Evidence for a Halide-binding Site in Halorhodopsin*

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In attempting to describe a halide-binding site in halorhodopsin (P_{580}), a light-driven chloride pump in halobacterial membranes, we have investigated the effects of chloride and bromide on flash-induced absorption changes in this pigment, and studied the effects of a diuretic drug, MK-473, on the photochemistry and the transport. We find that at high sulfate or phosphate concentrations, but in the absence of halide, the principal photointermediate is P_{580}, whose half-life is about 1.5 ms. When chloride or bromide are added, the production of P_{580} is depressed and its half-life becomes longer (up to ~10 ms). With increasing halide concentration, the cycle proceeds more and more via the alternative photointermediate, P_{520}, whose half-life varies with the halide concentration in a fashion similar to that of P_{580}. Transport activity, measured during sustained illumination, increases in a manner parallel to the accumulation of P_{520} up to about 400 mM halide, but declines at concentrations above this value. The transport is inhibited by MK-473 with competitive kinetics, and the effects of this inhibitor on the photocycle are also consistent with displacement of halide ions from their binding site. The observations reported here suggest that chloride and bromide bind to P_{580}, P_{560}, and P_{520}, and that this binding is to a distinct site on the protein.

We have recently shown that halorhodopsin, a light-driven electrogenic pump in the cytoplasmic membrane of Halobacterium halobium (14-15), will transport chloride ions inward across the membranes, against an electric and a concentration gradient (5). Up to about 300 mM, the dependency of the transport on chloride concentration obeyed Michaelis-Menten kinetics. Half-maximal transport rates, as determined from initial rates of the development of membrane potential or light scattering decrease in cell envelope vesicle suspensions, were at about 40 mM chloride. Bromide was the only other anion to replace chloride as a substrate of this transport system, although recently we found that iodide is also transported.†

Halorhodopsin was identified as a retinal protein similar to, but distinguishable from bacteriorhodopsin, with distinct spectroscopic (6-8) and photochemical properties (8-11). Chloride salts have a number of effects on the chromophore. The absorption band of halorhodopsin is red-shifted about 10 nm in the presence of chloride, relative to its position in water (7, 8). Its photocycle intermediates and kinetics are altered, in the presence of salt, a slower photocycle involving photointermediates P_{580}, P_{560}, and P_{520} (9, 10) was described, but in its absence a more rapid photocycle via photointermediates P_{520} and P_{580} was seen (10). We have studied the effects of chloride on the chromophore of halorhodopsin (12), but in high ionic strength salt solutions, such as 1.5 M Na_{2}SO_{4} or 3 M phosphate which preserve the integrity of the vesicle membranes, and allow correlating the light-dependent absorption changes with transport properties, recovery from flash-induced bleaching of the pigment under these conditions was much slower in the presence of chloride, and the amplitude of the absorption change was severalfold greater (12), i.e. the altered photocycle kinetics were consistent with the earlier described (10) effects of NaCl. We reported (12) that a chloride-dependent equilibrium exists between the 580-nm native form of halorhodopsin and a 410-nm form, which is formed in the dark probably by deprotonation of the Schiff’s base at pH values above 7. The apparent affinity constant for chloride in these effects was also about 40 mM (12).

The buffers we used contained high salt concentrations. Thus, the chloride-dependent effects were unlikely to be caused by the contribution of this anion to ionic strength. Rather, it would seem that it is the specific binding of the chloride to a binding site which was responsible for the effects on the chromophore. In the work reported here, we attempted to define further, the effects of chloride (and bromide) on the halorhodopsin photocycle, and relate these to transport. Our goal was to find out if a chloride-binding site exists in halorhodopsin, similar, for example, to the site in the band 3 anion transport protein in erythrocyte membranes (13).

**MATERIALS AND METHODS**

Halobacterium halobium strain L-33 was grown, and cell envelope vesicles prepared as previously described (14, 15). The vesicles were stored in 4 M NaCl, and dialyzed overnight against either 1.5 M sodium sulfate or 3.0 M potassium phosphate whenever removal of the chloride was desired. Most experiments were duplicated in both sulfate and phosphate solutions, and with added chloride or bromide. Chloride and bromide were added as 4 M sodium salts to the sulfate and the phosphate. The additions diluted the sulfate and the phosphate in proportion to the increasing halide concentrations, keeping the ionic strength of the salt mixtures nearly constant in all experiments.

