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Cation Binding to Halorhodopsin

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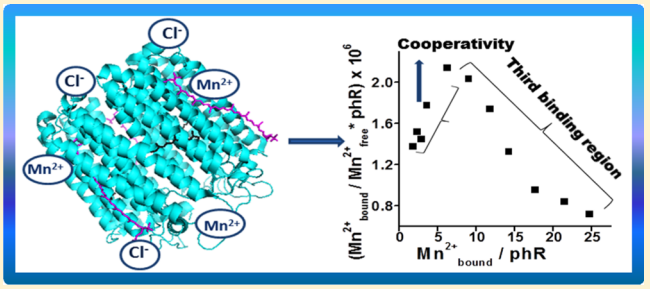
Supporting Information

ABSTRACT: A member of the retinal protein family, halorhodopsin, acts as an inward light-driven Cl^- pump. It was recently demonstrated that the *Natronomonas pharaonis* halorhodopsin-overproducing mutant strain KM-1 contains, in addition to the retinal chromophore, a lipid soluble chromophore, bacterioruberin, which binds to crevices between adjacent protein subunits. It is established that halorhodopsin has several chloride binding sites, with binding site I, located in the retinal protonated Schiff base vicinity, affecting retinal absorption. However, it remained unclear whether cations also bind to this protein. Our electron paramagnetic resonance spectroscopy examination of cation binding to the halorhodopsin mutant KM-1 reveals that divalent cations like Mn^{2+} and Ca^{2+} bind to the protein. Halorhodopsin has a high affinity for Mn^{2+} ions, which bind initially to several strong binding sites and then to binding sites that exhibit positive cooperativity. The binding behavior is pH-dependent, and its strength is influenced by the nature of counterions. Furthermore, the binding strength of Mn^{2+} ions decreases upon removal of the retinal chromophore from the protein or following bacterioruberin oxidation. Our results also indicate that Mn^{2+} ions, as well as Cl^- ions, first occupy binding sites other than site I. The observed synergetic effect between cation and anion binding suggests that while Cl^- anions bind to halorhodopsin at low concentrations, the occupancy of site I requires a high concentration.



Retinal proteins[1] represent a large group of integral membrane proteins containing transmembrane helices bound to a retinal chromophore. The retinal chromophore is covalently attached to a lysine residue of the protein via a protonated Schiff base linkage. Retinal proteins have various light absorption-triggered functions, such as proton pumping by bacteriorhodopsin (BR)[2−5] and transportation of chloride ion from the cell's extracellular side to its cytoplasm by halorhodopsin (hR).[6−10] BR and hR are structurally similar, with both possessing seven transmembrane helices (helices A−G) and a retinal chromophore bound to the ε-amino group of a lysine residue in helix G. The photoisomerization of the retinal C13═C14 bond leads to the pumping of the respective ions in and out of the cell.

The crystal structures of two hR homologues, one from *Halobacterium salinarum* (shR)[11] and one from a *Natronomonas pharaonis* mutant (phR),[12] have been determined. The X-ray crystal structure of phR from the hR-overproducing mutant strain KM-1 of *N. pharaonis* (DSM2160T) exhibits a lipid soluble chromophore, termed bacterioruberin (RN), that binds to crevices between the adjacent protein subunits in the trimeric assembly (Figure 1). In contrast, phR expressed in an *Escherichia coli* system does not bind the chromophore. Extensive studies have been conducted relating to chloride binding to regular halorhodopsin in *H. salinarum*[10,13−16] and *N. pharaonis*.[17−20] These have shown that Cl^- ions usually bind to the vicinity of the protonated Schiff base linkage, as studied by Raman spectroscopy.[21,22] The high-resolution structure of shR reveals that the chloride anion forms part of the retinal protonated Schiff base (PSB) counterion complex and replaces Asp85 of BR. In the case of halorhodopsin from *N. pharaonis*, Sato et al. proposed the existence of Cl^- binding or interacting sites in both the extracellular and cytoplasmic channels.[17] Removal of Cl^- from the phR KM-1 mutant induces a 20 nm red shift of the visible absorption band. It was also established that the blue, purple, and yellow forms of the pigment are interconvertible, depending on the pH and the presence of Cl^- ions in the medium.[23] Furthermore, it was suggested[23] that the trimeric phR−bacterioruberin complex possesses three or four anion binding sites per subunit. The primary chloride binding site (site I) is located in the vicinity of the retinal protonated Schiff base, while the secondary binding site (site II) is positioned at the interprotomer crevice near the cytoplasmic membrane surface (Figure 2). The binding affinity of Cl^- for site I is controlled by Cl^- occupancies at other sites in the KM-1 mutant.[23]

