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Hypothesis

Anion binding to the chloride pump, halorhodopsin, and its implications for the transport mechanism

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The light-driven chloride pump, halorhodopsin, binds and transports chloride across the membrane, and to a lesser extent nitrate. Binding and transport kinetics, and resonance Raman spectra of the retinal Schiff base, with these anions suggest the existence of two mutually exclusive binding sites. One of these may be the uptake site, and the other the release site during the transport. Plausible locations can be suggested for these sites, because halorhodopsin is a small protein with few buried positively charged residues, and the primary structure of a second pigment with similar function has recently become available for comparison.

Halorhodopsin; Retinal protein; Chloride transport; Chloride binding; Schiff base; Halobacteria

Halorhodopsin, a small retinal protein which functions as a light-driven electrogenic pump for chloride ions in *Halobacterium halobium*, will bind various anions; as a result its photoreactions are modified. These, and resonance Raman spectra of the chromophore suggest the existence of two anion binding sites. One of these (site I) is capable of binding only polyatomic anions, such as nitrate, while the other (site II) binds also monoatomic anions, such as chloride. It appears that only one of these binding sites can be occupied at a time. Binding of either chloride or nitrate to site II allows the photoproduction of the transient species, HR_L, and transport of the anion bound to this site. In this system chloride is transported at a higher maximal rate than nitrate. In contrast, in a second halorhodopsin, from *Natronobacterium pharaonis*, chloride and nitrate are equivalent with respect to transport and photoreactions, arguing that site I is modified in this protein. This may be either the replacement of Arg-103 with valine, or the reduced positive charge of the A–B cytoplasmic interhelical segment. The latter alternative is particularly interesting, because it implicates site I in chloride release during transport.

Chloride transport systems have been receiving much attention since the identification of cystic fibrosis as a defect in the transport of this anion [1,2]. The structural similarity of the putative product of the recently cloned cystic fibrosis gene to a bacterial anion (arsenate)-transport ATPase, as well as to phosphate and other bacterial translocation systems [3], suggests that there might be common principles shared by dif-

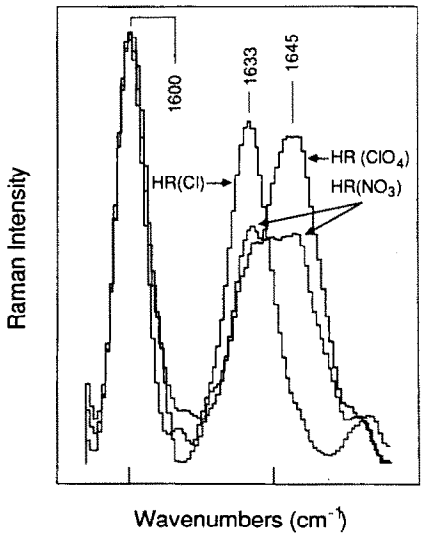

Fig. 1. Resonance Raman spectra of halorhodopsin in the presence of 200 mM NaCl, $NaNO_3$ or $NaClO_4$, as indicated. Only the Schiff base ($C{=}NH^+$ stretch) region is shown. Reprinted with permission from [14].

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ferent anion transporters. Halorhodopsin, a chloride pump, belongs to a family of small (about 15 kDa) retinal proteins with various light-dependent functions in the cytoplasmic membrane of halobacteria. Its light-driven cyclic transformations originate from the transient isomerization of the retinal, and cause the electrogenic import of chloride ions [4–6]. A simple system for the active transport of chloride, halorhodopsin might be regarded as a model for anion translocation, and following the transformations of the retinal chromophore by spectroscopic means provides a unique opportunity to study its mechanism. It might be expected that a chloride transport system would possess anion binding sites which participate in uptake and release during the translocation of the ion from one side of the membrane to the other. Some attempts to describe the properties and locations of these sites have been made earlier [7–13], since their transient occupancy during the transport cycle constitutes the basis for any model of the anion translocation.