Transport activity was determined as previously described (5), by following light-scattering decrease due to swelling of the vesicles, and by following the initial rate of fluorescence decrease (within the first 5 s) with added 5,5'-dipentyloxadecarbocyanine, a membrane potential-sensitive dye (5, 10, 17). The rates are expressed as per cent light-
intensity change/min and per cent fluorescence change/s for the two methods, respectively. In all studies, such as chloride dependency, the effects of inhibition, etc., the results with the two methods were in good qualitative agreement. With the inhibitor MK-473 present, the time-course of the light-scattering changes was not linear and for this reason the transport rates were estimated from the fluorescence changes.

The inhibitor MK-473 showed nonspecific binding to the halobacterial membranes. The concentration of free MK-473 in vesicle suspensions was estimated by centrifuging 1-ml assay mixtures in Amicon Centrifree micropartition tubes at 3,000 rpm for 5 min, collecting about 0.2 ml of filtrate, and after dilution with water, measuring the absorbance of the inhibitor at 277 nm.

Flash-dependent absorption changes were determined with the instrumentation described previously (12), but with modifications which allowed measurements at various wavelengths. All photocycle measurements were signal averaged over 512 flashes. For determination of flash-induced absorbance changes at and below 580 nm, the flash was through a 610-nm long-pass filter, and the photomultiplier was protected with a combination of interference and cut-off filters appropriate to the wavelength of measurement. For absorption changes at 620 nm and above, the flash was through a combination of a 580-nm interference filter and a 580-nm short-pass filter. The photomultiplier in the latter case was protected with a 610-nm long-pass filter, which did not completely eliminate flash artifact from the traces. Because the photointermediate of halorhodopsin was visualized by subtraction of two traces (as shown in "Results"), in these instances the matching of transmitted light in the two samples was by adjusting the high voltage of the photomultiplier. Thus, the flash artifact was the same in the two samples and the subtraction cancelled it.

The compound MK-473, [6,7-dichloro-2-cyclopropyl]-2-methyl-1-oxo-1,2-dihydroinden-5-siloxyacetic acid (mixture of two enantiomers), was synthesized as described earlier (18, 19).

RESULTS

The Two Alternative Photocycling Pathways of Halorhodopsin—Flash-dependent absorption changes in L-33 vesicles were measured at 570 nm in the absence and presence of halide (Fig. 1, A and B). Each trace shows the fast and the slow component described earlier (12), which correspond to halorhodopsin and slow rhodopsin, pigments present in membranes prepared from bacteriorhodopsin-negative H. halobium strains, such as L-33 (11, 20). The photobleaching of halorhodopsin, resolved from that of slow rhodopsin, shows greater increase in magnitude and its recovery becomes slower after 2 mM bromide is added (12 ms half-life in Fig. 1B versus 1.5 ms in Fig. 1A). This effect is even more evident in samples which had been partially bleached with hydroxylamine (Fig. 1, C and D) to remove most of the slow rhodopsin (12). Slow rhodopsin also responds to halide, but this effect is small (12).

A difference in the photocycling of halorhodopsin in the presence and absence of halide is seen also at other wavelengths. The rise and decay of a photointermediate at 500 nm (cf. Ref. 9) is evident in the presence of bromide (Fig. 2A). Only the bleaching of slow rhodopsin, which absorbs somewhat at this wavelength, is seen in the absence of the halide (Fig. 2B). The difference of these traces, shown in Fig. 2, A minus B, represents the absorption changes due to halorhodopsin alone. The photointermediate of halorhodopsin in Fig. 2, A and B decays with a half-life of 11 ms. This method of subtracting traces obtained with and without halide present was used throughout, because from Fig. 2B it appears that little or none of the photointermediate is produced in the absence of halide, and the subtraction produced a trace (Fig. 2, A minus B) with virtually no contribution from the slow pigment. At 660 nm, complex absorption changes are seen, because of overlapping with the halorhodopsin and slow rhodopsin absorption bands, and a flash artifact, both with and without bromide (Fig. 2, C and D). The difference between these traces, shown in Fig. 2, D minus C, suggests that halorhodopsin (designated as P580) gives rise to a red-shifted photointermediate in the absence of halide. This intermediate decays with a half-life of 1.5 ms. Thus, the recovery kinetics of P580 (e.g., in Fig. 1) match the decay of the 500-nm inter-

