, as compared to aerobically grown samples. These observations have also been confirmed by immunoblot analysis with polyclonal antibodies raised against purified pharaonis hR (data not shown).

In the first step of the purification, the membrane preparation was treated with Triton X-100 to eliminate peripheral membrane proteins, which leads to the separation of about 45% contaminating protein. After solubilization, the remaining membrane was chromatographed on DEAE-Sepharose, which removes about 90% of the protein material. At this stage, pharaonis hR is visible as a sharp purple band on the column. The rechromatography removes minor impurities. The last purification step, gel filtration on FPLC-Superdex 200, leads to a highly pure pharaonis hR preparation, with a ratio between the optical densities at 280 and 577 nm of 1.25 (in 150 mM NaCl). The yield of all purification steps is in the range 80–90%. From 45 L of cell culture, 17 mg of pure pharaonis hR could be isolated.

Preparation and Characterization of Pharaonis hR^{blue}. Lowering of the sodium chloride concentration of the buffer to 1 mM results in a conversion of the color of pharaonis hR

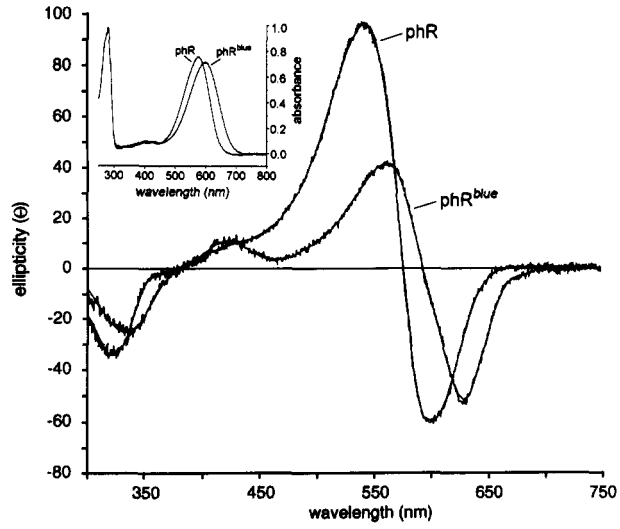

FIGURE 1: Circular dichroism spectra of the blue and purple forms of pharaonis hR: (phR) 1.5 mg/mL pharaonis hR in 0.1% DM and 10 mM citrate/phosphate buffer (pH 7.0); (phR^{blue}) after the addition of solid sodium chloride to a final concentration of 150 mM. A 0.5-cm path length cell was used; average of four scans. Inset: Absorption spectra of pharaonis hR and pharaonis hR^{blue}.

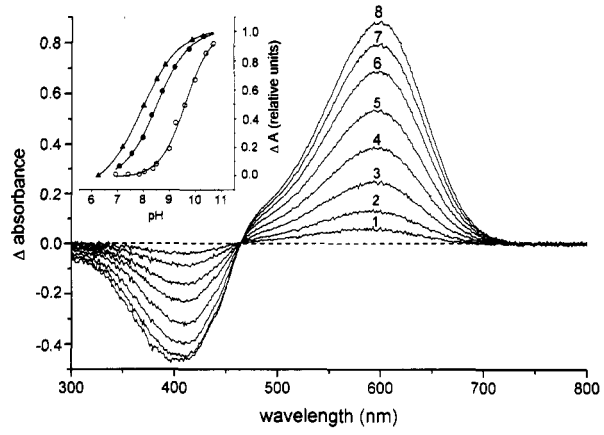

FIGURE 2: Difference absorption spectra of pharaonis hR (in 0.1% DM and 10 mM citrate/phosphate buffer). The differences were taken between the spectra at indicated pH values and the spectrum of pharaonis hR at pH 6.1. The final protein concentration was 0.8 mg/mL. (1) pH 7.08; (2) pH 7.56; (3) pH 8.00; (4) pH 8.33; (5) 8.75; (6) pH 9.22; (7) pH 9.78; (8) pH 10.30. Inset: Titration curves for pharaonis hR in 150 mM NaCl (○), pharaonis hR^{blue} (●), and pharaonis hR^{blue} in 50 mM Na_2SO_4 (▲). The absorbance changes in the maxima of the difference spectra are plotted against the pH. The inflection points are at pH 9.6, pH 8.5, and 8.0, respectively.