A number of recent observations suggest that in halorhodopsin there are two interacting anion binding sites of different specificities. (i) the C=N stretch vibrational mode of the halorhodopsin Schiff base is split into two frequencies, at 1633 and 1645 cm^{-1} [14]. With chloride or bromide the intensity was exclusively

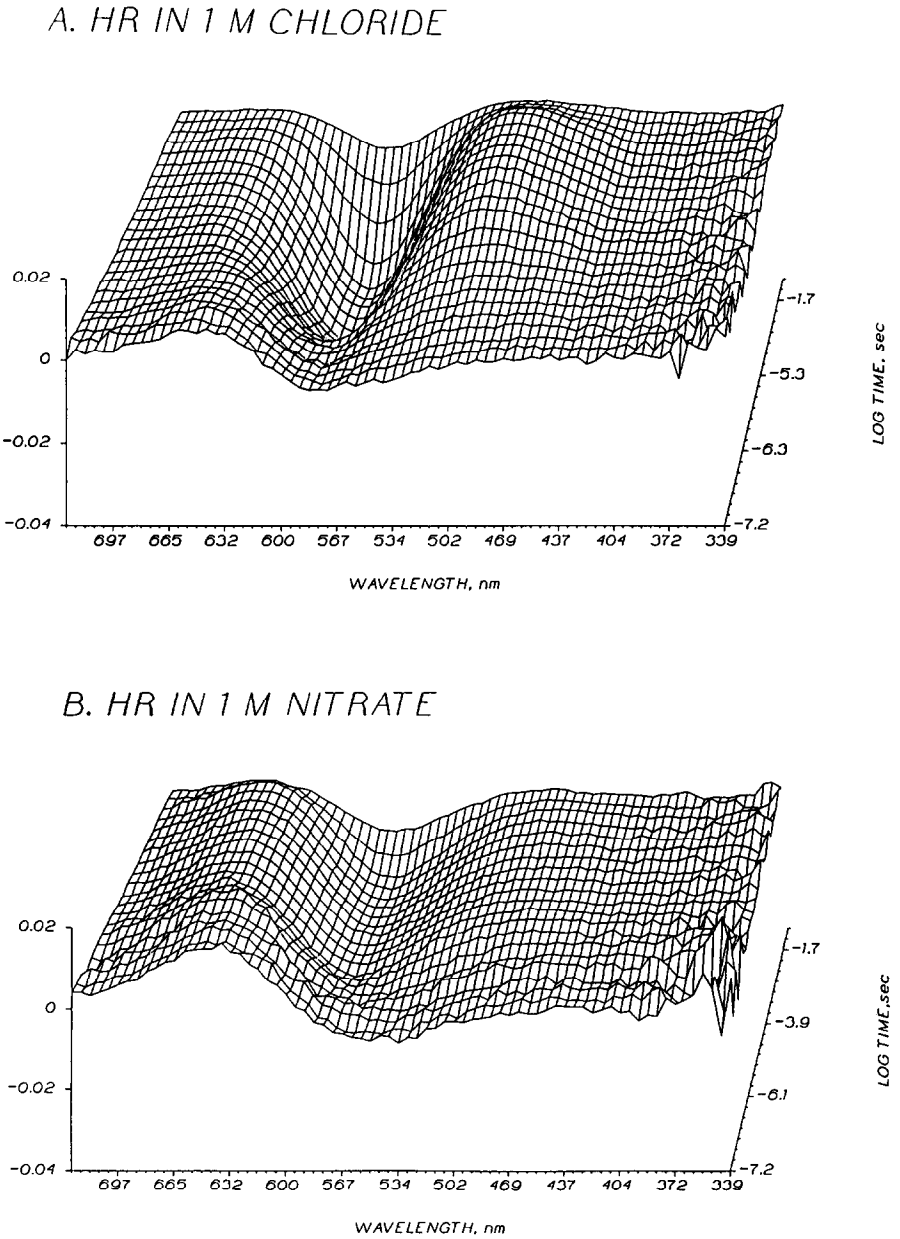

Fig. 2. Light-induced absorbance changes of the halorhodopsin chromophore in the visible region. (A) 1 M NaCl; (B) 1 M $NaNO_3$. The changes in absorbance (data from [21]) are displayed as functions of both the wavelength and the delay time between flash excitation and exposure of a gated diode array detector [20].