Previous reports have pointed to the participation of cations in various functional aspects of retinal proteins. A variety of experimental data, for example, indicate specific cation binding to BR. Potentiometric titration studies of BR deionized membranes using a Ca^{2+}-specific electrode have suggested two high-affinity cation binding sites and four to six low-affinity

Received: January 16, 2015
Revised: April 24, 2015
Published: April 24, 2015

DOI: 10.1021/acs.biochem.5b00039
Biochemistry 2015, 54, 3164−3172

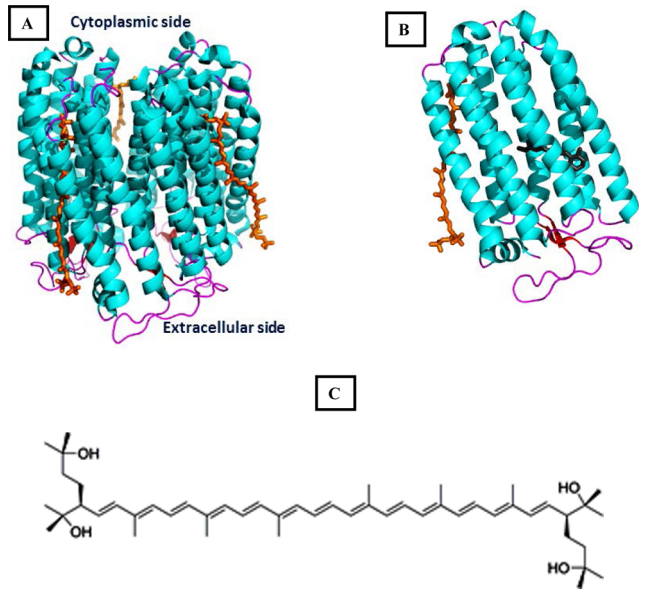


Figure 1. (A) Cartoon representation of trimeric phR (Protein Data Bank entry 3A7K) with bacterioruberin colored orange. (B) Cartoon representation of the phR monomer with bacterioruberin (orange) and retinal (gray). (C) Chemical structure of bacterioruberin.

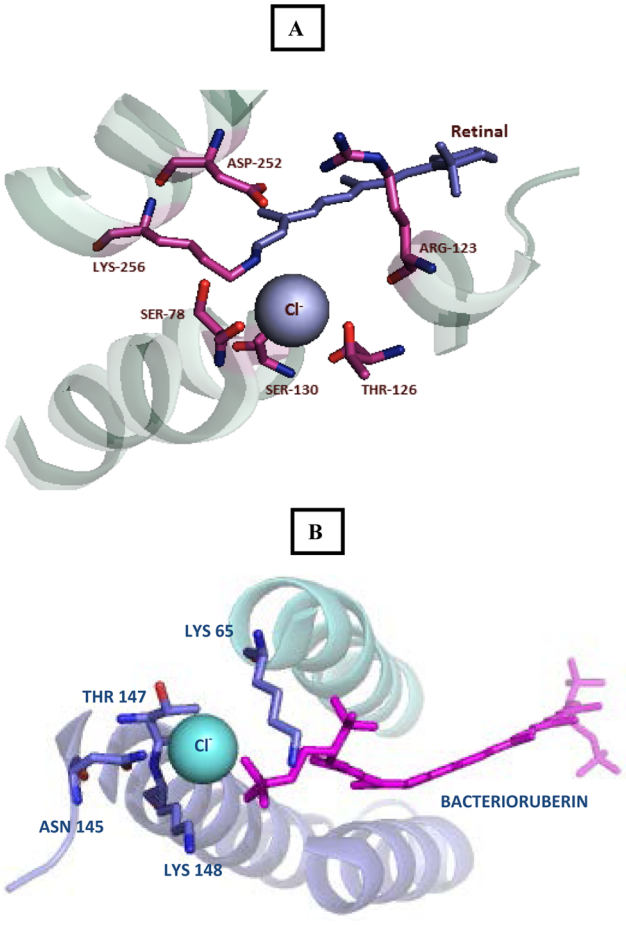


Figure 2. Chloride binding sites in phR. (A) Primary chloride binding site, with its interacting residues Thr126 and Ser130, near the protonated Schiff base. The Cl^- ion is colored purple. (B) Secondary binding site near the terminal end of the bacterioruberin of the phR trimer.

sites.[24−27] It was further proposed that cation binding to the secondary high-affinity site correlates with the blue-to-purple transition.[24,28] EPR measurement of Mn^{2+} indicated one high-affinity and three weaker binding sites, characterized by similar binding constants.[29]

Several studies have also suggested that the cation binding site, which determines the state of protonation of Asp85 and, thus, the color of the BR pigment, is located in the retinal binding pocket.[24,30−32] Another model has proposed that the cations bind to the membrane surface and control the apparent pK_a of Asp85 and, thereby, the purple-to-blue transition through a change in surface potential.[33−35] According to Guy Chapman's theory, free or bound metal cations on the membrane surface compete with protons and, thus, determine the local proton concentration around the membrane.

