Halide Binding by the Purified Halorhodopsin Chromoprotein

I. EFFECTS ON THE CHROMOPHORE*

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The halorhodopsin chromoprotein, a retinal-protein complex with an apparent molecular mass of 20 kilodaltons, exhibits all of the halide-dependent effects found for the chromophore of functional halorhodopsin in cell envelope vesicles. With increasing halide concentration (a) an alkali-dependent 580/410 nm chromophore equilibrium (attributed to reversible deprotonation of the retinal Schiff's base) is shifted toward the 580-nm chromophore and (b) the flash-induced photocycle proceeds increasingly via $P_{580}$, rather than via $P_{410}$. The halide-binding site(s) responsible for these effects must reside, therefore, in the chromoprotein. Chloride and bromide are about equivalent, but iodide is much less effective in these effects and in being transported. Several other anions, i.e., thiocyanate, nitrate, phosphate, and acetate, affect the absorption maximum of the chromophore but do not allow the production of $P_{580}$ upon flash illumination and are not transported. However, these ions appear to compete with chloride in the flash experiments. These observations suggest that binding of anions to a relatively nonspecific site affects the protonation state of the Schiff's base in the chromophore. Either this site directly or a more specific site, connected to the first one by a sequential pathway, is involved with the photocycle intermediates and with chloride transport by halorhodopsin.

Halorhodopsin has been recently described (1-5) as a retinal protein found in the cytoplasmic membrane of Halobacterium halobium, similar to, but not identical with, bacteriorhodopsin. The halorhodopsin system functions as a light driven inwardly directed electrogenic chloride pump (6). Studies with cell envelope vesicles (unfractionated membranes) prepared from bacteriorhodopsin-negative strains have shown (7, 8) that at high ionic strength halides affect the halorhodopsin chromophore in two ways: (a) halides shift a 580 nm/410 nm chromophore equilibrium from the 410-nm chromophore, which is favored at alkaline pH, toward the 580-nm chromophore, and (b) halides cause alterations in flash-induced absorbance changes so that the principal photointermediate is one which absorbs in the blue and is designated as $P_{580}$, rather than one which absorbs in the red and is designated as $P_{410}$. In addition, the decay of both of these intermediates is considerably slowed in the presence of halides (8). Furthermore, a diuretic drug, MK-473, acts as a competitive inhibitor of halide transport and halide-induced changes in the photocycle (8). All of these results have suggested that halorhodopsin contains at least one halide-binding site with an apparent affinity constant of about 40 mM.

As in bacteriorhodopsin, retinal is bound to a halorhodopsin apoprotein (halo-opsin) which on sodium dodecyl sulfate-polyacrylamide gels migrates with an apparent molecular mass of 20 kDa (3, 4). A method for the isolation of this protein (in its retinal-bound form it is termed the halorhodopsin chromoprotein) has been developed, and some of its properties have been described (9). Thus, the chromoprotein was seen to be reversibly bleached by illumination, a process which was accompanied by the release and uptake of protons (9). Since the photointermediate produced absorbed at 410 nm and its recovery time was very slow (half-life, 76 s at -6 °C), it probably corresponded to the product of the "alkaline photoreaction" observed with halorhodopsin in envelope vesicles (6). It also resembled the 410-nm chromophore obtained at pH > 7 in the dark when chloride was removed (7).

The complexity of the behavior of the halorhodopsin chromophore makes it necessary to use terms which distinguish among the various spectroscopic species produced in response to increased pH, removal of chloride, illumination, etc., but do not complicate the nomenclature unnecessarily. Thus, we refer to the chromophore of halorhodopsin with its absorption band near 580 nm as the "580-nm chromophore," in spite of the fact that the position of this band varies by 10-15 nm, depending on the conditions. We refer to the reversible pH-dependent shift of this band to near 410 nm as the "580-nm/410 nm chromophore equilibrium." Photocycle intermediates are designated as $P_n$, with the subscript denoting the estimated position of the absorption band, as used before (5). Similarly, work in this and future reports will mention the binding of halides (meaning here chloride, bromide, or iodide, but not fluoride) to halorhodopsin. The binding site(s) will be referred to as "halide-binding site(s)," except when specifically one of the halides, e.g., chloride, is used in which case we speak of chloride-binding site(s). It is to be understood that these terms mean the same site(s).