Fig. 1. Flash-induced absorbance changes in H. halobium strain L-33 vesicles at 570 nm. A and B, untreated vesicles in 3 M potassium phosphate, pH 7.0, without (A) and with (B) NaBr added just before the experiment so as to give a final concentration of 2 mM bromide and 1.5 M phosphate. C and D, vesicles bleached 6 h with hydroxylamine and washed as in Ref. 12, suspended in 3 M potassium phosphate, pH 7.0, without (C) and with (D) NaBr added as for B. Scale for the absorption changes is expressed as per cent change in light-intensity per flash. Upward deflection refers to absorption increase. Protein concentration in all cases, 10 mg/ml.

Fig. 2. Flash-induced absorbance changes in H. halobium strain L-33 vesicles at 500 nm (A and B) and 660 nm (C and D). B and D, vesicles suspended in 1.5 M sodium sulfate. A and C, vesicles suspended in sulfate with NaBr added just before the experiment. Scale for the absorption changes expressed as per cent change in light-intensity per flash. Protein concentration in all cases, 10 mg/ml.

\[\text{J. K. Lanyi, unpublished results with a halorhodopsin deficient strain.}\]
mediate in the presence of 2 M bromide, and the decay of the 660-nm intermediate in the absence of bromide.

Flash-induced absorbance changes were followed at wavelengths between 580 and 440 nm. At wavelengths of 500 nm and below, the subtraction method produced net traces which could be assigned, as a first approximation, to one intermediate because the decay was described largely by a single exponential. About 500 nm, however, subtraction of the trace without halide added caused a small error due to overlapping of the absorption changes reflecting the rapidly decaying component in the absence of halide (Fig. 1A). Correction for this error was by curve peeling and discarding the fast component. The absorption change amplitudes obtained in this way are shown as a function of the wavelength of measurement in Fig. 3. The data agree well with flash-induced difference spectra of halorhodopsin reported by other laboratories (9, 11).

Although the absorption maximum of halorhodopsin is near 580 nm, it will have some absorption and therefore show bleaching, at all wavelengths in Fig. 3. Likewise, the blue-shifted photointermediate will absorb, and therefore cause absorbance rise, at 580 nm. Thus, the measured flash-induced difference spectrum underestimates both the absorbance rise below 540 nm, and the absorbance decrease at higher wavelengths, and shifts the position of the absorbance rise maximum to lower wavelengths. In attempting to correct for this, we have taken an absorption spectrum for purified halorhodopsin, \(^\text{3}\) and scaled it to the absorption change at 580 nm. The absorption values obtained in this way for wavelengths below 580 nm were added to the measured changes (solid line in Fig. 3), so as to yield a first approximation for a corrected difference spectrum (small dashed line, Fig. 3). However, the blue-shifted photointermediate contributes to the observed change at 580 nm as well. To correct for this, we assumed that the absorption peak of the photointermediate is symmetrical, and that its contribution to the measured absorption change at 580 nm can be estimated from the mirror image of the peak across its maximum. The difference spectrum was then recalculated by rescaling the contribution of bleaching at all wavelengths to the new value of the change at 580 nm. Repeated iterations, with alternating corrections for absorption changes due to the bleaching of \(P_{\text{p}}\), and to the production of the photointermediate, yielded little further change in the difference spectrum beyond the third approximation (interrupted line) shown in Fig. 3. The result of these calculations is an estimate of the absorption spectrum of the blue-shifted intermediate, which appears to be centered near 520 nm (Fig. 3). Also included in Fig. 3 is the first order rate constant of the decay at different wavelengths, which is essentially constant between 440 and 580 nm, showing that under these conditions, the recovery of the absorption of \(P_{\text{p}}\) and the decay of the 520 nm photointermediate are well matched. On the basis of these results, we conclude that in the presence of halide there is one principal photointermediate in this wavelength region, and we designate it as \(P_{\text{p}}\).