at 1633 cm^{-1}, with nitrate at both frequencies with about equal magnitudes, and with perchlorate nearly entirely at 1645 cm^{-1}. These spectra are shown in Fig. 1. Consistent anion-dependent changes were seen at the C=N–H bending frequency as well. (ii) The light-induced spectroscopic changes were significantly different in chloride and nitrate containing buffers. Data which show this are illustrated in Fig. 2. While the photocycle of halorhodopsin in the presence of chloride could be described as the single reaction sequence, $HR - hv \rightarrow HR_K \longrightarrow HR_{KL} \longrightarrow HR_L \longleftrightarrow HR_O \longrightarrow HR$ [15–20], in the presence of saturating amounts of nitrate about half of the photoexcited pigment entered the same HR_L-containing sequence as in chloride, but the other half generated a second sequence, which consisted of the truncated cycle, $HR - hv \rightarrow HR'_K \longrightarrow HR_{KO} \longrightarrow HR_O \longrightarrow HR$ [17,20,21]. Thus, with respect to its photoreactions also, two states of the pigment were seen. (iii) Contrary to previous reports [22–24], we have found [25] that halorhodopsin transported not only chloride but also nitrate, at a maximal rate of about 30% that of chloride. (iv) However, in *pharaonis* halorhodopsin, an analogous pigment from *Natronobacterium pharaonis* whose primary structure and properties we described recently [25,26], nitrate was equivalent to chloride both in generating an HR_L-intermediate containing photocycle and in transport.

These findings are now rationalized in a model, whose elements are as follows. (i) Two binding sites exist, sites I and II; nitrate binds to both sites, while chloride binds only to site II and perchlorate binds only to site I. (ii) Binding of nitrate to site II is largely equivalent to chloride bound at this location, i.e. HR_L is produced during illumination, and the bound anions will be transported. (iii) Binding to the two sites is mutually exclusive, hence binding of an anion to site I (e.g. nitrate or thiocyanate) removes the effects of binding to site II. In the following we discuss the details and the justification of this model, point by point, as well as (iv) some data which do not yet fit into the model, (v) the possible locations of the anion binding sites, and (vi) its implications for how the anions might be transported.

(i) *Evidence for the existence of two sites and their anion specificities*

The resonance Raman spectra [14] clearly showed the existence of two modes in which anions can affect the Schiff base: the C=N stretch frequency assumed two discrete values, and the relative intensities of the two bands, but not their positions, were affected by the choice of the anion (Fig. 1). For consistency with an earlier model [9], we call the site which binds either chloride, bromide or nitrate, site II, and the other site, which binds nitrate and perchlorate, site I. The resonance Raman results are readily interpretable in terms of the proposed model. Occupancy of site II