Since reconstitution of chloride transport function in the chromoprotein has not been shown so far, it is not clear whether halorhodopsin consists of this protein alone, or of a complex with other, so far unidentified, components. Thus, it was important at this time to find out whether the halide-
binding site is in the chromoprotein or in a component lost during the purification.

The results obtained in this study show unequivocally that the purified chromoprotein is equivalent to the halorhodopsin in intact cell envelope vesicles with respect to the influence of halide on the chromophore. Thus, halide-binding sites which influence the properties of the chromophore are located in the chromoprotein. Results reported in the following paper on \textsuperscript{35}Cl nmr studies of chloride binding (10) suggest that at least one additional anion-binding site occurs in this protein.

**MATERIALS AND METHODS**

*Halobacterium halöbium* strains L-33 and S-9 were grown as described previously (11). The halorhodopsin chromoprotein was purified by low salt treatment of L-33 cells, followed by resuspension of the membrane fraction in 4 m NaCl, solubilization with Lubrol PX, and chromatography on phenyl-Sepharose CL-4B and hydroxyapatite, according to Stein and Oesterhelt (9). After chromatography the detergent was exchanged for octylglucoside\textsuperscript{1} (9), and the purified pigment could be stored for several months at 4 °C in the dark, in 1 m NaCl, 10 mM MOPS, pH 7.0, containing 1% octylglucoside. For spectroscopic experiments the preparation was dialyzed at 4 °C overnight (ca. 200 volumes of 1 m NaCl, 10 mM MOPS, pH 7.0, to remove excess octylglucoside.

Bacteriorhodopsin, which was used in this study as a reference, was isolated from S-9 cells according to Oesterhelt and Stoeckenius (12). The purified purple membrane was solubilized by stirring for 20 h at room temperature in the dark in 1 m NaCl, 10 mM MOPS, pH 7.0, containing 1% octylglucoside. After centrifugation at 10 °C at 200,000 × g for 1 h, the purple colored supernatant containing solubilized monomeric bacteriorhodopsin (9, 13) was collected.

For chloride-free media the halorhodopsin and bacteriorhodopsin chromoproteins were dialyzed at 4 °C in the dark against two changes of 1 liter of filtered 0.5 M Na\textsubscript{2}SO\textsubscript{4}, 10 mM MOPS, pH 7.0, for a total of 4 h. The concentration of chromoprotein was adjusted before the dialysis to give a final optical density of 0.1 at 580 nm. Higher sodium sulfate concentrations could not be used because of the limited solubility of the salt and the octylglucoside at low temperatures. The chloride concentration after dialysis was estimated to be less than 1 mM in all of these experiments. The halorhodopsin chromoprotein was unstable at very low chloride concentrations, and care was taken not to extend dialysis for longer times and not to expose the pigment to light or to room temperature for longer than required. However, all the experiments were carried out at room temperature. Protection from light, which is evidenced by decrease in absorption at 580 nm and appearance of absorption at 380-390 nm (as discussed in Ref. 9) was afforded by chloride concentrations as low as 0.5 m NaCl, 10 mM MOPS, pH 7.0, or 1% potassium phosphate (results not shown). If the chromoprotein were to behave similarly to halorhodopsin in intact membranes in sulfate (8) it should show low amplitude bleaching, with rapid (1-2 ms) recovery, and the principal photointermediate should be P\textsubscript{680}. Adding chloride should increase the bleaching amplitude, slow its recovery to near 10 ms, and redirect the photocycle pathway from P\textsubscript{680} to P\textsubscript{520}. In the presence of chloride, furthermore, the decay of any P\textsubscript{660} pro-
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Fig. 1. Flash-dependent absorption changes in H. halobium L-33 cell envelope vesicles, in cell envelope vesicles after addition of octylglucoside, and in purified halorhodopsin (HR) chromoprotein. Buffers: 1.0 M NaCl, 10 mM MOPS, pH 7.0, and octylglucoside in the cell vesicle experiments 1.0%, in the chromoprotein experiments 0.1%. Concentration of cell vesicle protein, 10 mg/ml. Concentration of halorhodopsin chromoprotein, 2 nmol/ml, which is roughly the same halorhodopsin concentration as in the cell vesicle experiments. Flash experiments were carried out at the indicated measuring wavelengths, with signal averaging over 512 repetitions, as under "Materials and Methods." Vertical bars correspond to per cent change in light intensity per flash.