The flash-induced absorption changes between 620 and 690 nm were determined in a similar way by subtracting traces obtained in the presence of bromide from traces obtained with bromide absent (as in Fig. 2, right). At 660 nm, the subtraction yielded a trace with a single exponential decay (as in Fig. 2), but at wavelengths below this, a contribution from the bleaching of \(P_{\text{p}}\) was also seen, with much slower decay than the principal photointermediate. Corrections for this error were made by curve peeling, as in the case of \(P_{\text{p}}\). The flash-induced difference spectrum obtained in this way is shown in Fig. 4. Successive approximations, carried out similarly to those in Fig. 3, produced a more symmetrical difference spectrum, but did not change the position of its maximum (not shown). In the absence of halide, therefore, the principal

\(\text{3} \) M. Steiner and D. Oesterhelt, personal communication.
photointermediate produced absorbs at 660 nm, and we label it $P_{560}$. This photointermediate may be the same as the one described earlier as $P_{540}$ in the absence of salt (10).

Effects of Halides on Photocycle Kinetics—The production and decay of $P_{540}$ and $P_{660}$ were followed at different intermediate bromide concentrations, and visualized by the subtractive method used in Fig. 2. The traces obtained at 500 nm and at 660 nm are shown in Fig. 5, A and B, respectively. It is evident from these traces that addition of increasing amounts of halide results in the increasing production of $P_{540}$ and the decreasing production of $P_{660}$. Furthermore, the rate of decay for both of these photointermediates becomes progressively slower with increasing concentrations of the halide.

Experiments, such as shown in Fig. 5 were carried out both in phosphate and sulfate, and with chloride and bromide, in order to more completely describe the effects of halides on photocycle kinetics. Fig. 6A shows the amplitude of absorption changes at 500 nm, as well as the decay rate constant for $P_{540}$ in phosphate containing various concentrations of bromide. Fig. 6B contains the same parameters for the absorption changes at 570 nm, as well as the initial rates of transport in the same vesicles. Transport activity was determined by both light-scattering and fluorescence change (former not shown), as described under “Materials and Methods.” As found before (5, 12), half-maximal absorption changes and transport activity is at about 40 mM halide. In addition, at bromide concentrations higher than 0.5 M, inhibition of the transport is seen, a finding similar to what has been observed with the anion transport system in erythrocytes (21). The rate constant of decay decreases with increasing halide concentration at both wavelengths, as shown already for 500 nm in Fig. 5A. At the higher bromide concentrations, the decay rate constant at 500 nm agrees with the recover constant rate at 570 nm.

The same general pattern is seen with chloride (Fig. 6, C and D), and with bromide and chloride in sulfate (Fig. 7), but some significant differences are evident. In sulfate, the rate constants are higher at low chloride concentrations (for example, for $P_{540}$ in sulfate, Fig. 7C), and the $P_{540}$ decay rate varies with halide concentration differentially from the recovery rate (compare the decay rate-constant at 500 nm in sulfate and chloride, Fig. 7C, with the recovery rate constant at 570 nm with the same salts, Fig. 7D). Most significantly, at low halide concentrations, the decay of $P_{540}$ is faster than the recovery of $P_{540}$, a finding which suggests the existence of an additional photointermediate arising from the decay of $P_{540}$, and whose decay rate constant should match the recovery rate constant at 570 nm. We have not been able to find such a photointermediate between 400 nm and 690 nm, however. In addition, we could not detect $P_{540}$, described earlier as a photointermediate of halorhodopsin with a rise time of 0.4 ms and a decay time of 10 ms (9, 10). $P_{540}$, an early photointermediate of the photocycle, decays too rapidly to be detected with $\mu$s resolution at room temperature (9, 10), and therefore cannot be placed between $P_{540}$ and $P_{560}$.

We considered the possibility that the effect of halides on the decay of $P_{540}$ and the recovery of $P_{540}$ might be due to binding to a site on the vesicle interior. The half-life of a chloride gradient in sulfate was earlier found to be about 30 min for these vesicles (5). Thus, the permeability would allow for substantial accumulation of chloride during the few min required for the experiments shown in Figs. 6 and 7 at the higher chloride concentrations, but not at the lower chloride concentrations. Vesicles equilibrated with sulfate were therefore suspended in sulfate containing 200 mM chloride at 0 min and absorption changes were followed at 500 nm, as in Fig. 7C, at various times up to 80 min. No changes in the
decay rate constant were observed (data not shown), even though during the experiment, the internal chloride content of the vesicles should rise from 0 to near 200 mM. This finding would seem to rule out a role for a site internal to the vesicles in the effects described in Figs. 6 and 7.