Recent kinetic studies of Asp85 reprotonation as a function of cation size have indicated that the color-controlling cation binding site in BR is in an exposed location on or near the membrane surface.[36] Furthermore, it was shown that the titrations of Asp85 and of the cation binding residues in BR are uncoupled, excluding direct binding of the cation to Asp85.[37] Further supporting the notion that the binding occurs at the membrane surface are solid-state NMR analyses indicating that cation binding effects Ala196, located in the E−F interhelical loop.[38] A study using eosin dye covalently bound to the protein suggested that the cations do not bind to specific sites and bind equally to both membrane surfaces and negatively charged lipids.[39] While much knowledge exists regarding cation binding to BR, the possibility of cations binding to hR and their interaction with anions within the pump remains to be resolved.

To shed new light on the mode of operation of phR, we investigated the possible binding of cations to mutant phR. Our EPR spectroscopy studies reveal that divalent cations like Mn^{2+} and Ca^{2+} bind to the protein. We further observed a high degree of cooperativity in the binding process of Mn^{2+} ions, with the binding of the cation and anion being mutually related. The presence of a chloride anion increased the affinity of Mn^{2+} for the protein, and vice versa. The bacterioruberin chromophore was also found to contribute to cation binding, as the number of bound cations declined considerably once the RN structure was modified.

MATERIALS AND METHODS

phR Membrane Preparation. The KM-1 strain of *N. pharaonis* was grown in a culture medium (pH 9) as described by Ihara et al.[40] The phR membranes were isolated using a reported protocol,[40] washed with 100 mM NaCl, suspended in 100 mM NaCl, and subjected to several rounds of centrifugation. The deionized phR membrane was prepared by passing the washed phR suspension through a Dowex 50WX2 (Sigma) cation-exchange column. The resulting deionized membrane was obtained at pH 4−4.5.

Oxidation of the Bacterioruberin Chromophore. The aqueous suspension of phR membranes was treated with potassium persulfate for 6 h under dark conditions.[41] The decrease in bacterioruberin band intensity at 545, 505, and 490 nm was monitored using a UV−vis spectrophotometer. Following the disappearance of bacterioruberin absorption, the sample was dialyzed against doubly distilled water overnight.

Preparation of the Apoprotein. The apoprotein was produced by incubating the phR membranes with freshly

DOI: 10.1021/acs.biochem.5b00039
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prepared hydroxylamine (pH 7.2) and subsequently irradiating for 2 h with a Schott 250 W cold light source (Carl Zeiss Microscopy, Jena, Germany) equipped with a heat-absorbing filter and an optic fiber (level 4B). The light was filtered through a long-pass cutoff filter with a λ of >550 nm (Schott, Mainz, Germany). The samples were dialyzed against doubly distilled water.

Preparation of Fe(II)–*o*-Phenanthroline(OP) Complexes. The $Fe(OP)_3^{2+}$ complex was obtained by mixing 1 equiv of a freshly prepared $FeCl_2$ solution with 4 equiv of the ligand *o*-phenanthroline. An orange-red-colored complex absorbed at 500 nm.

EPR Measurements. EPR spectra were recorded by CW EPR spectrometer ELEXSYS -500 (Bruker) at room temperature in flat quartz cell. Mn^{2+} ions at increasing concentrations were added to deionized membrane samples, and the EPR spectra of the free Mn^{2+} ions were monitored. The pH was adjusted to 4.8 (in most cases) or higher, as required. The binding of Mn^{2+} ions to the deionized phR and the bleached samples was analyzed using the Scatchard equation:

$$\frac{r}{c} = nK - rK$$

where r is the ratio between the concentration of bound ligand and the total number of available binding sites, c is the concentration of free ligand, and n is the number of binding sites per protein molecule.

UV–Vis Absorption. All UV–vis absorption measurements were taken using an Agilent 4583 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA) equipped with an Agilent 89090A thermostated cuvette holder. Absorption spectra were corrected for light scattering.