from purple to blue. This can also be accomplished by the removal of sodium chloride by gel filtration on a Sephadex G25 column. During filtration, the absorption maximum of pharaonis hR undergoes a shift from 577 to 600 nm at pH 7 (Figure 1, inset). The original purple form can be regained by the addition of anions such as chloride.

The circular dichroism spectra of pharaonis hR and pharaonis hR^{blue} in the visible range are also shown in Figure 1. Both forms exhibit a bilobe in the 450–700-nm region with crossover points at 575 nm for pharaonis hR and 595 nm for pharaonis hR^{blue} resembling the absorption maxima. Similar bilobe behavior has already been described for hR (Duschl et al., 1988; Hasselbacher et al., 1988), which has been attributed to intermolecular exciton interactions between neighboring chromophores. Interestingly, the magnetic transition of pharaonis hR at 318 nm also shifts bathochromically to 335 nm in pharaonis hR^{blue}. On the addition of chloride to the blue form, ellipticity is increased mainly in the positive arm of the bilobe. The chloride-free form also exhibits a positive band at around 420 nm, which is probably caused by the deprotonation of the Schiff base at this pH.

Titration of Pharaonis hR^{blue} with Base. Pharaonis hR^{blue} can be titrated with citrate/phosphate buffer to a yellow species. Figure 2 shows the difference absorbance spectra in the range 300–800 nm, in which the spectrum at pH 6.1 has been taken for comparison. A distinct isosbestic point is observed at 464 nm. The pK_a is determined to be 8.5 (Figure 2, inset). Titration pharaonis hR in the presence of NaCl (150 mM) shifts the titration curve to higher pH values (pK_a = 9.6). The addition of Na_2SO_4 (150 mM) decreases the pK_a to 8.0. If pharaonis hR is passed through an anion exchange column (counterion, OH), the yellow form of pharaonis hR can be obtained directly. This species can be converted to pharaonis hR^{blue} by the addition of, for example, HEPES buffer.

Titration of Pharaonis hR^{blue} with Anions. The addition of different anions (*except* sulfate) to pharaonis hR^{blue} results in a hypsochromical shift of the absorption maximum (Figure 3). Table 1 summarizes the λ_{max} values of specific anion-loaded pharaonis hR. The absorption maxima are dependent not only on the nature of the anion but apparently also on the

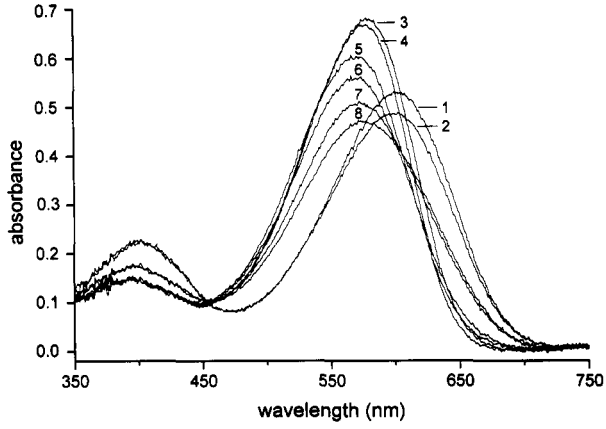

FIGURE 3: Absorption spectra of pharaonis hR^{anion}. Samples of pharaonis hR^{blue} in 10 mM sodium citrate/phosphate buffer and 0.1% DM (pH 7.5) were treated with indicated salts to final concentrations of 250 mM: (1) pharaonis hR^{blue}; (2) Na_2SO_4; (3) NaJ; (4) NaCl; (5) $NaNO_3$; (6) NaN_3; (7) $NaBrO_3$; (8) NaF.