Fig. 2. Chloride dependence of the photointermediates produced by flash illumination in the halorhodopsin chromoprotein. The pigment, in 1.0 M NaCl, 10 mM MOPS, pH 7.0, was dialyzed against two changes of 0.5 M Na2SO4, 10 mM MOPS, pH 7.0, with precautions to minimize denaturation as described under "Materials and Methods." Chromoprotein concentration, 2 nmol/ml. Conditions of flash experiments as in Fig. 1. Samples without added chloride were used only once, as they were partly (up to 20%) after the contents of the cuvette were mixed) bleached during the flash regime. Samples with added chloride (200 mM) were stable and could be reused.

Produced should be slower. The flash-induced absorption changes in the halorhodopsin chromoprotein are in good agreement with this behavior. As shown in Fig. 2, in the absence of chloride the amplitude of the bleaching is small, its recovery is rapid, and an intermediate (P380) is observed at 660 nm. No intermediate is seen at 500 nm (P500). Adding chloride to 200 mM causes large changes in the photocycling behavior. Fig. 2 shows that the bleaching at 570 nm proceeds with greatly increased amplitude, slower recovery time, the appearance of a photointermediate at 500 nm, and slower decay of the photointermediate at 660 nm. A complication in these experiments, which precludes quantitative evaluation, is the photosensitivity of the pigment in the absence of chloride, which leads to irreversible destruction. Such an effect had been observed already with cell envelope vesicles, but it is particularly noticeable during the flash regime performed on the solubilized chromoprotein for the measurements at 660 nm. The amplitude of absorbance change at 660 nm with 200 mM chloride present should be smaller than in the absence of chloride (8). We attribute the higher than expected relative amplitude (Fig. 2) to protection from photodestruction by chloride. The dependence of P380 amplitude on the chloride concentration, determined in experiments similar to that shown in Fig. 2, is described below.

The apparently poor photoactivity of halorhodopsin chromoprotein in the absence of chloride may be caused by a combination of three effects: (a) photodestruction of the pigment, which is significant without protection by chloride; (b) decreased absorption of actinic light by the blue-shifted 580-nm chromophore in the absence of chloride; and (c) inherently less photoconversion efficiency for the chromophore without chloride binding.

Octylglucoside-solubilized bacteriorhodopsin, as well as purple membrane suspensions, were tested for chloride dependence under identical conditions to those in Fig. 2, but only at 570 nm. No influence of chloride on the amplitude of the bleaching or on the recovery kinetics was observed (not shown).

Chloride-dependent Shift in the Absorption Spectrum of the Halorhodopsin Chromoprotein—The effects of pH and chloride on the absorption spectrum of the purified halorhodopsin chromoprotein are shown in Fig. 3. At pH 6 the pigment is largely in its 580-nm chromophore form even in the absence of chloride, and addition to chloride up to 200 mM has only a minor effect on the spectrum. A chloride-dependent shift in the absorption band of the 580-nm chromophore, from 565 to 577 nm, is evident, however. Such a shift has been described already for H. halobium membranes (20). Partial denaturation of the chromophore in the absence of chloride (cf. under "Materials and Methods") caused the appearance of a small absorption band near 380 nm, which was unaffected by the chloride.

At pH 8 a major shift of the absorption band to near 400 nm is evident, which is reversed upon addition of chloride. The reaction is a simple equilibrium between P380 and the blue-shifted form as indicated by the single isosbestic point at 470 nm (Fig. 3). In addition to this large change, the shift from 565 to 577 nm upon chloride addition is also seen at pH 8. With excess chloride (up to 200 mM), which produces the maximal amount of the 580-nm chromophore, significant
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The positive absorption band at 577 nm and the negative one at 420 nm correlate well with the chloride-dependent absorption changes in cell envelope vesicles, reflecting the 580-nm/410-nm chromophore equilibrium state described for halorhodopsin (7).