RESULTS

EPR Measurements. *Multiple Cation Binding Sites.* The binding of Mn^{2+} to deionized halorhodopsin membranes from the KM-1 strain at pH 4.8 (obtained by passing the pigment through Dowex) was determined by EPR spectroscopy with respect to the free signal of Mn^{2+} ions. The deionized phR samples were gradually titrated with an increasing concentration of $MnCl_2$, starting with 0.2 equiv of $MnCl_2$. The pattern of binding of Mn^{2+} to the deionized phR was demonstrated by a Scatchard plot (Figure 3). We detected a free Mn^{2+} signal only after adding 2 equiv of Mn^{2+}, suggesting that the first 2 equiv of Mn^{2+} ions formed a tight binding region, a reflection of the existence of extremely strong binding sites. The next noted region was at up to ~7 equiv of Mn^{2+}. Here, the Mn^{2+} cations bind phR in a positive cooperative manner. The third region was detected following the further addition of Mn^{2+} ions (up to 40 equiv of Mn^{2+} added), as shown in Figure 3. The binding constant calculated from the Scatchard plot for this region is 1.1×10^5 M^{-1}. Thus, the EPR results suggest that multiple sites exist for the binding of Mn^{2+} to deionized phR.

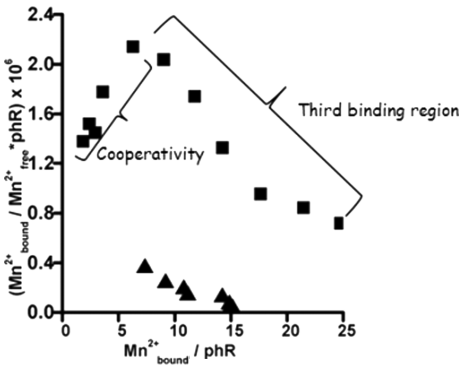

Figure 3. Comparative Scatchard plot of binding of $MnCl_2$ to 5 μM deionized halorhodopsin (phR) (pH 4.8) at starting Mn^{2+} concentrations of (■) 0.16 equiv and (▲) 11.3 equiv.

The binding strength of Mn^{2+} ions drastically reduced once 11 equiv of Mn^{2+} ions had been added in one portion, i.e., skipping the region of positive cooperativity [Figure 3; (▲)]. The calculated binding constant is on the order of 10^4 M^{-1}, which is lower than that measured for the gradual addition of Mn^{2+}. We propose that the gradual addition triggers a change in the protein conformation that induces the formation of binding sites characterized by a high affinity for Mn^{2+}. In contrast, the addition of 11 equiv of Mn^{2+} all at once induces an alternative protein conformation, one that affords weaker Mn^{2+} binding.

We found that the pH of the medium markedly affects the strength of binding of Mn^{2+} ions to deionized phR. As the pH increased, $MnCl_2$ bound more tightly to the protein (Figure 4).

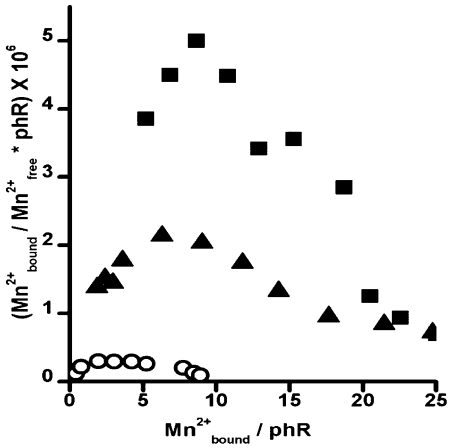

Figure 4. Binding of $MnCl_2$ (0.1–40 equiv of Mn^{2+}) to 5 μM deionized phR at (■) pH 8, (▲) pH 4.8, and (○) pH 3.3.

The binding constant values for the third binding region are presented in Table S1 of the Supporting Information. At pH 8, the first 5 equiv of Mn^{2+} ions is tightly bound to phR, as no free signal of Mn^{2+} ions could be detected by EPR spectroscopy. As noted above, we observed that the first 2 equiv of Mn^{2+} is strongly bound at pH 4.8. In contrast, binding is much weaker at pH 3.3, and a signal of free Mn^{2+} ions was detected even following the addition of only 0.1 equiv of Mn^{2+}. The stronger binding of Mn^{2+} ions at higher pH may reflect the involvement of acidic residues, such as Asp or Glu, in the Mn^{2+} binding process.

Cation Binding to Apo-phR and Oxidized Bacterioruberin phR. The phR contains a RN chromophore in addition to the retinal chromophore. To pinpoint the role of these two chromophores in binding of cation to phR, we studied the nature of binding of Mn^{2+} ion to apo-phR (i.e., phR lacking the retinal protonated Schiff base) and to the oxidized RN pigment.[41] The Scatchard plot (Figure S1 of the Supporting Information) indicates that the binding strength of Mn^{2+} is considerably reduced in both the apoprotein and the oxidized RN relative to that of the native protein. These results can be related to the effect of both the protonated Schiff base linkage

DOI: 10.1021/acs.biochem.5b00039
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and RN in maintaining the protein conformation that leads to the creation of Mn^{2+} cation binding sites (see Table S2 of the Supporting Information for binding constant values).