pH. On lowering of the pH, the maximum of pharaonis hR^{blue} is shifted from 601 nm at pH 7.5 to 592 nm at pH 4.5. A similar effect is observed for all other anions tested at the three pH values, with $NaNO_3$ having the biggest influence. For this anion the absorption maximum is found at 549 nm for pH 4.5. It should be noted that at this pH sulfate also affects the absorption, which is shifted from 600 nm at pH 7.5 to 583 nm at pH 4.5. Fluoride also binds to pharaonis hR^{blue}. The resulting reconstituted pigment has its absorption at 576 nm (pH 7.5). The half-width of the absorption band is 123 nm compared to 97 nm for pharaonis hR^{chloride}. A similar effect is also observed for bromate (Table 1). It should be noted that higher concentrations of NaF shift the absorption maximum to lower wavelengths (557 nm at 1 M NaF).

The color shift is accompanied by a change in the extinction coefficient measured at λ_{max}. For pharaonis hR^{blue}, ϵ = 50 000 M^{-1} cm^{-1}. In the purple form this value increases to 54 000 M^{-1} cm^{-1} at 150 mM NaCl. At higher ionic strength (4 M NaCl), where any shift in the absorption maximum is no longer observed, the extinction coefficient increases to 59 000 M^{-1} cm^{-1}. This observation might indicate an additional binding

Table 1: pH-Dependent Absorption Maxima (nm) of Pharaonis hR^{blue} (First Row) and Pharaonis hR^{anion} [a]

	pH		
added salt	4.5	6.0	7.5
no addition	592 (105)	599 (108)	601 (108)
Na_2SO_4	581 (108)	597 (109)	600 (113)
NaF	570 (121)	576 (120)	576 (123)
NaCl	568 (96)	576 (95)	576 (97)
KCl	568 (96)	576 (95)	576 (95)
NaBr	571 (96)	578 (95)	579 (95)
NaI	563 (98)	577 (100)	578 (100)
NaN_3	568 (104)	569 (104)	569 (105)
$NaNO_3$	549 (102)	568 (103)	569 (108)
$NaBrO_3$	551 (112)	570 (113)	572 (117)

[a] To obtain specific anion-loaded pharaonis $hR^{anion's}$, samples of pharaonis hR^{blue} were diluted with stock solutions of the indicated salts to a final concentration of 250 mM. All preparations and salt solutions were prepared by using the same buffer system (10 mM citrate/phosphate and 0.1% DM) at the appropriate pH. The half-widths (nm) of the absorption bands are given in parentheses.

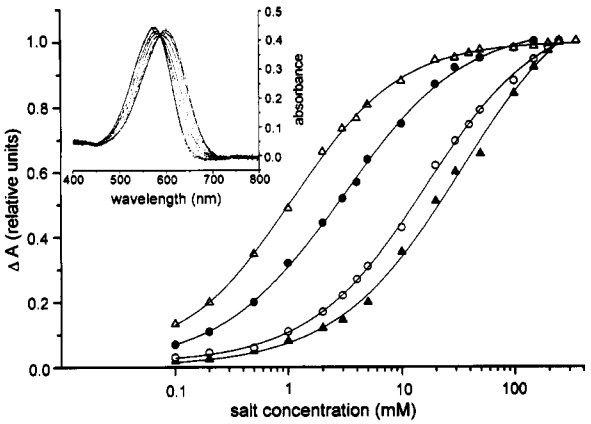

FIGURE 4: Saturation curves of pharaonis hR^{blue} (0.3 mg/mL in 10 mM citrate/phosphate buffer (pH 6.0) and 0.1% DM) with NaBr (△), NaCl (●), $NaNO_3$ (○), and NaN_3 (▲). The maximal absorbance changes of the difference spectra between the titrated form and the salt-free blue form were plotted against the corresponding salt concentrations. The maximal absorbance change is set as 1. Inset: Absorption spectra of pharaonis hR^{blue} at various chloride concentrations. The spectra were corrected for volume changes.

site for anions. However, the increase might also be due to incomplete titration at lower Cl^- concentrations. The shift of λ_{max} may have been too small to be detected.

