Chloride Concentration Dependency of the Electrogenic Activity of Halorhodopsin†

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Received November 6, 1998; Revised Manuscript Received February 8, 1999

ABSTRACT: The capacitive photoelectric current responses of the halorhodopsins from *Halobacterium salinarum* (shR) and from *Natronobacterium pharaonis* (phR) were studied using membrane fragments adsorbed onto a thin polyester film. The electric current of shR was not much affected by ionic strength or cations present in the medium (Na^+, K^+, Li^+, Mg^{2+}, or Ca^{2+}), but was greatly influenced by the Cl^- concentration. It increased biphasically as the Cl^- concentration increased from 0 to 5 M, then decreased and almost vanished at around 10 or 12 M. Apparent K_d's of about 0.1 and 6 M were deduced for the K_d of Cl^- uptake sites. We had to assume a sigmoidal increase of Cl^- binding with a Hill coefficient of about 8 at the cytoplasmic, Cl^- release site(s). The half-maximum Cl^- concentration for the sigmoidal binding was about 7.5 M. The electric current of phR had a maximum around 30 mM Cl^- and biphasically decreased at higher Cl^- concentrations. The apparent K_d for the Cl^- uptake site was 5 mM. The biphasic decrease in the transport activity was explained by assuming a sum of simple hyperbolic type binding (K_d = 0.2 M) and sigmoidally increasing binding with a Hill coefficient of 10 on the cytoplasmic side. The half-maximum concentration of the latter cooperative binding was 5.6 M. This great difference between the apparent affinity of the release site of shR and that of phR can explain the previously reported difference between the Cl^- dependency of their photocycles. These results also suggest that there may be multiple Cl^- binding sites in the Cl^- transport pathway. A simple sequence of Cl^- transport steps based on a multiion channel model is proposed.

In a general mechanism for ion pumping, there must be at least three steps. The first is to take up an ion from a medium where its concentration is low. The second is to translocate it within the pump protein. The final step is to release it to the medium which contains the ion at a higher concentration. As a very simple scheme of active transport, we recently proposed the "Stochastic Energization−Relaxation Channel Model", in which the difference in the affinity for the transported ion between the uptake site (the entrance) and the releasing site (the exit) plays an essential role (*1*). The ΔpH dependency of the proton translocation of bacteriorhodopsin (bR)[1] was well explained in terms of the pK_a of the key amino acid residues at the cytoplasmic and extracellular side of bR (*1*). Irrespective of the details of the particular mechanism, the asymmetry of the binding affinity for the transported ion at the uptake and release sites seems critical for a unidirectional translocation of the ion. Thus, knowledge of the affinities of the transported ion is very important. However, in the case of the Cl^- transporter halorhodopsin (hR), there is not much experimental data upon which to base an estimate of the Cl^- ion affinity for a specific binding site (*2*, *3*). NMR spectroscopy has been used for this purpose for hR from *Halobacterium salinarum* (shR) (*4*), but the pumping activity of shR has not been examined in a sufficiently wide range. A K_m of about 40 mM was obtained using cell envelope vesicles (*5*), 80 mM was obtained by the Cl^- dependency of the photocycle (*6*), and the black lipid membrane technique gave a K_m of 8 mM (*7*). These values are much smaller than the physiological cytosolic Cl^- concentration (about 4 M, a supersaturated concentration) or the extracellular Cl^- concentration (>2.5 M). In addition, as stated above, if Cl^- release is an important step in the transport process, we can expect a decrease in the transport activity at an extremely high Cl^- concentration due to the saturation of the Cl^- binding site responsible for Cl^- release. However, this has never been observed.

In the present study, we have applied our recently developed method (*8*) to examine the light-driven Cl^- pumping by hR from *Halobacterium salinarum* (shR) and *Natronobacterium pharaonis* (phR) and have examined the concentration dependency of their transport activity over a wide range of Cl^- concentrations (0−12 M). The Cl^- dependency gave a distorted bell-shaped curve from which we estimated affinities of the Cl^- binding sites which affect Cl^- transport. These results could explain the previously reported difference in Cl^- dependency in the photocycle of these hR's (*6*, *9*, *10*). In addition, the results suggest that Cl^- movement is not strictly limited to a single pathway, but may occur along multiple pathways at the entrance and the exit of hR. The results were also discussed in terms of a theory of multiion channel (*11*), and a simple Cl^- transfer

† This work was supported in part by Grant-in-Aid for Scientific Research (C) 09833001 (to E.M.) from the Ministry of Education, Science, Sports and Culture of Japan.

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[1] Abbreviations: bR, bacteriorhodopsin; hR, halorhodopsin; shR, hR from *Halabacterium salinarum*; phR, hR from *Natronobacterium pharaonis*; K, L, N, O, and HR′ are intermediates of the photocycle of halorhodopsin, in analogy to the intermediates of bacteriorhodopsin.

10.1021/bi9826456 CCC: $18.00
Published on Web 04/03/1999

sequence based on a multiion channel model is proposed.

EXPERIMENTAL PROCEDURES

Materials. The overproducing strains of shR and phR were kind gifts from Dr. R. Needleman (Wayne State University School of Medicine). The membrane fragments containing hR were isolated by sucrose density gradient centrifugation (*12*). The shR-overproduced membranes were suspended in 20 mM Tris−maleate buffer containing 4 M NaCl and 2 mM $MgCl_2$ (pH 7.0) (Buffer A). Typically, the concentration was adjusted to $A_{570} = 1.5$. The phR membranes were concentrated using a Centricon 10 apparatus (Amicon, Beverly, MA) and used without removing sucrose.