Effect of Anions on Cation Binding. The binding of Mn^{2+} cations to the phR pigment raises the question of whether it is related to anion binding. As it was reported that SO_4^{2-} binds more weakly than chloride anions bind to phR,[23] we decided to assess the binding of $MnCl_2$ and $MnSO_4$ to the deionized protein under similar conditions. A free Mn^{2+} signal appeared following the gradual addition of 2 equiv of $MnSO_4$, similar to the result obtained for $MnCl_2$. It, therefore, appears that the tightest $MnCl_2$ binding region bears a close resemblance to that of $MnSO_4$ (up to 2 equiv). The anion type does not seem to influence Mn^{2+} binding in this region. Our comparative analysis of the Scatchard plots of $MnCl_2$ and $MnSO_4$ did, however, reveal a major change in the pattern of binding of $MnSO_4$ (Figure S2 of the Supporting Information) in the form of the disappearance of the positive cooperative effect. This suggests that Mn^{2+} binding is associated with binding of anion to phR, reflecting a synergism of the affinity of Cl^- and Mn^{2+} ions for the protein.

Binding of Ca^{2+} and Na^+ Cations. To explore the patterns of binding of other cations to deionized phR and to obtain comparative binding data with respect to Mn^{2+}, we conducted competition studies of Mn^{2+} binding with $CaCl_2$ and NaCl. These experiments were conducted by incubating deionized phR with various concentrations of Mn^{2+} followed by the addition of $CaCl_2$. The titrations were performed with preincubated phR−Mn complexes (the Mn^{2+} ions were added gradually to the protein sample in these cases). Our results, presented in parts A and B of Table S3 of the Supporting Information and plotted in Figure 5A, indicate that Ca^{2+} ions compete efficiently with Mn^{2+} ions for the first equivalent, but ∼200 equiv of Ca^{2+} is needed to displace the second Mn^{2+} equivalent (Table S3A of the Supporting Information and Figure 5A). However, the Ca^{2+} ions compete weakly in the presence of a greater number of equivalents of Mn^{2+} ions added gradually, as noted by the extremely high concentration of Ca^{2+} ions (as much as 100 times greater than that of Mn^{2+}) required to displace Mn^{2+} ions from the Mn^{2+}−phR complexes (Table S3B,C of the Supporting Information). Furthermore, the Scatchard plot (Figure 5B) derived from the free signal of Mn^{2+} ions from the competition experiments with Ca^{2+} ions indicates that Ca^{2+} ions possess two binding regions: a stronger binding region (∼30 equiv of Ca^{2+} ions added) with a binding constant of 6.4×10^4 M^{-1} and a weaker binding region (∼225 equiv of Ca^{2+} ions added) with a binding constant of 6.49×10^2 M^{-1}. Both binding constants were calculated in the presence of Mn^{2+} ions.

We also conducted reverse titration experiments, in which the protein was incubated with different concentrations of Ca^{2+} (2.5 and 13.2 equiv) followed by titration with $MnCl_2$ (Table S4A,B of the Supporting Information). The presence of $CaCl_2$ appears to somewhat hamper the binding of Mn^{2+} ions. The plot of the free Mn^{2+} ions versus total equivalents of Mn^{2+} added to the Ca−phR complex (Figure S3A of the Supporting Information) indicates that the initial concentration of free Mn^{2+} ions decreases as the concentration of $MnCl_2$ increases. This observation indicates that the Mn^{2+} ions bind to the Ca−phR complex but in a manner weaker than that of native phR, as also deduced from the Scatchard plot (Figure S3B of the Supporting Information).