On the addition of anions to pharaonis hR^{blue}, the absorption changes follow a sigmoidal curve. As representative examples, the titrations with bromide, chloride, azide, and nitrate are shown in Figure 4. The equilibrium constants (half-maximal binding) increase in the order bromide < chloride < nitrate < azide < bromate, with values of 1, 3, 16, 33, and 165 mM, respectively. A preliminary analysis of the binding curves provides evidence that the bimolecular reaction needs only one anion to cause the wavelength shift. However, more detailed experiments must be performed for a final answer.

Photocycle of Pharaonis hR^{blue} and Pharaonis hR Loaded with Specific Anions. The excitation of pharaonis hR^{blue} by light at 530 nm displays a very simple photocycle. Only one intermediate is observed at times slower than 10 μs, the time resolution of the equipment. It decays back to the ground state with a half-life time of 530 μs (Figure 6A). The reaction was followed every 10 nm in the range between 430 and 670 nm. The combined traces were analyzed using the "global fit" method of Müller et al. (1991). The corresponding amplitude spectrum (Figure 5) is characterized by a positive peak at 520 nm that is indicative of the formation of the sole

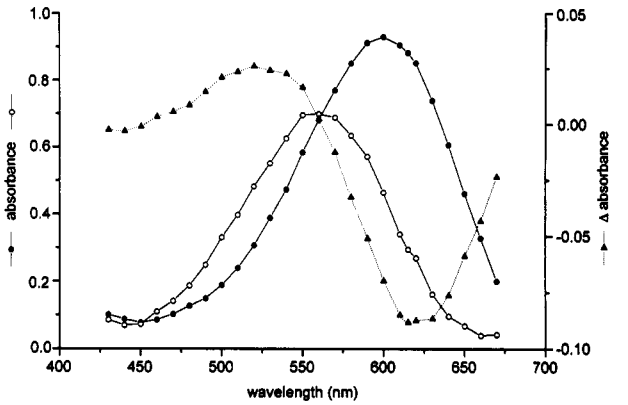

FIGURE 5: Amplitude spectrum of the L-type intermediate of the pharaonis hR^{blue} photocycle (▲). Also included are the spectra of pharaonis hR^{blue} (●) and the calculated intermediate (○). The samples were measured in 10 mM citrate/phosphate buffer and 0.1% DM (pH 6.0). The optical density was about 1.

Table 2: Time Constants of the Photocycle of Purified Pharaonis hR^{anion}

salt	τ_1 (μs)	τ_2 (μs)	τ_3 (μs)	τ_4 (μs)
NaCl (4 M)	9	652	704	1407
NaCl (150 mM)	18	196	481	3469
$NaNO_3$ (150 mM)	180	251	542	9509
NaN_3 (150 mM)	30	280	1976	5161

intermediate. The minimum at 620 nm is due to the depletion of the ground state. Under the assumption that the half-width of the absorption maximum of the intermediate is not totally different from that of the ground state, one can calculate the absorption spectrum of the new intermediate, which has a maximum at about 560 nm. This calculation corresponds to a fraction of photocycling molecules of about 15%. In Figure 5, the amplitude, the ground, and the calculated intermediate spectra are shown.

The photocycles of specific anion-loaded pharaonis hR's were studied at six different wavelengths. In Figure 6, the traces of the corresponding absorbance changes of pharaonis $hR^{chloride}$, pharaonis hR^{azide}, and pharaonis $hR^{nitrate}$ are depicted as representative examples. The data could be analyzed with four apparent rate constants of first-order reactions, which are summarized in Table 2. The turnover is generally in the millisecond range, with chloride being shorter than all other anions tested. Higher chloride concentrations substantially accelerate the photocycle.