The pHs of the buffers containing high salt concentrations were adjusted to 7.0 using 50 mM Tris−maleate. All the salts were guaranteed grade purchased from Nacalai Co. (Kyoto, Japan). When necessary, more than 50 mM Tris base was added to neutralize the buffer. pH was measured by a Beckman pH meter type Phi 10 equipped with a pH electrode (39849) and also confirmed by pH test paper (Merck Neutralit pH 5−10).

Adsorption of hR Membranes onto a Thin Film and Electric Measurement. The adsorption of the shR and phR membranes to the film was carried out as described for purple membranes (*8*). The setup for the measurement was similar to that developed by Drachev et al. (*13*) or Holz et al. (*14*). Briefly, a 0.9 μm thick polyester film (Lumirror; Toray Co., Tokyo) was placed between the two chambers of an apparatus for the electrical measurement. Eighty microliters of the membrane suspension was applied directly on one side of the film, and was left for 40 min at room temperature. Excess membranes were removed by pipetting, and 1.5 mL of Buffer A was added to both chambers. For measurements of shR, the buffer in the membrane-adsorbed side chamber was exchanged twice with NaCl-free Buffer A to wash out residual unbound membranes and Cl^- ions. Subsequently, after a 1−3 min incubation with Buffer A containing 0.5% octylglucoside, the chamber was washed several times and filled with original Buffer A. In the case of phR, the adsorbed membranes were incubated with 50 mM Tris−maleate (pH 7.0) containing 0.1 M $CaCl_2$ and 0.1% octylglucoside for 2 min, and washed with the same buffer without octylglucoside. The peak height of the electric current upon continuous illumination was measured (*8*). When the effect of different medium components (Cl^- concentration, cation species, etc.) was examined, the old solution was removed and the solution of the new composition was added by simple pipetting. We waited for 5 min to let the membrane system reach equilibrium with the new solution before electrical measurements.

RESULTS

Adsorption of hR-Containing Membranes onto Lumirror. The hR-containing membrane fraction was adsorbed onto Lumirror in a very similar way to that used for the bR purple membrane as described under Experimental Procedures. In the case of shR, the magnitude of the photoelectric response was relatively small just after adsorption and usually increased after exchanging the buffer in the compartment of the membrane-adsorbed side. After washing the membrane with octylglucoside, the magnitude of the photoresponse became large and highly reproducible. These properties were the same as those observed for bR (*8*). The direction of the electric current corresponded to the movement of positive charges from the aqueous phase to Lumirror. This direction indicates that Cl^- was released from the space between Lumirror and the adsorbed membrane to the medium. In case of phR, the magnitude of the electric current did not increase after octylglucoside treatment, but the reproducibility was improved. The direction of the electric current was sometimes reversed in separate experiments in the case of phR. We did not investigate the cause of this polarity change. Recently, Kalaidzidis et al. reported a similar phenomenon (*15*). They attributed the cause of the different direction of the electric currents to the different Cl^- binding states of phR. Anyway, the polarity was constant in each experiment, and the same Cl^- dependency was obtained irrespective of the direction of the electric current.

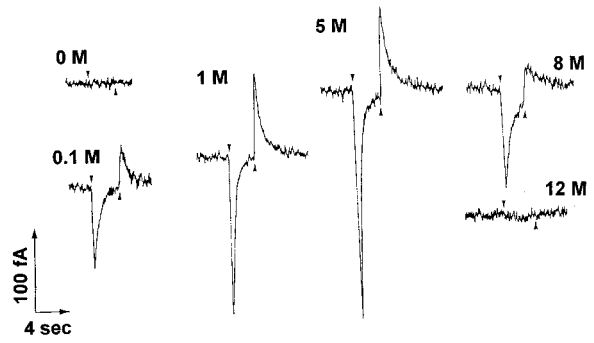

FIGURE 1: Traces of the capacitive electric current upon illuminating shR-containing membranes adsorbed onto Lumirror at the indicated Cl^- concentrations. $CaCl_2$ was used. Downward arrowheads indicate ON and upward arrowheads indicate OFF of the illumination. The peak height of the downward electric current corresponds to the initial (maximum) rate of charge movement upon illumination.

Cl^- Concentration Dependency of the Electrogenic Activity of shR. Figure 1 shows the photoelectric responses of the shR-containing membrane−Lumirror film system at various Cl^- concentrations. As Lumirror is impermeable to ions and acts as an electric capacitor, the photoelectric current by hR becomes capacitive (downward peak). Its peak height corresponds to the initial (maximum) rate of the charge movement. The upward peak upon light-off corresponds to the system discharge and does not reflect the activity of hR. We did not observe protein polarization charge movement at 0 M Cl^-. The polarization signal accompanying the isomerization of the retinal moiety might be too fast or too small to be detected in our system where the signal was filtered at 50 Hz. Alternatively, at 0 M Cl^-, the chromophore might have lost bound Cl^- and did not adsorb actinic light due to spectral change. Figure 2 shows the peak height plotted against the concentration of several Cl^- salts. In Figure 2A, we chose $CaCl_2$ in order to change the Cl^- concentration over a wide range. First, the Cl^- concentration was increased from 0 to 12 M (open circles) and then decreased from 12 to 0 M (filled circles) using the same film (Figure 2A). It is clear that the electrogenic activity of shR increased biphasically as the Cl^- concentration increased from 0 to 5 M, and then decreased and almost vanished at around 10 or 12 M. When the effect of Cl^- concentration was examined, the solutions of different Cl^- concentration were exchanged by pipetting as described under Experimental Procedures. The shR-containing membranes which might