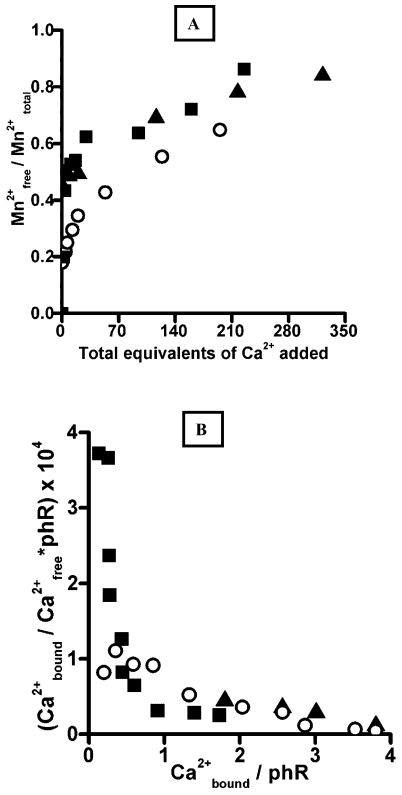

Figure 5. Competition assay with $CaCl_2$ in which 5 μM deionized phR incubated with different concentrations of $MnCl_2$ (gradually added) was titrated with $CaCl_2$. (A) Mn^{2+}_{free} vs Ca^{2+}_{total} equivalents. (B) Findings represented as a Scatchard plot: (■) 2.3 equiv of Mn^{2+}, (○) 6 equiv of Mn^{2+}, and (▲) 11.3 equiv of Mn^{2+}.

The two sets of competition studies we performed (incubation with Mn^{2+} and competition with Ca^{2+} and the reverse one) produced different results. In the first set, in which we incubated deionized phR with 2 equiv of Mn^{2+} ions, we observed strong competition by Ca^{2+} ions. The Ca^{2+} ions efficiently displaced the first equivalent of the Mn^{2+} ions but did so in a much weaker manner for the second equivalent. It is conceivable that initially both the Ca^{2+} and Mn^{2+} ions share the same binding sites in this tight binding region. However, Ca^{2+} ions can displace the Mn^{2+} ions from phR only at high concentrations. The Ca^{2+} ions offer weak competition to the Mn^{2+} ions for binding to the second and third regions. In the case of reverse titration experiments, in which Mn^{2+} ions are added to the Ca^{2+}−protein complex, the Mn^{2+} ions can still bind to phR but not as strongly as to the native protein. Nevertheless, the pattern of binding of the Mn^{2+} ions to the protein strongly resembles that of regular binding of Mn^{2+} ions to phR, and positive cooperativity is retained (Figure S3B of the Supporting Information). Thus, in the case of reverse titration experiments in the presence of Ca^{2+} ions, the binding sites are not fully available for the Mn^{2+} ions, which is why much weaker binding is observed compared to that of the native protein.

To shed light on the binding of Na^+ ions to the protein, we titrated $MnCl_2$−phR complexes with increasing concentrations of NaCl. We observed that, even at very high concentrations of NaCl, the free Mn^{2+} signal did not increase (data not shown), reflecting the failure of Na^+ ions to displace the Mn^{2+} ions from the Mn^{2+}−phR complex. Just like was observed for bacteriorhodopsin, Na^+ cations have a binding affinity lower

DOI: 10.1021/acs.biochem.5b00039
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than that of divalent cations. We also evaluated the binding of Mn^{2+} ions to deionized protein preincubated with different concentrations of NaCl. Our findings show that while micromolar concentrations of NaCl do not affect the binding of Mn^{2+} to the protein, at 1 mM NaCl (238.2 equiv of Na^+), the strength of Mn^{2+} binding is somewhat reduced and the region of positive cooperativity is absent (Figure S4 of the Supporting Information). This suggests that at 1 mM NaCl, the protein's conformation is altered, as opposed to the case of the deionized protein, resulting in the loss of the Mn^{2+} binding positive cooperative effect, though the Mn^{2+} ions still compete for the binding sites already occupied by the Na^+ ions. It appears that a high NaCl concentration lowers the binding affinity of Mn^{2+} ions, as reflected in the binding constant values ($K_b = 5.53 \times 10^4\ M^{-1}$).

Competition Experiments with Large Cation $Fe(OP)_3^{2+}$. To ascertain whether large cations bind to phR, we performed competition studies with $Fe(OP)_3Cl_2$ (ferrous tris-*o*-phenanthroline chloride) at pH 4.8. It is a reasonable assumption that this cation's huge dimensions will limit its binding solely to the protein's surface. Deionized phR was incubated with different concentrations of $MnCl_2$, added gradually, and subsequently titrated with a solution containing the $Fe(OP)_3Cl_2$ complex. The free signal of Mn^{2+} was monitored by EPR spectroscopy, and the results are presented in Table S5 of the Supporting Information. As can be gathered from Figure S5 of the Supporting Information, there is no competition at pH 4.8 between Mn^{2+} and $[FeOP_3]^{2+}$ ions in the tight binding region that exists at up to 2 equiv of Mn^{2+} ions. In contrast, the $[FeOP_3]^{2+}$ ions compete and efficiently displace the Mn^{2+} ions from the second, positive cooperativity region. This may suggest that both ions share a common binding site, probably on or near the protein surface in this region.