The traces of the absorbance changes at the six wavelengths chosen provide a first insight into the nature and the individual states of the photocycle (Figure 6). The photocycle of hR has been studied extensively [summarized in Lanyi (1990) and Oesterhelt et al. (1992)]. Ames et al. (1992), applying kinetic resonance Raman spectroscopy, connected the intermediates of the photocycle to the following kinetic scheme: $hR_{578} \rightarrow hR_{520} \leftrightarrow hR_{640} \rightarrow hR_{578}$. In their model, chloride is released to the cytoplasm during the transition from hR_{520} to hR_{640}. Another chloride is taken up from the outside during the reformation of the ground state (hR_{578}).

The photocycle of pharaonis $hR^{chloride}$ at both chloride concentrations (Figure 6B and Table 2) contains intermediates absorbing at 520 and 640 nm. The same is true for pharaonis $hR^{nitrate}$, indicating that nitrate is also pumped by this pigment, which had already been demonstrated by Duschl et al. (1990). The accumulation of pharaonis hR_{640} is dependent on the chloride concentration. At 150 mM NaCl, pharaonis hR_{640} is formed with an apparent rate constant corresponding to

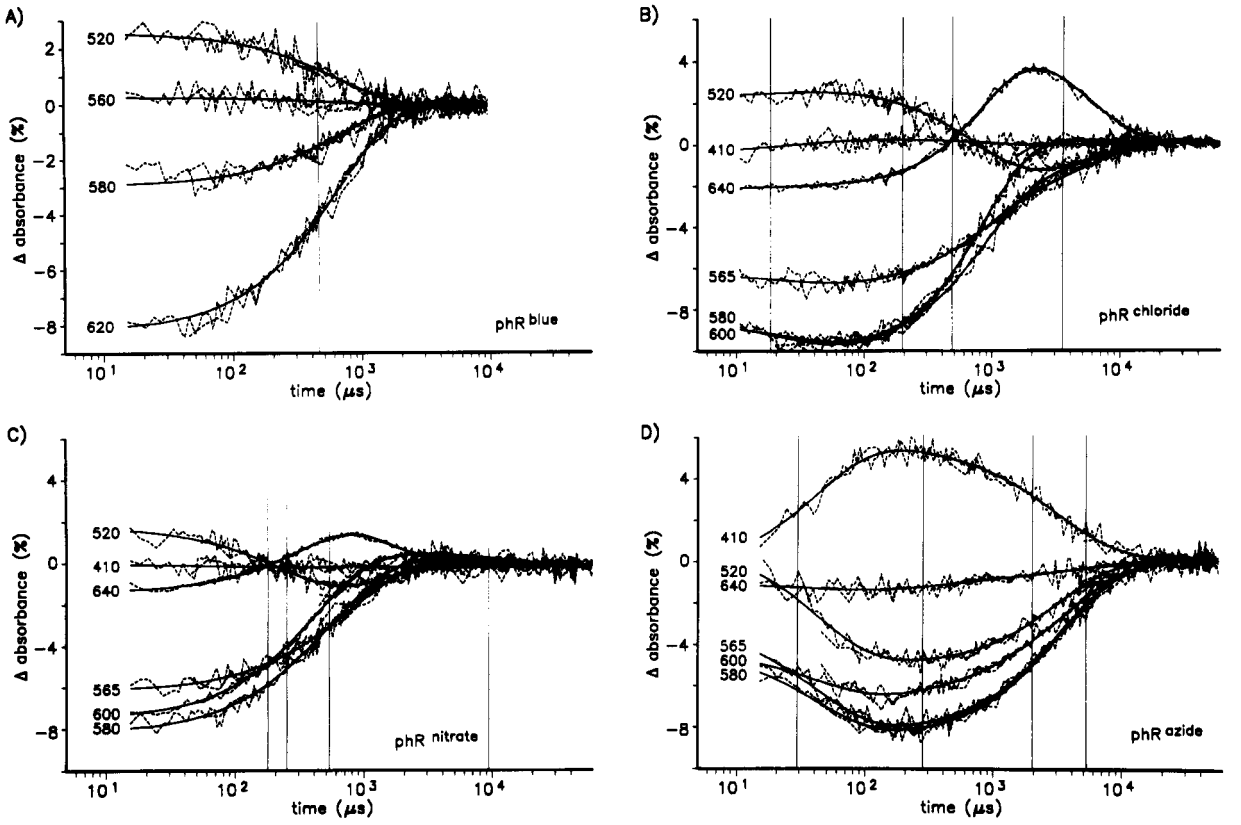

FIGURE 6: Traces of absorbance changes at indicated wavelengths (nm): (A) pharaonis hR^{blue}; (B) pharaonis $hR^{chloride}$; (C) pharaonis $hR^{nitrate}$; (D) pharaonis hR^{azide}. The vertical bars denote the calculated half-life times. Samples of pharaonis hR^{blue} in 10 mM sodium citrate/phosphate buffer and 0.1% DM (pH 6.0) were treated with the corresponding salts to final concentrations of 150 mM.

481 μs (τ_3). It decays back to the ground state with a half-life time of about 3.5 ms (τ_4). However, at 4 M NaCl the ratio of τ_3:τ_4 equals 1:2, so that pharaonis hR_{640} does not accumulate considerably. In accordance with the photocycle model given by Ames et al. (1992), the decay (τ_4) of hR_{640} increases with higher chloride concentrations, whereas at 150 mM NaCl the formation of hR_{640} (τ_3) decreases. It should be noted that for pharaonis $hR^{chloride}$ as well as for pharaonis $hR^{nitrate}$, the intermediate absorbing at 640 nm can be observed (Figure 6B,C). Duschl et al. (1990), in their publication on the