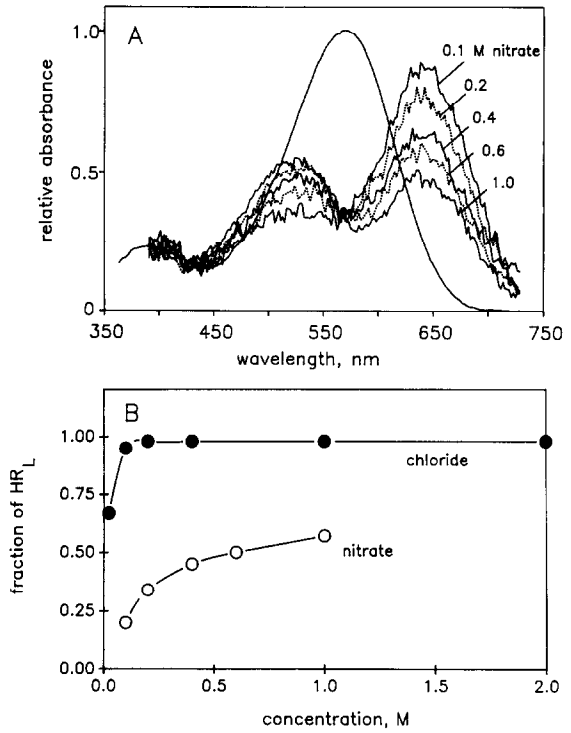

Fig. 3. Yield of HR_L in the HR photocycle, as a function of chloride and nitrate concentrations. (A) Reconstructed absorption spectra for the mixtures of intermediates observed 136 μs after the flash, at the indicated nitrate concentrations. The peaks at 520 nm and 640 nm are attributed to HR_L and HR_O, respectively. (B) Fraction of photocycling HR which produced HR_L in experiments, such as in (A), in the presence of chloride and nitrate, as indicated. Reprinted with permission from [21].

shifts the Schiff base frequency to 1633 cm^{-1}, a value which represents less coupling between the C=N stretch and the N–H rock than is seen for bacteriorhodopsin, i.e. weaker Schiff base counterion interaction. Occupancy of site I results in a higher frequency, 1645 cm^{-1}, i.e. increased coupling relative to bacteriorhodopsin, and consequently stronger counterion interaction. Hence, an anion at site I affects the Schiff base more strongly than at site II. This could be related to the distance of the two sites to the Schiff base, or to the angle of the N–H bond vector relative to the sites.

There are complexities in other anion effects on the pigment which cannot be explained with a single binding site. Nitrate converts just half the pigment, but not more, to the form which produces the HR_L containing photocycle [21], while with chloride all of the pigment is converted. This was inferred from the amplitude of HR_L at different concentrations of the anions (Fig. 3). It will be shown below how this observation is consistent with the resonance Raman results. Additionally, we find that chloride and nitrate behave competitively in eliciting absorption shifts, which produce characteristic positive or negative difference spectra for the chromophore [9,11,12], and these kinetics are also

too complex to be accounted for by a single site (unpublished experiments).

The dissociation constant of site II for chloride is unambiguous, since this binding involves a single site only. Measurements of spectroscopic and pK shifts indicated that the K_d in this case was abot 8 mM [10,11]. Half-maximal transport using a reconstituted system was also at about 8 mM chloride [23]. The dissociation constants of the two sites for nitrate are probably somewhat higher than for chloride, but they are more difficult to evaluate because of competitive effects between the two sites where nitrate can bind.

(ii) *Consequences of anion binding to sites I and II*

In the absence of added anions, the pigment produces only the truncated photocycle, lacking HR_L [15]. When an anion (either chloride or nitrate) is bound to site II, the HR_L containing reaction sequence is produced upon illumination [7,15–18,21], and the rate of transport is in proportion to the transient production of HR_L [7]. When an anion (nitrate, thiocyanate, perchlorate) is bound to site I, it appears to produce no effect other than increasing the proportion of the truncated photocycle, at the expense of the HR_L containing photocycle. With nitrate, which binds to both sites, the outcome is determined by the balance of these two effects. Thus, it appears that an anion is transported only when bound to site II, and transport is always accompanied by the photocycle which contains HR_L. Occupancy of site I, on the other hand, suppresses both transport and the production of HR_L.

(iii) *Evidence for mutual competition between the two binding sites*

As suggested under (ii), when site II is occupied, addition of anions which bind to site I will have the effect of increasing the fraction of halorhodopsin which cannot produce HR_L. Furthermore, since this kind of photocycle is seen also in the absence of bound anions, occupancy of site I appears to influence the photoreaction *only* in that it will decrease the effect of site II. This concept is developed further by considering the effects of anions on the halorhodopsin photoreactions. As shown in Fig. 3, while in the presence of saturating concentrations of chloride only the HR_L-containing photocycle is observed, at saturating concentrations of nitrate both photocycles are seen, and to about equal (half) extents [21]. The latter finding corresponds to the persistence of two stretching frequencies for the Schiff base, with about equal (half) amplitudes (Fig. 1), at saturating nitrate concentrations [21]. The two-site model accounts for these results only if nitrate can bind to both sites, but *only one site can be occupied at a time*. As expected from this, addition of nitrate to a chloride-containing sample will decrease the amount of HR_L produced (our unpublished observations). The extent of the transport of nitrate relative to chloride, and the inhibition of nitrate on chloride transport [25] are also consistent with this idea.