Affinity of the Halorhodopsin Chromoprotein for Halides and Other Anions—Studies with L-33 cell envelope vesicles had shown (6-8) that the apparent affinity of halorhodopsin for chloride and bromide was about 40 mM in each of three kinds of measurements: light-dependent transport, influence on the 580-nm/410-nm chromophore equilibrium, and appearance of P500 during flash illumination. Apparent affinity constants for the halorhodopsin chromoprotein could be obtained for the last two of these halide-dependent effects. The absorbance increase at 577 nm as a function of chloride concentration at pH 6, 7, and 8 is shown in Fig. 5A. Half-maximal saturation of the effect is observed near 10 mM chloride at each pH value, but the extent is greatly increased with increased pH. In Fig. 5B the dependence of the amplitude of flash-induced absorbance change at 500 nm (○—○) as well as the rate of decay of the P500 photointermediate (●—●) are shown as functions of chloride concentration. Half-maximal amplitude change at 500 nm occurs also near 10 mM chloride. Although an apparent affinity constant cannot be calculated for the chloride effect on decay rate, its maximal changes are also at concentrations below 50 mM chloride.

A large number of other anions was tested for the two effects on the halorhodopsin chromophore and for whether or not absorption remains at 380 nm, which is due to denatured chromoprotein.

Octylglucoside-solubilized bacteriorhodopsin was tested for chloride-dependent effects under identical conditions to those in Fig. 3, and no shift or change in absorbance was seen after addition of chloride up to about 200 mM (not shown).

A chloride-dependent difference spectrum, calculated from the absorption changes at pH 8 (Fig. 3) is shown in Fig. 4.

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**Fig. 3.** pH-dependent equilibrium of the visible absorption band of halorhodopsin chromoprotein between the 580-nm chromophore and a 410-nm chromophore, and the stabilization of the 580-nm chromophore by added chloride. The pigment was dialyzed against sulfate, as described in Fig. 2, to a final concentration of 2 nmol/ml, at pH 6.0 (upper) and pH 8.0 (lower). Each sample was freshly prepared and used immediately after dialysis. Chloride additions from a 4 M NaCl stock solution; final concentrations as follows: upper (pH 6), 1, zero; 2, 10 mM; 3, 200 mM; lower (pH 8), 1, zero; 2, 10 mM; 3, 20 mM; 4, 40 mM; 5, 100 mM; 6, 200 mM. The amplitude of the traces was corrected for the dilution of the samples by the additions.

**Fig. 4.** Chloride-dependent difference spectrum of the halorhodopsin chromoprotein. Conditions as in Fig. 3 at pH 8.0. The spectrum in the absence of chloride was subtracted from the spectrum with added 200 mM chloride.

**Fig. 5.** Dependence of the 580-nm/410-nm chromophore equilibrium (A) and flash-induced absorbance change at 500 nm (B) on chloride concentration. A, absorption change at 577 nm upon addition of chloride to halorhodopsin chromoprotein, determined as in Fig. 3, but at pH 6.0, 7.0, and 8.0. B, absorption change after flash illumination, followed at 500 nm in halorhodopsin chromoprotein solutions as described in Fig. 2, but at various chloride concentrations. ○—○, amplitude of absorption change expressed as per cent decrease in light intensity per flash; ●—●, first order rate constant of decay for the absorption change.
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TABLE I
Anion-dependent 580-nm/410-nm equilibrium and flash-induced production of $P_{520}$ in the halorhodopsin

The halorhodopsin chromoprotein ($A = 0.1$) was dialyzed against 0.5 M Na$_2$SO$_4$/10 mM MOPS pH 7.0 (+100 mM NaCl for competition experiments, last column). The absorbance changes and flash experiments were carried out immediately after addition of 200 mM anions as 4 M sodium salts adjusted to pH 7.0 (or as 1 M sodium fluoride, 2 M sodium citrate, 3 M potassium phosphate). For estimation of the $K_m$ values, different amounts of anions up to 200 mM were added.