properties of pharaonis hR, could not detect a hR_{640}-like intermediate at either high or low chloride concentrations. The discrepancy may be found in the different experimental conditions used, which might alter the rise and decay times of hR_{640}. Indeed, the appearance of this intermediate was suggested by an earlier work on pharaonis hR (Bivin & Stoeckenius, 1986) and in experiments with Tween-washed membranes mentioned by Duschl et al. (1990).

Another conclusion can be drawn from a trace at 410 nm that is representative for an intermediate with a deprotonated Schiff base, like the M state in the bR photocycle. The small increases in absorbance at 410 nm for pharaonis $hR^{chloride}$ and pharaonis $hR^{nitrate}$ might indicate that under physiological conditions a fraction of molecules undergoes a photocycle containing pharaonis hR_{410}. This kind of photocycle is fully established in the pharaonis hR^{azide} reaction cycle (Figure 6D). This so-called azide effect was first described by Hegemann et al. (1985). The kinetic parameters are quite similar to those of the bacteriorhodopsin photocycle (Müller et al., 1991). Two apparent rate constants (see Table 2) with half-life times of 1.9 and 5.2 ms are connected with the decay of pharaonis hR_{410}, and the formation of this intermediate is also in the same time range.

DISCUSSION

Pharaonis hR was first detected by Bivin and Stoeckenius (1986) and subsequently characterized biochemically by Duschl et al. (1990) and sequenced by Lanyi et al. (1990a). A comparison of its functional properties with those of the halobacterial pigment hR revealed close resemblances concerning their principal function as a chloride pump. Differences were found in connection with anion binding and the transport of nitrate, which is pumped by pharaonis hR almost as well as chloride (Duschl et al., 1990). The amino acid sequences also showed extensive similarities, although with characteristic variances. These are found particularly in association with the putative binding sites of anions to hR. Duschl et al. (1990) proposed that the replacement of Arg103 by Val and the decrease in charge of the loop connecting helices A and B (from +4 to +1) might account for the reduced discrimination between chloride and nitrate.