(iv) *Data which do not fit*

Although the presence of two C=N stretch bands of about half-maximal intensity at saturating concentrations of nitrate is clearly related to the observation that half of the halorhodopsin enters the HR_L containing photocycle while the other half enters the HR_L lacking photocycle under these conditions, the dependencies of the two kinds of effects on the nitrate concentration do not quite agree. The two C=N stretch frequencies were observed already at 50 mM nitrate, and their ratio did not change greatly up to 400 mM nitrate [14]. The yield of the HR_L intermediate, in contrast, approximately doubled in this concentration range, before reaching a plateau (Fig. 3 and [21]). Thus, the simple model described above, which requires a 1:1 correspondence between the state of the Schiff base and the photoreaction of the pigment, is probably not correct. A possible modification of the model might be the introduction of structured binding sites, which allow binding equilibria among multiple species and thereby dissociate the state of the Schiff base somewhat from the photoreaction of the pigment.

(v) *Possible locations of sites I and II in the protein*

There is evidence that chloride binds to halorhodopsin on the extracellular side of the membrane [7,27], and this defines the probable location of site II: it is almost certainly where chloride and nitrate bind prior to their translocation. The location of site I is more problematical. There are two alternatives: (a) sites I and II are in close proximity, or (b) sites I and II are

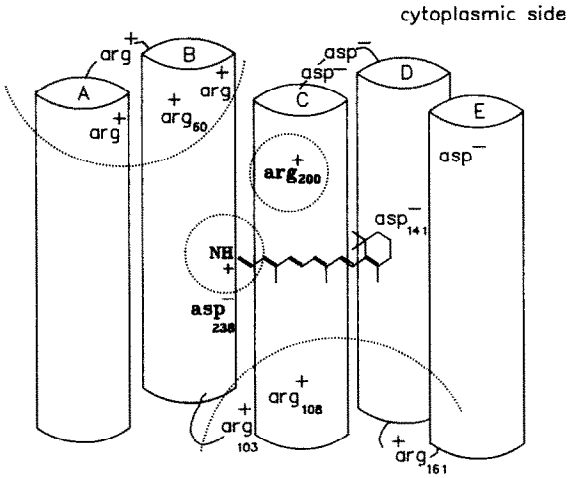

Fig. 4. Possible structural model for the anion binding sites of halorhodopsin. The arrangement of helices is as in [28]. Helices F and G were removed for clarity; residues located on these helices are shown in bold characters. Residues or positively charged regions, which might participate in anion binding as discussed in the text, are indicated with dotted arcs and circles.

not in close proximity, but are coupled by an allosteric mechanism. In alternative (a) both sites face the extracellular side, but in alternative (b) site I might face the cytoplasmic side. There is no evidence as yet which would favor either of these, but the small number of positively charged residues in the two halorhodopsins allows only a few possibilities. The plausible secondary and tertiary structure of halorhodopsin [28] includes 3 buried arginine residues, Arg-60, Arg-108, and Arg-200. Fig. 4 shows a crude structural model for halorhodopsin (with helices F and G removed for clarity), in which the locations of these residues relative to the retinal are shown. The structure is justified by the similarities of halorhodopsin and bacteriorhodopsin [13,28]. The majority of positively charged residues (8 out of a total of 11 arginines) in the protein are excluded from consideration, since their chemical modification did not significantly alter chloride binding [29]. As shown in Fig. 4, Arg-103, Arg-108, and Arg-161 are located on the extracellular side of the Schiff base, and are thus the primary candidates for site II. The two other buried positively charged residues, Arg-60 and Arg-200, are closer to the cytoplasmic side, and are surrounded by several exposed arginine residues. Direct binding to the Schiff base seems to be excluded by the pH independence of the anion binding [11], the way the apparent pK_a of the Schiff base increases with anion concentration [9], and the absence of expected downshifts in the Raman frequencies with varying the anion [14]. However, it is possible that during the translocation cycle the anion interacts directly with the Schiff base [30].