It is evident in Figs. 6 and 7 that half-maximal transport rates are seen at halide concentrations which are near 40 mM, but are somewhat higher under some of the four experimental conditions used; chloride in sulfate and phosphate. The production of P_{	ext{seg}} paralleled the transport activities.

Effects of a Chloride-Transport Inhibitor on Halorhodopsin—We tried a number of commonly used inhibitors of chloride transport in eukaryotic membranes (18, 22) and cerebral tissues, were inhibitory to halorhodopsin. This includes loop diuretics (e.g. ethacryninc acid, furosemide, bumetanide, osolzone, and mesalyl), compounds that react with SH-groups (e.g. 1-chloro-2,4-dinitrobenzene, N-ethylmaleimide, and 5,5'-dithiobis-(2-nitrobenzoic acid)) and others (e.g. tizoileimide, dipiridamole, and pyridoxal-5-phosphate). Fig. 8 shows Lineweaver-Burk plots for the chloride dependence of transport without inhibitor up to 400 mM, and at two concentrations of MK-473. It is evident that the V_{	ext{max}} is unchanged, but the apparent K_{m} for chloride increases with increasing inhibitor concentration. The data for MK-473 thus fit competitive inhibition kinetics, suggesting that this compound functions as an analogue of chloride. This idea was tested by examining the inhibitory effect of MK-473 at 50 and 200 mM chloride. Fig. 9A shows per cent inhibition as a function of free MK-473 concentration. The inhibition curves are sigmoid, and Hill plots of the data (Fig. 9B) reveal a high degree of cooperativity (slopes between 3.5 and 4). The affinity of the inhibitor for halorhodopsin is clearly higher at 50 mM

Other commonly used inhibitors of chloride transport, such as 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonate and 4,4'-disothiocyanato-2,2'-stilbenedisulfonate which contain a sulfonate group (13), and are based on the affinity of the chloride exchange system of erythrocyte membranes to sulfate, had no effect on halorhodopsin. Likewise, many other inhibitors of the erythrocyte system (13), of chloride transport, and of other systems were not inhibitory to halorhodopsin. This

The abbreviation used is: MOPS, 3-(N-morpholino)propanesulfonic acid.

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**Scheme 1**

Cl

CH3

CH2COOH

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**Fig. 7. Effects of bromide (A and B) and chloride (C and D) on halorhodopsin photocycle kinetics and transport activity.** L-33 vesicles were suspended in 1.5 M sodium sulfate and in sulfate plus halide as in Fig. 2, to a final concentration of 10 mg/ml protein. Photocycle parameters and transport activity determined as in Fig. 6. Symbols as in Fig. 6.

**Fig. 8. Lineweaver-Burk plot of the chloride dependence of transport in the presence and absence of the inhibitor MK-473.** L-33 vesicles were suspended (to 0.13 mg/ml protein) in 1.5 M sodium sulfate, 100 mM MOPS, pH 6.6, with added chloride. Transport was determined from the initial rate of fluorescence quenching of a cyanine dye (added to 1.3 p~), as described under "Materials and Methods." A large portion of MK-473 was nonspecifically absorbed to the membranes, and the concentrations shown (10 and 20 mM) refer to free inhibitor concentration in the assay mixtures, determined as described under "Materials and Methods."
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chloride relative to that at 200 mM. Thus, chloride and MK-473 appear to be competitive with one another, supporting the idea that a site exists which binds both species.

Fig. 10 shows the effects of MK-473 on the flash-induced absorption changes at 570 nm and at 500 nm at different bromide concentrations. Surprisingly, the amplitude of the photobleaching of slow rhodopsin at 570 nm is reduced by the inhibitor, an effect seen particularly clearly in the absence of halide. The flash-induced changes due to halorhodopsin are affected in a manner which suggests decreased halide binding; at both 570 nm and 500 nm, the amplitudes are reduced and the decay rates are increased. This result is consistent with the competitive displacement of chloride by MK-473 from its binding site, the same conclusion we could draw from the inhibition kinetics (Figs. 8 and 9). In the absence of bromide, the bleaching of halorhodopsin is greater in the presence of the inhibitor (but with no change in decay rate), a finding for which we have no explanation at present.