Effect of Cations on the UV–Vis Absorption Spectrum. It was previously reported that the absorption maximum of phR in the KM-1 membrane is red-shifted following removal of chloride ions by extensive pigment washing.[23,40] Our analysis of the effect of adding NaCl to washed phR revealed a gradual blue shift of the retinal absorption along with a change in bacterioruberin absorption (data not shown). To pinpoint the influence of cations on the absorption of the two chromophores in phR, we monitored the effect of gradually adding $MnCl_2$, NaCl, and $CaCl_2$ to the deionized protein. We also examined the outcome of introducing one portion of the salts to deionized phR to corroborate our EPR results indicating that Mn^{2+} ions exhibit different binding behaviors in response to the two different ways of addition.

Gradual Addition of Salts. A sharp decrease in the retinal absorption band appeared at 610 nm following the addition of $MnCl_2$ to the deionized phR, as compared to other salts (Figure 6A and Figures S6 and S8A of the Supporting Information). We also noted a decrease in the RN band intensity (Figures S6, S7, and S8B of the Supporting Information). However, it appears that there is no direct correlation between the two, as evident from the $\Delta A_{610}/\Delta A_{540}$ ratio (Table S6 of the Supporting Information), which was not constant during the titration process. It can, therefore, be concluded that the two chromophores are affected by two different processes and probably also by different binding sites.

The absorption spectra indicated a decrease in the intensity of the retinal 610 nm absorption band up to addition of 35 equiv of Mn^{2+} (Figure S8A of the Supporting Information). The detected change in retinal band intensity up to ~10 equiv

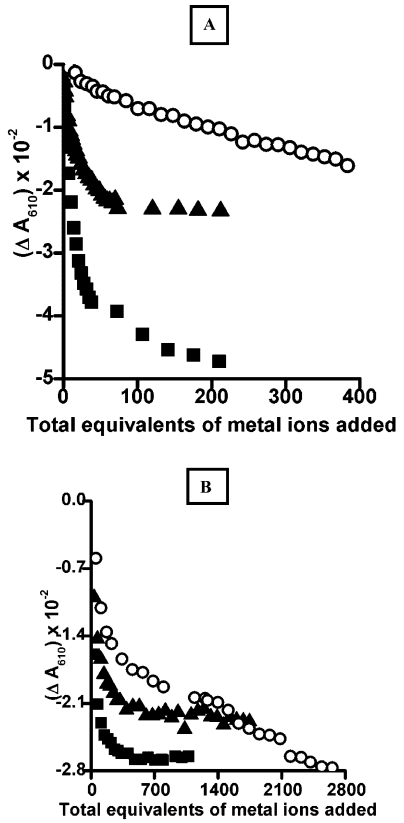

Figure 6. UV–vis titrations of 5 μM deionized phR with different concentrations of NaCl, $CaCl_2$, and $MnCl_2$. ΔA_{610} plotted vs equivalents of metal ions for (A) gradual addition and (B) one-portion addition: (■) $MnCl_2$, (▲) $CaCl_2$, and (○) NaCl.

of Mn^{2+} is an outcome of both the tight binding region and the positive cooperativity region, yet we could not differentiate between these two regions from the absorption measurements. Importantly, the binding of the first 2 equiv of $MnCl_2$ had but a minor effect on retinal absorption, indicating that the chloride anion did not occupy its binding site close to the retinal protonated Schiff base. Similarly, occupation of the cooperative region did not significantly affect retinal absorption. Our data point to a greater change in retinal absorption intensity for $MnCl_2$ than for other ions, such as Ca^{2+}, Na^+, and $MnSO_4$. We assume that the change in the absorption is due to the binding of a Cl^- ion in the vicinity of the protonated Schiff base; however, our EPR results indicate that the cations bind to the protein, as well, and the nature and binding constant are interlinked to the type of counteranion. The binding of $MnCl_2$ also affects bacterioruberin absorption (Figure S8B of the Supporting Information); the initial decrease in RN band intensity can be correlated with the binding of Cl^- ions to the secondary binding site (site II) at the interprotomer crevice.[23]

One-Portion Addition of a High Concentration of Salts. The addition of higher concentrations of salts to the deionized phR sample in one major dose led to changes in both the retinal and RN absorption bands (Figure 6B and Figures S9 and S10 of the Supporting Information). It appears that the transition to the purple form, for both $MnCl_2$ and $CaCl_2$, consists of two phases: a significant change in the first phase of up to 200 equiv and a minor change in the second phase. In the case of NaCl, however, both phases contribute equally to the transition. The difference in spectra obtained from the titration

DOI: 10.1021/acs.biochem.5b00039
Biochemistry 2015, 54, 3164–3172

experiments indicates that, especially for Mn^{2+} and Ca^{2+} ions, the RN bands are perturbed differently under these two distinct phases. Also, the gradual addition of $MnCl_2$ and $CaCl_2$ leads to a significantly larger decrease in the retinal band intensity compared to the introduction of a single major dose (Figure S11A,B of the Supporting Information).