<table>
<thead>
<tr>
<th>Anion (200 mM)</th>
<th>$K_m$ ($\mu$M)</th>
<th>Relative absorbance change/flash at 580 nm</th>
<th>$\lambda_{max}$ of the 580-nm chromophore*</th>
<th>$K_m$ (mM)</th>
<th>Relative absorbance change/flash at 500 nm</th>
<th>Relative absorbance change/flash at 500 nm in presence of chloride</th>
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<tr>
<td>Cl$^-$</td>
<td>8-12</td>
<td>(100)</td>
<td>577</td>
<td>10</td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>Br$^-$</td>
<td>8-12</td>
<td>103</td>
<td>577</td>
<td>5-10</td>
<td>99</td>
<td>ND</td>
</tr>
<tr>
<td>I$^-$</td>
<td>10-14</td>
<td>90</td>
<td>572</td>
<td>5-10</td>
<td>35</td>
<td>ND</td>
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<tr>
<td>F$^-$</td>
<td>9</td>
<td>557</td>
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<td>0</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>25</td>
<td>73</td>
<td>567</td>
<td>0</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>SCN$^-$</td>
<td>13</td>
<td>107</td>
<td>567</td>
<td>0</td>
<td>20</td>
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</tr>
<tr>
<td>Acetate</td>
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<td></td>
<td>563</td>
<td>0</td>
<td>80</td>
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<tr>
<td>Citrate</td>
<td>0</td>
<td>567</td>
<td></td>
<td>0</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>16</td>
<td>73</td>
<td>560</td>
<td>0</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>

* The $\lambda_{max}$ of chloride-free halorhodopsin chromoprotein was 565 nm after dialysis against sulfate.

b ND, not determined.

c The 580-nm/410-nm equilibrium depends linearly on acetate up to the highest concentration (200 mM) tested.

At this concentration the relative amplitude of the absorption increase was about 46%.

not they are transported by this system. Table I shows that in addition to chloride, bromide, and iodide, the following anions were effective in shifting the 580-nm/410-nm chromophore equilibrium, described in Fig. 3, toward the 580-nm chromophore: nitrate, thiocyanate, phosphate, and acetate, but not fluoride and citrate. Interestingly, the non-halide anions caused no red-shift of the 580-nm chromophore band, unlike the halides. Fluoride which did not change the 580-nm/410-nm chromophore equilibrium, caused the remaining 580-nm chromophore band to move 10 nm to lower wavelengths. As expected, chloride plus the non-halide anions produced maximal effects on the chromophore equilibrium similar to those with chloride alone. In contrast with the relative lack of specificity among anions for this effect, the production of $P_{520}$ after flash illumination (Table I) and transport (6, 8) was observed only with the halides. In the transport experiments the maximal rates with iodide were about 25% of those with chloride or bromide, but the half-maximal rate with iodide was at <10 mM, much below the 40 mM obtained with chloride or bromide. Table I shows that chloride and bromide are about equivalent, and iodide is about a third as effective, but at the same affinity. Although the other anions tested did not cause the appearance of flash-induced absorbance change at 500 nm, they diminished the magnitude of these changes in the presence of chloride (Table I). Thicyanate and nitrate are most interesting in this respect; they clearly influence the 580-nm/410-nm chromophore equilibrium and act inhibitory to the chloride-dependent appearance of $P_{520}$, but they are not competent by themselves to cause the production of $P_{520}$. The behavior of phosphate and fluoride are different; phosphate changes the chromophore equilibrium, but does not inhibit the generation of $P_{520}$ and fluoride has no effect on the equilibrium but inhibits the formation of $P_{520}$ (Table I).

It should be mentioned that the apparent affinity constant for chloride and bromide in this report (about 10 mM) does not agree with the value found earlier for transport (6, 8) and for effects on the chromophore (7) in cell envelope vesicles (about 40 mM). For reasons of solubility at 4 °C 0.5 M sulfate was used in this work, while 1.5 M sulfate or 3 M phosphate was used in the earlier studies with cell envelope vesicles. It is, therefore, possible that weak competition for the halide-binding site(s) by sulfate and phosphate raised the apparent $K_m$ for chloride in the presence of high sulfate or phosphate concentrations.