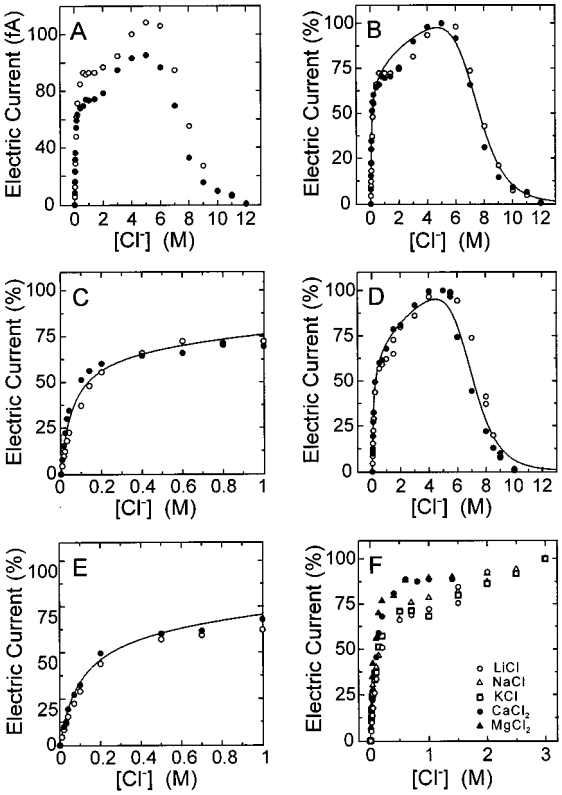

FIGURE 2: Cl^- concentration dependency of the photoelectric current generated by shR-containing membranes adsorbed onto Lumirror. (A) The Cl^- concentration dependency was examined using $CaCl_2$. Open circles represent an experiment with increasing $CaCl_2$ concentration from 0 to 6 M. Filled circles represent an experiment with decreasing $CaCl_2$ concentration from 6 to 0 M after the experiment using an increase in the concentration with the same membrane. (B) The same as (A), but the data are normalized with the maximum photoelectric current. (C) The same as (B), but the abscissa below 1 M Cl^- was expanded for clarity. (D) The same as (B), but LiCl was used instead of $CaCl_2$. (E) The same as (D), but the abscissa below 1 M Cl^- was expanded for clarity. (F) Effect of various cations on the Cl^- concentration dependency of the photoelectric current. The data obtained by different films were normalized at 3 M Cl^-. The solid lines in (B)−(E) were drawn according to eq 3 using the parameters listed in Table 1.

have dissociated from Lumirror were removed upon exchange of the medium, and the increase in the electrogenic activity was not due to the increase of the adsorbed membranes. Due to the inactivation or dissociation of the shR-containing membrane from Lumirror, the activity obtained in the decreasing path (filled circles) was smaller than that in the increasing path (open circles) in Figure 2A. When the activity was normalized by the maximum value in each path and replotted in Figure 2B,C, they coincided fairly well. Therefore, we concluded that possible denaturation or dissociation of shR-containing membrane from Lumirror during experiments did not affect the shape of the Cl^- dependency curve considerably. The coincidence also means that the Cl^- concentration between the Lumirror film and adsorbed hR-containing membranes reached equilibrium with that of the bulk solution before electrical measurements. A very similar Cl^- concentration dependency was obtained using LiCl instead of $CaCl_2$ (Figure 2D,E). Thus, the suppression of the electric current at high Cl^- concentration (>6 M) was not due to a specific effect of Ca^{2+} or Li^+, but it reflects the effect of high Cl^- concentration. In Figure 2F,

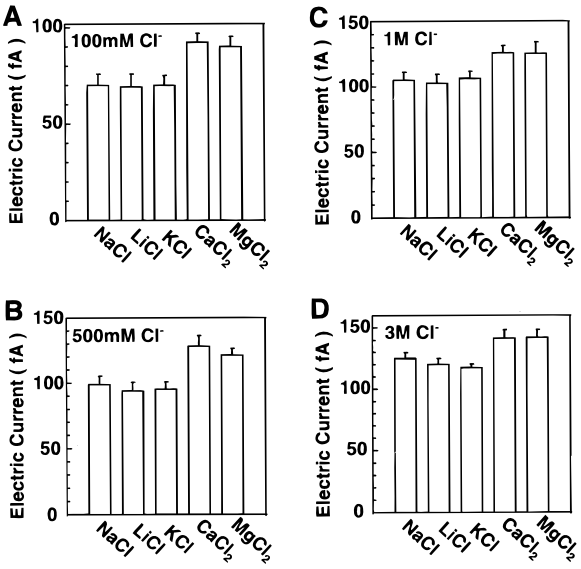

FIGURE 3: Cation dependency of the photoelectric current generated by shR-containing membranes adsorbed onto Lumirror. Various chloride salts were compared using the same membrane at fixed Cl^- concentrations. (A) 100 mM Cl^-. (B) 500 mM Cl^-. (C) 1 M Cl^-. (D) 3 M Cl^-.

the effects of other Cl^- salts ([Cl^-] <3 M) were examined. Due to the limited solubility, the electric current at Cl^- concentrations of less than 3 M was used. Again, it is clear that the electrogenic activity did not significantly depend on the cations, within experimental error, but depended mainly on Cl^- concentration.

Cation Specificity of the Electrogenic Activity of shR. In Figure 2F, the shape of the Cl^- concentration dependency on the electrogenic activity was very similar irrespective of the cation species; however, the magnitude of the electrogenic activity could not be compared between different Lumirror films in separate experiments. To examine the effect of different cations on the magnitude of the electric response, we compared different Cl^- salts using the same Lumirror film at fixed Cl^- concentrations (0.1−3 M) in Figure 3. There was a slight tendency for divalent cations (Ca^{2+}, Mg^{2+}) to stimulate the electrogenicity compared with monovalent cations (Na^+, Li^+, K^+). However, the extent of stimulation by divalent cations was at most 20%, and we do not regard this difference as significant.