Since *pharaonis* halorhodopsin hardly discriminates between chloride and nitrate [25], it contains, presumably, a modified site I. There are two major differences in the primary structure of these proteins, which might account for this. One possibility is that the absence of site I type binding is related to the replacement of Arg-103 with valine in the *pharaonis* pigment [26]; on the basis of its accessibility to trypsin cleavage [31], Arg-103 is localized at the extracellular end of helix C. The 3 arginine residues, located on the extracellular side of the retinal in the space enclosed by the seven-helical structure in halorhodopsin (shown with a dotted arc below the retinal in Fig. 4), would thus form both sites I and II. Competition in this case would arise because of steric or electrostatic exclusion of the binding of a second anion in this region. The second possibility is that the absence of site I in *pharaonis* halorhodopsin originates from the decrease of the net charge of the A–B helical end-region from +4 to +1 [26]. This segment, containing most of the aqueous arginine residues and located on the cytoplasmic side, is indicated with a dotted arc above the retinal in Fig. 4. Arg-251 and Arg-258 are located near this region, on the cytoplasmic end of helix G (not shown); in *pharaonis* halorhodopsin these residues are also missing. The functional role of these residues in transport is very likely in the release of chloride after the translocation [13], since the rest of the cytoplasmic surface is negatively charged (Fig. 4). Although the available evidence suggests that binding at the A–B helical connection does not have the properties expected for site I (strict selectivity against chloride and influence on the C=N stretching frequency of the Schiff base), access of an anion to Arg-60 and/or Arg-200 may require this positively charged domain. In this case, site I would consist of Arg-60 and/or -200, residues oriented toward the cytoplasmic side, and competition with the distant site II could be only by an allosteric effect.

(vi) *Implications for chloride transport*

The possibility that site I is located on the cytoplasmic side is attractive since the site for the uptake of the transported anion (site II) would then alternate with the site at or near the release of the anion (site I), as proposed in many schemes for the translocation of substrates by membrane carriers. The implication is that, as required in these models, *halorhodopsin exists in two conformations whose equilibrium is determined by occupancy of the uptake and release sites, and the protein passes through these conformations during the transport cycle.* This kind of mechanism was not invoked for proton transport by the related retinal protein, bacteriorhodopsin [6], and may be required for the translocation of larger ions, such as chloride, only. The apparent absence of site I in *pharaonis* halorhodopsin would mean that in this system the anion is released differently during the transport than in halorhodopsin. The fact that an HR_O-like intermediate, produced in halorhodopsin by chloride release in the $HR_L \longrightarrow HR_O$ reaction [16–19], was not observable in the photocycle of *pharaonis* halorhodopsin [25] also suggests this.

The strategic location of the Schiff base between the two regions facing the two membrane surfaces, and its movements during the isomerization of the retinal, suggest its direct involvement in the translocation mechanism. A proposed mechanism for chloride transport [6,30] includes the transient association of the chloride ion with the Schiff base, which during the 13-*cis*/13-*trans* isomerization of the retinal assumes the role of the 'switch' in the pump.

Remarkably, both buried arginine residues 108 and 200 in Fig. 4 are found also in bacteriorhodopsin, but this pigment does not exhibit chloride dependent effects. As recognized before [6,13,28], the critical difference between the two proteins may be the scarcity of negatively charged residues in halorhodopsin, which would allow the binding of anions to the arginines. Indeed, at low pH where at least some of its acidic residues are protonated, bacteriorhodopsin will bind chloride, and its photoreactions resemble those of halorhodopsin [32–34]. Recent results [35] indicate

that oriented bacteriorhodopsin sheets at pH near 1 produce a sustained photocurrent in the presence of chloride, but not in its absence. It is an intriguing possibility that this observation might originate from chloride transport by bacteriorhodopsin under these conditions.

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