MK-473 which is a competitive inhibitor of chloride binding to halorhodopsin in L-33 cell envelope vesicles (8) had little or no effect on the chloride dependence of flash-induced absorbance changes and chromophore equilibrium in the purified chromoprotein. We have no explanation at this time for the lack of inhibition by this agent in the purified preparation.

Essentially all of the experiments in Table I were repeated with octylglucoside-solubilized bacteriorhodopsin. No significant effects were seen on the absorption spectrum on this pigment by the anions used nor were any effects found on the flash-induced absorbance changes at 570 nm (not shown).

DISCUSSION

The results described here fully support the idea that anion-binding sites, with some specificity for halides, are present in the halorhodopsin chromoprotein. Apart from minor differences, probably due largely to the instability of the chromophore in halide-free octylglucoside, the chloride-dependent effects of halorhodopsin seen in cell envelope vesicles (7, 8) are thus observed also in the purified chromoprotein. Therefore, halorhodopsin may well consist of the 80-kDa chromoprotein described here, is transported during the illumination.

Earlier work with L-33 cell envelope vesicles (7) had shown that between pH 7 and 9 the halorhodopsin absorption band exists in an equilibrium between the 580-nm chromophore and a 410-nm species. The equilibrium is shifted toward the

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5. Schobert, unpublished results.
580-nm chromophore by chloride. During sustained illumination of the cell envelope vesicles (7) or the solubilized chromoprotein (9) in NaCl a 410-nm form is produced, which is similar to that obtained at alkaline pH in the absence of chloride. In the purified preparation the light-induced production of the 410-nm species was shown to be accompanied by the loss of a proton (9). These findings most likely reflect the lowering of the pK of the Schiff’s base and subsequent deprotonation, both during the illumination and in the absence of chloride. It is worthwhile to point out that a protonated (605 nm) form of bacteriorhodopsin seems also to bind anions, notably chloride, in the vicinity of the chromophore (21).

The effects of anions on the chromoprotein are best discussed by comparing chloride (representative of halides) and thiocyanate (representative of several other anions). The former anion shifts the 580-nm/410-nm chromophore equilibrium toward the 580-nm chromophore at pH above 6 (Figs. 3 and 4, Table I) and causes the production of P520 after flash illumination (Fig. 2, Table I). The latter anion influences the chromophore equilibrium in a similar way but does not cause the appearance of P520 (Table I). However, thiocyanate competes effectively with chloride and inhibits the production of P520 (Table I). These observations can be rationalized in a model in which a variety of anions, including chloride and thiocyanate, etc., are bound to a relatively nonspecific site and influence the pH-dependent equilibrium exhibited by the chromophore. An electrostatic effect which increases the pK of the Schiff’s base proton upon anion binding would account for the stabilization of the 580-nm chromophore and imply that the binding site is located in the vicinity of the Schiff’s base. Alternatively, a conformational change upon binding could change the environment of the Schiff’s base so as to raise its pK. If the transport of the anion across the protein proceeds via a series of binding sites, both transport and the appearance of P520 would require that the anion move to a next site whose specificity is greater. Hence, only chloride, bromide, and iodide will show an effect on the photocycle (Table I). However, thiocyanate (and other similarly active anions) will be inhibitory because they displace chloride from the first site and thus block its access to the second site. Phosphate and fluoride do not fit this scheme (Table I). Another possible scheme would include only one site, with affinity for all the anions in Table I but citrate. Transport and the production of P520 would proceed only when chloride, bromide, or iodide occupy this site, however. The observed red shifts (Table I) indeed argue for specific interaction by these ions with the chromophore upon binding. The multiple binding site model is made somewhat more likely by the findings in the following paper (10), in which two more anion-binding sites are described in the halorhodopsin chromoprotein from ScyC1 nmr line broadening. These have dissociation constants of 100 mM and greater than 1 M for chloride. Our tentative proposal of the existence of several binding sites in the halorhodopsin chromoprotein must be still confirmed by chemical methods. It is clear from the results in this paper, however, that the complex effects of halides on halorhodopsin distinguish it from bacteriorhodopsin, which does not respond to halides but in a similar way responds to protonation changes with changes in its absorption maximum as well as in the intermediates of its photocycle (21).

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