Effects of Ionic Strength and Viscosity on shR. Under the conditions of Figures 2 and 3, ionic strength changed significantly as Cl^- concentrations changed. It was impossible to maintain a constant ionic strength while changing the Cl^- concentrations over a wide range due to the limited solubility of these salts. To examine the effect of ionic strength, we added various concentrations of $(NH_4)_2SO_4$ while keeping Cl^- concentration at 1 M. The electrogenic activity was slightly suppressed at high $(NH_4)_2SO_4$ concentration (Figure 4A), but the observed dependency was clearly different and smaller than the Cl^- concentration dependency in Figure 2.

At high $CaCl_2$ concentrations (>3 M), we noticed that the viscosity of the medium increased. To examine the effect of the viscosity on the electrogenic response by hR, we added glycerol while keeping the $CaCl_2$ concentration at 2 M (Figure 4B). Addition of glycerol activated the electrogenic activity only slightly.

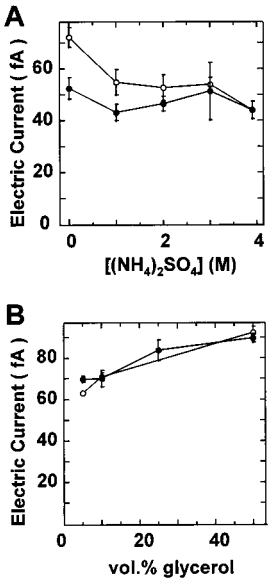

FIGURE 4: Effect of ionic strength and viscosity on the photoelectric current generated by shR-containing membranes adsorbed onto Lumirror. (A) Ionic strength was changed by adding $(NH_4)_2SO_4$. Open circles represent an experiment in which $(NH_4)_2SO_4$ concentration was increased. Filled circles represent a subsequent experiment in which $(NH_4)_2SO_4$ concentration was decreased. (B) Viscosity was changed by adding glycerol. The meaning of open and filled circles is the same as in (A).

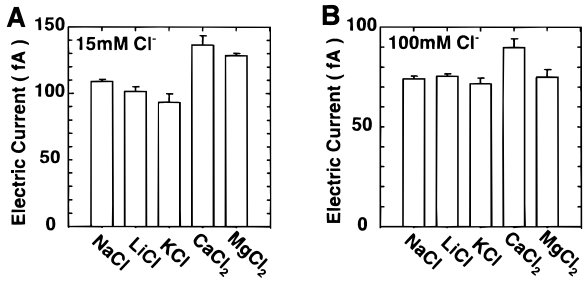

FIGURE 5: Cation dependency of the photoelectric current generated by phR-containing membranes adsorbed onto Lumirror. Various chloride salts were compared using the same membrane at fixed Cl^- concentrations. (A) 15 mM Cl^-. (B) 100 mM Cl^-.

From these data, we conclude that the effects on the electric response by ionic strength and viscosity are small, if any, and that the data in Figure 2 reflect the Cl^- concentration dependency of the transport process.

Cl^- Transport Activity of phR. The Cl^- concentration dependency of the transport activity was examined using another well-studied hR from *Natoronobacterium pharaonis* (phR) (*16*). Similar to shR, the transport activity was not significantly dependent on the cations examined (Na^+, Li^+, K^+, Ca^{2+}, and Mg^{2+}) (Figure 5). To our surprise, however, the transport activity of phR showed a considerably different Cl^- dependency than shR. As shown in Figure 6A, we increased the [Cl^-] from 0 to 8 M (filled circles) and then decreased it from 8 to 0 M (open circles). We used $CaCl_2$ because the adsorbed phR-containing membranes seemed somewhat unstable in LiCl solutions. In the case of phR, the activity in the decreasing path (open circles in Figure 6A) was smaller than that in the increasing path (filled circles in Figure 6A), and they did not coincide with each other well even after normalization (Figure 6B). However, the significant difference in the transport activity from shR is obvious. First, the Cl^- transport activity showed a steep

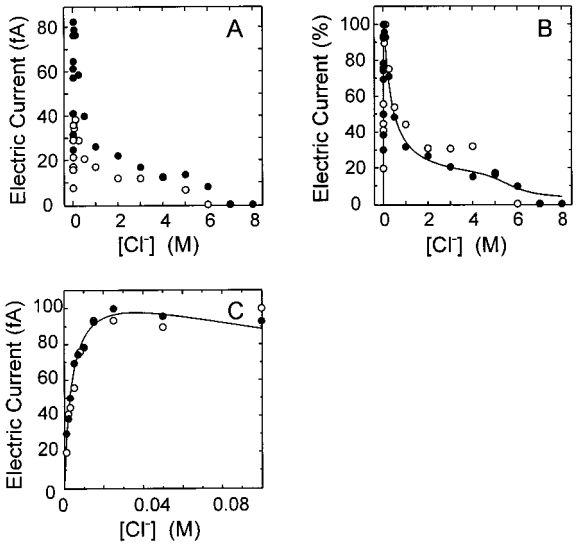

FIGURE 6: Cl^- concentration dependency of the photoelectric current generated by phR-containing membranes adsorbed onto Lumirror. (A) Cl^- concentration dependency was examined using $CaCl_2$. Filled circles represent an experiment with an increasing $CaCl_2$ concentration from 0 to 4 M. Open circles represent a subsequent experiment with a decreasing $CaCl_2$ concentration from 4 to 0 M using the same membrane. (B) The same as (A), but the data are normalized with the maximum photoelectric current. (C) The same as (B), but the abscissa below 0.1 M Cl^- was expanded for clarity. The solid lines in (B) and (C) are drawn according to eq 3′ using the parameters listed in Table 1.

increase as the Cl^- concentration increased, and it reached a maximum at around 30 mM (Figure 6C). Second, above 30 mM Cl^-, the transport activity steeply decreased with a reproducible shoulder around 4 M Cl^-, and almost vanished at 8 M Cl^- (Figure 6B).