**DISCUSSION**

Earlier results on the effects of chloride salts on the halorhodopsin photocycle (10, 12) are here extended under conditions which allow the comparison of absorption changes with chloride transport activity. There are two significant findings: (a) When halide is added at concentrations near its apparent affinity constant for transport, the principal photointermediate is identified as 
P520, but in the absence of halide it is found to be 
P360 (Figs. 2, 3, and 4). As described before (12), the photobleaching amplitude is severalfold greater in the presence of halide, consistent with this change (Figs. 1, 6, and 7). At high halide concentrations, the decay of 
P520 matches the recovery of 
P360 (Fig. 3). At intermediate halide concentrations, however, the decay of 
P520 is more rapid than the recovery of 
P360 (Figs. 6 and 7), suggesting the existence of another photointermediate, which should accumulate under these conditions. This intermediate has not been found yet. (b) The decay rates of both 
P520 and 
P360 become slower at increasing concentrations of halide (Figs. 5, 6, and 7). This observation suggests that rapidly decaying halide-free forms of these photointermediates are in rapid equilibria with slowly decaying halide-bound forms. Thus, the effects of halides on halorhodopsin are of two kinds: diversion of the photochemical events from the production of 
P520 to 
P360 upon halide binding, and decrease of the decay rates of the photointermediates upon halide binding. We have no reason to suppose that these effects are due to different halide-binding sites.

The results, together with earlier ones which suggested a chloride-dependent equilibrium for halorhodopsin in the dark (12), lead to a model for the photochemical changes in halorhodopsin (Fig. 11). In this model, chloride binding causes only small (7, 8, 10) band shifts, but has readily observable effects on the pathway of photochemical changes for halorhodopsin (shown as 
P560) and on the rates of thermal decay for 
P520 and 
P360. For clarity, the postulated additional photointermediate, to be placed between 
P520 (and possibly 
P360) and 
P560, is not included. The model in Fig. 11 raises a number of questions, which cannot be answered at present: (a) Is the binding of the halide at a single site? (b) Are the effects observed for the photolysis caused by the binding of the same halide ion which is transported? (c) Are any of the effects of the halides due to secondary consequences of the translocation (e.g. conformational changes in the protein), rather than to halide binding per se? (d) Are there chloride-dependent equilibria which involve chloride on the inside of the vesicles, rather than on the outside, as implied by the model? (e) At what point in the model is the halide ion transported across the membrane?

The existence of a specific halide-binding site in halorhodopsin is strongly supported by the finding that MK-473, a chloride analogue with hydrophobic properties, shows competitive inhibition kinetics for the transport (Figs. 8 and 9), and reverses the halide-dependent effects on the photocycle (Fig. 10). The analogy between halorhodopsin and the erythrocyte chloride transport system is uncertain. Some compounds bearing structural similarities to MK-473 (e.g. ethacrynic acid) are inhibitory to chloride translocation by the band 3 anion transport system in erythrocytes (25), but this

**Fig. 11. Proposed model for the effects of halides on the photocycle of halorhodopsin.** Halorhodopsin is shown as 
P560. Halide-dependent equilibria are proposed for 
P560 and for the photointermediates 
P520 and 
P360. Half-lives of decay are shown for halorhodopsin suspended in sodium sulfate with added chloride (cf. Fig. 7). Halide binding to 
P560 diverts the photocycle from 
P520 to 
P560. Halide binding to the photointermediates causes significant slowing of the decay rates. hv refers to the action of light.
system functions as a passive anion exchange system, rather than as an electrogenic pump. Indeed, most of the inhibitors of the band 3 chloride transport system have no effect on halorhodopsin. It is surprising therefore, that the concentration dependence of the chloride transport by halorhodopsin resembles that in the erythrocyte system (21), showing half-maximal saturation around 50 mM chloride and significant inhibition at higher concentrations, between 0.5 and 2 M (Figs. 6 and 7). In the case of band 3, the inhibition is attributed to a second, low affinity site for chloride (21, 26), but we have no evidence so far for this in halorhodopsin (27). Since the transport activity is higher in 4 M NaCl than the maximum attainable in sulfate or phosphate with chloride added (5), the intrinsic activity of halorhodopsin without the chloride inhibition must be considerably higher than what we have detected. It should be mentioned, however, that the physiological chloride concentration in halobacterial cultures is always greater than 3 M, well above the concentrations at which halorhodopsin exhibits the increase and the decrease in transport activity described in this work. The apparent affinity constant of halorhodopsin for chloride thus cannot reflect a physiological requirement, but might originate in the constraints which apply to the specific binding of anions by proteins altogether.

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