Another difference is the insensitivity of pharaonis hR to low salt concentrations. This property allowed the removal of intrinsic anions, which resulted in the isolation of the blue pigment pharaonis hR^{blue}. The blue form of pharaonis hR has striking similarities to the blue membrane of bacteriorhodopsin (bR^{blue}) (Kimura et al., 1984), the absorption maximum of which is also found at 600 nm. The photocycles of bR^{blue} (Mowery et al., 1979; Fahmy & Siebert, 1990) and pharaonis hR are also quite alike. Both pigments undergo reaction cycles with a blue-shifted intermediate, which in the case of bR^{blue} bears a close resemblance to the L intermediate. These observations indicate that the environments of the retinal chromophore and the charge distribution around the protonated Schiff base in bR^{blue} and pharaonis hR^{blue} match each other. Since the projected structures of hR and bR at a resolution of 6 Å are also almost identical (Havelka et al.,

1993), it can be infered that the counterion complexes in bR^{blue} and pharaonis hR^{blue} resemble each other. For bR, it was proposed that the two negative charges of Asp85 and Asp212 and the positively charged Arg82 participate in the counterion complex with the protonated Schiff base (Dér et al., 1991).

On the acidification of the medium or the removal of cations from the purple membrane bR^{blue} is formed (Fischer & Oesterhelt, 1979; Kimura et al., 1984; Chang et al., 1985). It could be shown that the protonation of Asp85, which is in close vicinity to the protonated Schiff base, is responsible for the purple–blue transition (Subramaniam et al., 1990; Metz et al., 1992). In other words, the removal of one negative charge produces this red shift. A similar blue species absorbing at 600 nm is obtained if Asp85 is replaced by a neutral amino acid, such as Ala (Otto et al., 1990). In both pharaonis hR and halobacterial hR, the corresponding position of Asp85 is replaced by Thr (Lanyi et al., 1990a; Soppa & Oesterhelt, 1993), giving rise to a charge distribution identical to that in bR^{blue} or those in Asp85Aax bR mutants. One would therefore predict that hR absorbs around 600 nm. However, contrary to this reasoning, the absorption maximum of hR is observed at 578 nm, and only the removal of chloride bathochromically shifts the absorption maximum. This observation can be explained unequivocally by the assumption that in pharaonis hR chloride takes over the role of Asp85 in bR. The removal of this anion would then induce a Schiff base counterion complex very similar to that in bR^{blue}.

Additional support for a close interaction between the protonated Schiff base and chloride is found in the difference between the pK_a's of pharaonis hR and pharaonis hR^{blue}. For pharaonis hR a pK_a of 9.6 was determined, whereas for the blue form a pK_a of 8.5 was measured. It should be noted that this difference is not due to the ionic strength because titration of pharaonis hR^{blue} dissolved in 150 mM Na_2SO_4 reveals a pK_a of 8.0, which is opposite the result observed in 150 mM NaCl. Marti et al. (1992) investigated the pK_a of the Schiff base deprotonation in bR mutants in which, for example, Asp85/Asp212 were exchanged with Asn. They described a remarkable reduced pK_a of the Schiff base and a logarithmic dependency of the apparent pK_a on the NaCl concentration. These findings were interpreted in the framework of a model in which the deprotonation is accompanied by loss of the anion, in their example chloride. This model implies that the pK_a becomes dependent on the concentration of the bound anion. These results are corroborated by Steiner et al. (1984), who found a shift of the protonation/deprotonation equilibrium toward the protonated hR_{578} upon increasing halide concentrations.