Analysis of the Cl^- Concentration Dependency of the Electrogenic Activity by hR. A minimal mechanism for ion pumping contains at least three steps. The first is to capture an ion from a low ion concentration medium. The second is to translocate it within the protein. The last is to release it to a medium where it is at a higher concentration. If we assume a simple channel model with the three ion binding sites shown in Figure 7A, the ion binding sites at the entrance of the channel (Site A) must have a high affinity for the transported ion in the first step, and the ion binding site at the exit (Site C) must have a low affinity for transported ion in the last step. Otherwise, ion transfer from Site B to Site C and ion transfer from Site A to Site B cannot occur in this translocation step. Thus, the rate of ion translocation should be proportional to the probability of the occupied state of Site A and the probability of the empty state of Site C. As it is likely that Site A and Site C are in equilibrium with the bulk medium through an electrical double layer, the ion concentration dependency of the pumping activity may be expressed by the apparent affinities of these sites in these steps and the maximum velocity of the transport process through the channel as schematically shown in Figure 7B. This is analogous to the analysis of a bell-shaped pH profile of an enzyme reaction. The actual concentration dependency of the Cl^- binding which affects transport activity may be quite complex (see Discussion); however, based on this assumption, we tried to fit the data obtained for hR using simple equations.

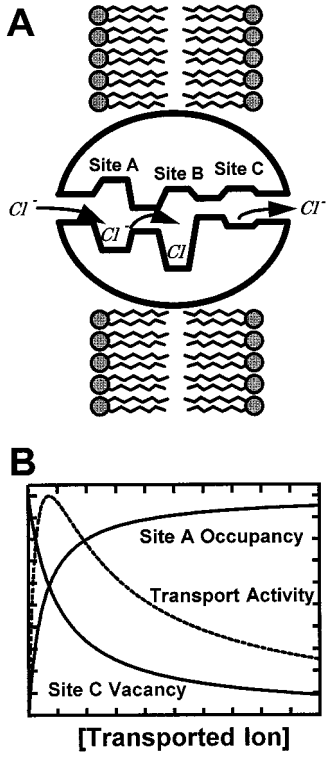

FIGURE 7: (A) A three-site model of an ion pump. Site A should have high affinity for transported ion in the ion-taking-up step whereas Site C should have low affinity in the ion-ejecting step. The dynamic change of the affinity of Site B can induce vectorial ion translocation. See text for details. (B) Schematic representation to show that the total ion transporting activity is proportional to the product of Site A occupancy in the taking-up step and Site C vacancy in the releasing step. In this case, the concentration of the transported ion is assumed to be the same on either side of the membrane.

In the case of shR, data below 4 M Cl^- could be easily fitted when we assumed a combination of two Michaelis−Menten type dependency (eq 1):

$$I_{max}\left(s\frac{[Cl^-]}{K_1+[Cl^-]}+(1-s)\frac{[Cl^-]}{K_2+[Cl^-]}\right) \quad (1)$$

The simplest explanation of this equation is that there are two kinds of Site A in Figure 7 (or two different states of a single Cl^- binding site) with different affinity for Cl^- (K_1 and K_2) (however, see Discussion). The contribution from the two sites is defined by s and $1 - s$ with a maximum velocity of I_{max}. As for the data from 6 to 12 M, it was impossible to fit them with any simple combination of the hyperbolic Michaelis−Menten type saturation at Site C. Rather, the data suggested that the Cl^- binding at Site C increased sigmoidally as the Cl^- concentration increased. Such a Cl^- concentration dependency could be expressed with an equation such as

$$\frac{[Cl^-]^n}{K_3{}^n+[Cl^-]^n} \quad (2)$$

where n is a Hill coefficient.

In total, the Cl^- transport activity by shR is proportional to the product of eq 1 and (1 − eq 2) (eq 3):

$$I_{max}\left(s\frac{[Cl^-]}{K_1+[Cl^-]}+(1-s)\frac{[Cl^-]}{K_2+[Cl^-]}\right)\times\left(1-\frac{[Cl^-]^n}{K_3{}^n+[Cl^-]^n}\right) \quad (3)$$

In the case of phR, the increasing phase of the transport activity (saturation of Site A) was well expressed by a simple hyperbolic Michaelis−Menten equation (eq 1′):

$$\frac{I_{max}[Cl^-]}{K_1+[Cl^-]} \quad (1')$$

However, any simple combination of the hyperbolic Michaelis−Menten type saturation failed to express the decreasing phase (saturation of Site C) with a shoulder around 4 M. After some trial and error, we found that a combination of a hyperbolic saturation curve and a sigmoidal saturation curve could express the data (eq 2′):

$$s\times\frac{[Cl^-]}{K_2+[Cl^-]}+(1-s)\times\frac{[Cl^-]^n}{K_3{}^n+[Cl^-]^n} \quad (2')$$

This equation assumes two kinds of Site C in Figure 7A, one of which exhibits hyperbolic saturation and the other exhibits sigmoidal saturation. s and $1 - s$ determine the ratio of Cl^- transport through these sites.