A third argument for direct binding of the anion to the protonated Schiff base of pharaonis hR is derived from the binding curves (Figure 4) and the absorption maxima (Figure 3) of different pharaonis hR^{anion}. Firstly, it should be noted that, with chloride as the anion, the wild-type absorption maximum and the photocycle kinetics of pharaonis hR obtained indicate that the original state has been reconstituted. Thus, a tool is provided to study the nature and structure of anion binding site(s) in pharaonis hR. As shown in Figure 3 and Table 1, the absorption maxima are strongly dependent on the nature of the anion and also on the pH of the buffer. A comparison of the present data with published absorption maxima of hR, acid bR^{purple}, and bR mutants (Table 3) reveals similar behavior for almost all anions tested. An exception can be recognized for fluoride, which shifts the maxima in the various species to wavelengths around 552 nm. However, pharaonis $hR^{fluoride}$ absorbs at 576 nm (at pH 7.5). The reason for this discrepancy may be found in the higher fluoride concentrations used for the binding studies as compared to the present investigations. Indeed, by using 1 M NaF the maximum is shifted to 557 nm.

Table 3: Absorption Maxima of Protonated Schiff Bases with Different Halides as Counterions[a]

salt	phR	hR[b]	bR_{mutant}[c]	bR_{acid}[d]	PSB_{ret}[e]
NaF	576	552	556	545	468
NaCl	576	572	565	565	460
NaBr	578	575	574	570	468
NaI	577	571	580	580	478

[a] phR: pharaonis hR. bR_{mutant}: In this mutant, Asp85 and Asp212 were exchanged against Asn. bR_{acid}: the acid purple form of bR. PSB_{ret}: the protonated retinylidene Schiff base of butylamine. A similar table was compiled by Walter and Braiman (1994). Data were taken from [b] Walter and Braiman (1994), [c] Marti et al. (1992), [d] Fischer and Oesterhelt (1979), and [e] Blatz et al. (1972).

Accumulated evidence indicates that the wavelength regulation of the absorption maximum (opsin shift) in bacterial and vertebrate rhodopsins is primarily due to a weak electrostatic interaction of the positively charged Schiff base with its counterion [for a summary, see Albeck et al. (1992)]. The electrostatically weak, interacting counterion complex can be obtained by increasing the distance between the charges (Blatz et al., 1972) and/or by hydrogen-bonded networks (Baasov & Sheves, 1985). The data presented in this work are in agreement with both of the above considerations. It is interesting to note that neutralization of the Schiff base counterions in bR by either mutation (Marti et al., 1992) or protonation of Asp85 (Dér et al., 1991) allows anions to bind to the protonated Schiff base.

In plots of the absorption maxima against crystallographic, hydrated, or Stokes (hydrodynamic) radii, no linear or reciprocal relationship could be recognized, as has been described for hR by Schobert and Lanyi (1986). They found a linear dependence of the affinity of one of the postulated anion binding sites (site I) for various anions on their reciprocal Stokes radii. At pH 4.5, a linear dependency of the crystal radii on the absorption maxima of various specific anion-loaded pharaonis hR's exists (data not shown). However, the interdependence is opposite what one would expect: with increasing radii, the maxima shift toward blue. These data point to direct, though complex interactions of the anions with the Schiff base. Furthermore, because of the strong difference between the λ_{max} and their half-band-widths of halides on the one hand and of the polyatomic anions on the other hand, they may also reflect the proposal of Walter and Braiman (1994) that binding of polyatomic anions involves a conformational change of the protein, resulting in an altered binding site.

The question now arises whether a set of two binding sites exists in pharaonis hR, as was proposed for hR by Lanyi et al. (1990b), or whether the data are more compatible with the model of Walter and Braiman (1994). The binding curves (Figure 4) do not support two different sites (with different affinities) since they can be linearized under the assumption of one binding site. However, an additional site with a binding constant in the molar range cannot be excluded. These data, the formation of the hR^{blue}, and the wavelength regulation by anions are congruent with the hypothesis of Walter and Braiman (1994), who proposed one binding site in close proximity to the protonated Schiff base. The different behavior of polyatomic anions was explained by a one-site, two-state model. Anions such as nitrate or bromate would bind to the same sites as halides, but would invoke an additional

conformational change of the protein. Additional experiments using resonance Raman and Fourier transform infrared spectroscopies should supply data on the nature of the interaction of anions with the Schiff base.

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