In total, the Cl^- transport activity by phR is expressed by the product of eq 1′ and (1 − eq 2′) (eq 3′):

$$\frac{I_{max}[Cl^-]}{K_1+[Cl^-]}\times\left[1-\left(s\times\frac{[Cl^-]}{K_2+[Cl^-]}+(1-s)\times\frac{[Cl^-]^n}{K_3{}^n+[Cl^-]^n}\right)\right] \quad (3')$$

The parameters obtained based on these equations are listed in Table 1.

DISCUSSION

Any ion pump should possess an active center which contains the critical ion binding site whose affinity changes upon energization. In addition, ion binding sites which facilitate the migration of ion between the medium and the active center are indispensable. Our recent results on bR suggested that the pH gradient and membrane potential dependencies of proton transport were kinetically different and largely controlled by the affinity and the position of these sites. Actually, simple simulation estimating the pK_a and electric distance between these sites could reproduce the properties of proton transport (*1*). In the case of bR, the pH dependency of the capacitive photoelectric current gave a half-maximum at pH 4.6 at the acidic side and pH 10 at the basic side, respectively (*8*). They coincided well with the pK_a values of Glu204 (pK_a = 4.7) and Asp96 (pK_a = 11), which are important for proton release (*17*, *18*) and uptake (*19*−*21*), respectively. Thus, from the Cl^- concentration dependency of hR, the apparent affinity of Cl^- binding at the uptake and release sites may be estimated. In this context, the bell-shaped Cl^- concentration dependency was interpreted

Table 1: Parameters for Cl^- Dependency of the Cl^- Translocation by HR[a]

	K_1 (M)	K_2 (M)	K_3 (M)	n	s	eq
shR ($CaCl_2$)	0.06	6	7.5	8	0.5	3
shR (LiCl)	0.1	6	7.5	8	0.41	3
phR ($CaCl_2$)	0.005	0.21	5.6	10	0.91	3′

[a] K_1 and K_2 for shR stand for the apparent affinity of the Cl^- binding site(s) at the entrance of the Cl^- pathway whose contribution is defined by s and $1 - s$. K_3 for shR stands for the apparent affinity with a Hill coefficient of n at the exit of the Cl^- pathway. These parameters are defined in eq 3. K_1 for phR stands for the apparent affinity of the Cl^- binding site at the entrance of the Cl^- pathway. K_2 and K_3 for phR stand for the apparent affinity of Cl^- binding sites at the exit of the Cl^- pathway whose contribution is defined by s and $1 - s$. n for phR is a Hill coefficient associated with K_3. These parameters are defined in eq 3′.

in terms of the number and affinity of the binding sites at the entrance and exit of the transport pathway.

In the case of shR, the maximum transport activity was attained at around 5 M Cl^-, which is reasonable for a Cl^- pump in a halophilic bacterium. As for the Cl^- binding at the extracellular side, two kinds of binding were suggested from the biphasic increase in the transport activity below 5 M Cl^-. We cannot completely exclude the possibility that this biphasic increase was caused by the heterogeneous adsorption of the shR-containing membrane fragments onto Lumirror or by the contribution of other membrane proteins. However, judging from the high reproducibility under many experimental conditions in many separate experiments, we think that this possibility is rather unlikely and that this biphasic increase reflects the inherent nature of shR itself. The apparent parameters obtained for Cl^- binding using $CaCl_2$ (Figure 2B and Table 1) were a K_1 of 0.06 M and a K_2 of 6 M with an accompanying I_{max} of 145 (I_{max} is in arbitrary units) and an s of 0.5. Similar values were obtained using LiCl (Figure 2C and Table 1). The coincidence of the K_1 with the previously reported K_m for Cl^- transport (0.04 M) using membrane vesicles indicates that the interfacial effect of narrow space between Lumirror and adsorbed membranes was small, if any. On the other hand, the discrepancy between the K_1 in the present study and a K_m of 8 mM reported previously (*7*) may reflect the difference in sample preparations. The previous study used Tween-washed membrane fragments prepared from a bR-deficient mutant, and the contribution from other membrane proteins might have been larger than the present study. The inhibition of Cl^- transport above 5 M Cl^- was first reported here. To explain the inhibition of Cl^- transport in terms of Cl^- binding to the exit site, we had to assume a sigmoidal increase of the occupancy with increasing Cl^- concentration. Such binding cooperativity was also observed for a competitive inhibitor of hR, MK 473 (*22*).

What then is the origin of the multiple apparent affinities for Cl^- uptake or the sigmoidal dependency for Cl^- release? One possible explanation is that there may be multiple Cl^- pathways at the surface of hR. This intriguing possibility was first pointed out for bR by Kimura et al. (*23*) based on a precise structural study. Another possibility is an application of the theory of a multiion channel (*11*). In a multiion channel, a concept of multiple ion occupancy was introduced to explain the high permeability and high selectivity. According to this concept, ion flux is slow when only one ion is in the channel molecule but the flux is accelerated by the mutual repulsion when the second ion comes into the channel. Indeed, the crystal structure of the K^+ channel was consistent with that concept (*24*). The biphasic increase in Cl^- transport in shR may reflect such an acceleration of Cl^- transfer by multiple Cl^- binding. The theory of multiion channel may also account for the decrease of Cl^- transport by shR at high Cl^- concentration. It was shown that the conductance of a multiion channel decreases at an extremely high ion concentration because the flux depends on the existence of vacant sites for ions within the channel to move. At high concentrations, any vacancy formed by an ion jumping into the solution is immediately canceled by another ion coming back from the solution, and the net flux is inhibited (*11*). The high Hill coefficient derived for the sigmoidal binding at the exit site of hR may be hardly explainable assuming a single-file pore, but such a strong cooperativity may arise from complex interactions near the surface of the protein. However, none of the possibilities raised above has been proved at present, and we cannot reach any decisive conclusion about the origin of the multiple K_d values for Cl^- uptake or a sigmoidal dependency for Cl^- release. Further studies are required for the elucidation of the interaction between transported ions and proteins. At an extremely high concentration of Cl^- (12 M), even the collapse of the hydration shell should be taken into account.

It is notable that the Cl^- concentration dependency of Cl^- transport by shR is consistent with the previously reported Cl^- concentration dependency of the photocycle. In the case of shR, the value of 0.06 M for K_1 is consistent with the K_m for Cl^- transport (0.04 M) reported previously using cell membrane vesicles (*5*). The value of 6 M for K_2 was not reported before, but it may correspond to the Cl^- binding which was not saturable at concentrations above 1 M reported using NMR spectroscopy (*4*). Váró et al. carefully separated the photocycles which start from *all-trans*-retinal from those that start from 13-*cis*-retinal with or without bound Cl^-, and analyzed both of them (*6*). However, they could not find any Cl^--dependent rate constants. The reason that they could not find any Cl^--dependent rate constant in the reverse reaction (Cl^- release step) is now clear from the present results. The decrease in the Cl^- transport activity by shR was observed only above 5 M Cl^-, whereas the highest concentration examined by Váró et al. was 2 M (*6*). Because of the low affinity of the Cl^- releasing site and the apparent sigmoidal increase in the occupancy, the Cl^- dependency of the rate constant escaped from detection. Interestingly, the apparent K_d for the photocycle amplitude was reported to be 80 mM, which was significantly higher than the K_d for the Cl^--dependent spectral change of the unphotolyzed chromophore (20 mM) (*6*). The K_1 obtained in this study (0.06 M for $CaCl_2$ and 0.1 M for LiCl) seems to correspond to the former K_d. Combined with the data from the literature, we suggest that there are two kinds of Cl^- binding with apparent affinity constants of 60−100 mM and 5 M for Cl^- uptake near the extracellular surface, a Cl^- binding site with an apparent K_d of 20 mM in the vicinity of the retinal, and a Cl^- binding site(s) responsible for releasing Cl^- near the cytoplasmic surface whose K_d is as high as 7 M with a high Hill coefficient. These may be regarded as Sites A, B, and C in Figure 7A. The affinity change in Site B upon energization can induce vectorial Cl^- transport (*1*).

By studying specific mutants, Rüdiger and Oesterhelt (*25*) concluded that Arg108, together with Thr111, constitutes a specific Cl^- binding site on the extracellular side which affects anion uptake and chromophore absorption. This binding site may be regarded as Site B in Figure 7A. On the cytoplasmic side, Thr203 facilitates Cl^- transfer which is associated with N intermediate formation (L intermediate decay) (*25*). Thr203, together with Arg200 and other weakly interacting amino acid residues or water molecules, may constitute a cooperative weak binding site corresponding to Site C in Figure 7A. As for Site A in Figure 7A, there is no candidate at the molecular level now. It may not necessarily consist of a single amino acid residue.

The Cl^- transport activity of phR showed a strikingly different Cl^- concentration dependency in comparison to shR. The activity sharply increased with increasing Cl^- concentration, and this increasing phase was approximated by a single Michaelis−Menten type reaction with a K_m value of 5 mM. It decreased above 30 mM Cl^-, and this decreasing phase was expressed by a Michaelis−Menten type Cl^- binding ($K_2 = 0.2$ M) in combination with a small contribution of cooperative binding ($K_3 = 5.6$ M with $n = 10$) (Table 1).

In the photocycle of phR, Váró et al. identified N to O and O to HR′ reactions as the step where Cl^- release and uptake occur during the transport (*9*, *10*). The apparent binding constant for the step where Cl^- is taken up was calculated to be 15 mM from the rate constants of the O to HR′ reaction and the HR′ to O reaction. The apparent binding constant for the Cl^- release step was also calculated from the rate constants of the N to O reaction and the O to N reaction, and it was 0.15 M. The binding constant for the Cl^--dependent spectral change of the unphotolyzed chromophore and the photocycle amplitude was reported to be 1 mM (*9*, *10*). The K_d values of 15 mM, 0.15 M, and 1 mM may be regarded as the K_d of the Cl^- binding site near the extracellular surface, the K_d of the Cl^- binding site near the cytoplasmic surface, and the K_d of the Cl^- binding site near the retinal, respectively. In the present study, the apparent binding constant for taking up Cl^- ion (K_1) was around 5 mM, and the apparent K_d of the Cl^- releasing site (K_2) was 0.2 M. In view of the different experimental conditions and different way to calculate these parameters, our data are in surprisingly good agreement with the K_d values of 15 mM and 0.15 M reported by Váró et al. (*9*, *10*). These sites may be regarded as Sites A, B, and C in Figure 7A as in the case of shR. Recently, time-resolved photovoltage analyses were reported for phR (*15*, *26*) and shR (*26*). In both phR and shR, major charge movement was observed in the millisecond time scale, and it was proposed that N (and/or O) intermediate formation was associated with charge movement (*26*).

Together with these literature data, we tentatively suggest the following Cl^- transport sequence for phR based on Figure 7A. In the unphotolyzed state, Cl^- binds to Site A and Site B whereas Site C remains empty according to their affinity for Cl^-. In the case of phR, the apparent affinities are 5 mM, 1 mM, and 0.2 M, respectively.[2] Upon photoenergization, a conformational change of the protein induces a drastic decrease in the affinity of Site B. Thus, the interaction between Cl^- and chromophore changes in the K and L intermediates. Subsequently, Cl^- moves from Site B to Site C. Site A cannot accept Cl^- because it is already occupied. This step may correspond to the N intermediate formation which accompanies charge movement in hR. Then, Cl^- release occurs from Site C due to its low affinity to the cytoplasmic side during O intermediate formation (*9*, *10*) before the slow relaxation to the high-affinity state of Site B. A slow conformational change of the protein may occur to recover the high affinity of Site B after the dissociation of Cl^- from Site C, and Cl^- moves from Site A to site B. This step may be the second electrogenic process. Finally, Site A takes up Cl^- from the extracellular side during HR′ formation (*9*, *10*), and further relaxation occurs to the original unphotolyzed state, phR. This is in accordance with our previously proposed Energization−Relaxation Channel Model (*1*). In the case of shR, it was proposed that Cl^- is released during the N intermediate decay and taken up during the decay of the O intermediate based on resonance Raman study (*27*). Although the contribution of the O intermediate to the normal photocycle coupled with Cl^- transport in shR is somewhat controversial (*6*, *25*, *27*), combined with the data of time-resolved photovoltage measurement, we suspect that a sequence of Cl^- transfer similar to phR may also hold for shR.

In conclusion, we have examined the Cl^- concentration dependency of the transport activity of hR over a wide range. An apparent affinity and cooperativity in Cl^- binding was derived. The data suggest multiple pathways of Cl^- transfer near the extracellular and cytoplasmic surface of hR. Taking the literature data into account, a simple sequence of Cl^- transfer steps is proposed.

[2] For simplicity's sake, we ignored the minor cooperative binding of Cl^- at Site C.

ACKNOWLEDGMENT

We thank Dr. R. Needleman at the Wayne State University School of Medicine for the shR- and phR-overproducing strains and their membranes and for carefully reading the manuscript. We also thank Messers. K. Morizumi, S. Nishino, and T. Ohtaki of Toray Industries, Inc., for Lumirror and Dr. Janos K. Lanyi at the University of California for critical and helpful comments on this work.

REFERENCES

1. Muneyuki, E., Ikematsu, M., and Yoshida, M. (1996) *J. Phys. Chem. 100*, 19687−19691.
2. Lanyi, J. K. (1990) *Physiol. Rev. 70*, 319−330.
3. Oesterhelt, D. (1995) *Isr. J. Chem. 35*, 475−494.
4. Falke, J. J., Chan, S. I., Steiner, M., Oesterhelt, D., Towner, P., and Lanyi, J. K. (1984) *J. Biol. Chem. 259*, 2185−2189.
5. Schobert, B., and Lanyi, J. K. (1982) *J. Biol. Chem. 257*, 10306−10313.
6. Váró, G., Zimányi, L., Fan, X., Sun, L., Needleman, R., and Lanyi, J. K. (1995) *Biophys. J. 68*, 2062−2072.
7. Bamberg, E., Hegemann, P., and Oesterhelt, D. (1984) *Biochim. Biophys. Acta 773*, 53−60.
8. Muneyuki, E., Okuno, D., Yoshida, M., Ikai, A., and Arakawa, H. (1998) *FEBS Lett. 427*, 109−114.
9. Váró, G., Brown, L. S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R., and Lanyi, J. K. (1995) *Biochemistry 34*, 14490−14499.
10. Váró, G., Needleman, R., and Lanyi, J. K. (1995) *Biochemistry 34*, 14500−14507.

11. Hille, B. (1991) in *Ionic channels of excitable membranes*, 2nd ed., pp 362−389, Sinauer, Sunderland, MA.
12. Oesterhelt, D., and Stoeckenius, W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 853−2857.
13. Drachev, L. A., Kaulen, A. D., Semenov, A. Yu., Severina, I. I., and Skulachev, V. P. (1979) *Anal. Biochem. 96*, 250−262.
14. Holz, M., Lindau, M., and Heyn, M. P. (1988) *Biophys. J. 88*, 623−633.
15. Kalaidzidis, I. V., Kalaidzidis, Y. L., and Kaulen, A. D. (1998) *FEBS Lett. 427*, 59−63.
16. Duschl, A., Lanyi, J. K., and Zimányi, L. (1990) *J. Biol. Chem. 265*, 1264−1267.
17. Brown, L. S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R., and Lanyi, J. K. (1995) *J. Biol. Chem. 270*, 27122−27126.
18. Richter, H.-T., Brown, L. S., Needleman, R., and Lanyi, J. K. (1996) *Biochemistry 35*, 4054−4062.
19. Holz, M., Drachev, L. A., Mogi, T., Otto, H., Kaulen, A. D., Heyn, M. P., Skulachev, V. P., and Khorana, H. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2167−2171.
20. Souvignier, G., and Gerwert, K. (1992) *Biophys. J. 63*, 1393−1405.
21. Szaraz, S., Oesterhelt, D., and Ormos, P. (1994) *Biophys. J. 67*, 1706−1712.
22. Schobert, B., Lanyi, J. K., and Cragoe, E. J., Jr. (1983) *J. Biol. Chem. 258*, 14158−15164.
23. Kimura, Y., Vassylyev, D. G., Miyazawa, A., Kidera, A., Matsushima, M., Mitsuoka, K., Murata, K., Hirai, T., and Fujiyoshi, Y. (1997) *Nature 389*, 206−211.
24. Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science 280*, 69−77.
25. Rüdiger, M., and Oesterhelt, D. (1997) *EMBO J. 16*, 3813−3821.
26. Muneyuki, E., Shibazaki, C., Ohtani, H., Okuno, D., Asaumi, M., and Mogi, T. (1999) *J. Biochem. 125*, 270−276.
27. Ames, J. B., Raap, J., Lugtenburg, J., and Mathies, R. A. (1992) *Biochemistry 31*, 12546−12554.

BI9826456