To pinpoint the effect of the Cl^- anion, we titrated the deionized halorhodopsin with $MnSO_4$ and Na_2SO_4 and monitored the changes in UV−vis absorption. We noted a gradual decrease in the intensity of the retinal band for $MnSO_4$, which was significantly higher compared to that of Na_2SO_4 (Figure S12C of the Supporting Information). The ΔA_{600} up to around 15 equiv, which forms the first phase of the transition, can be correlated with the Scatchard plot (Figure S2 of the Supporting Information) derived from the EPR measurements, where it is observed that phR can bind up to ∼17 equiv of Mn^{2+} ions. The binding is much weaker and negligible beyond 17 equiv of Mn^{2+} ions. However, we were unable to differentiate the tight binding region in the UV−vis titration experiments. Our comparison of results for $MnCl_2$ and $MnSO_4$ indicates that retinal absorption is more significantly affected by the addition of $MnCl_2$. Kanada et al. reported that the absorption peak of the retinal chromophore in the blue form of the KM-1 mutant is practically unaffected by the addition of sulfate ions. The decrease in the bacterioruberin band intensity, however, is related to the binding of a sulfate ion to the secondary binding site (site II) at the interprotomer crevice.[23] Our finding that the change in the retinal absorption band is significantly larger for $MnSO_4$ than for Na_2SO_4 may stem from the synergistic binding effect of the SO_4^{2-} and Mn^{2+} ions close to site I. In contrast, the Cl^- ions that occupy both site I and site II promote stronger binding of the counterion Mn^{2+} compared to that of $MnSO_4$ to the blue membrane. It is clear that the strong affinity of Cl^- ions for sites I and II facilitates the capture of more Mn^{2+} ions to the blue membrane compared to the sulfate ion.

DISCUSSION

Existence of Multiple Cation Binding Sites. The phR protein is a well-known ion pump with a high affinity for chloride ions, which bind to multiple sites of the protein. We were interested in ascertaining whether cations also bind to this protein. Our EPR measurements revealed multiple binding sites in the deionized phR for Mn^{2+} ions. At pH 4.8, the first 2 equiv of Mn^{2+} ions bind strongly, which is why we were unable to detect a free Mn^{2+} signal in this region by EPR spectroscopy. An additional 5 equiv binds in a positive cooperative manner and the next 20 equiv with a somewhat lower affinity ($k = 1.1 \times 10^5$ M^{-1}). The protein can accommodate many more equivalents of Mn^{2+} ions than bacteriorhodopsin, particularly for the strong positive cooperative binding. This positive cooperativity is observed only when the Mn^{2+} ions are added gradually starting with minute concentrations (<1 equiv). Surprisingly, the binding of Mn^{2+} cations is significantly weakened when ∼11 equiv of Mn^{2+} cations is added all at once rather than gradually. We propose that the slow increase in concentration assists in creating binding sites with a high affinity for Mn^{2+} ions. This is due to protein conformational alterations induced by the occupation of strong binding sites that, in turn, facilitates cooperative binding of additional Mn^{2+} cations and, possibly, the formation of Mn^{2+} clusters.[42,43] It is plausible that the addition of higher concentrations of Mn^{2+} ions leads them to first occupy weaker binding sites due to a favorable kinetic effect. This binding process prevents the conformational change from occurring, and therefore, the cooperative effect and the binding to high-affinity binding sites do not take place. It is possible that the proteins adopt distinct conformations in each of these two cases.

The question of why the addition of <1 equiv induces the cooperative effect remains. It was suggested that bacteriorhodopsin has two high-affinity cation binding sites and four to six low-affinity sites.[24−27] It was also proposed that the binding of a cation to the second high-affinity site correlates with the blue-to-purple absorption transition.[24,28] This absorption transition is associated with the protonation of the Asp85 residue, which serves as a main component of the counterion complex. The protonation state of Asp85 is crucial for the biological function of bacteriorhodopsin, because it functions as the proton acceptor residue from the retinal protonated Schiff base following light absorption. It was demonstrated that the first cation equivalent binds in a cooperative manner.[44] We observed in the work presented here a similar phenomenon, in which the addition of <1 equiv to halorhodopsin mutant KM-1 induces a specific conformational alteration that allows Mn^{2+} ions to assume a cluster formation. It is well-established that bacteriorhodopsin is arranged in trimers, with a similar structure detected for halorhodopsin mutant KM-1.[12,23,45−49] We propose that the addition of <1 equiv of Mn^{2+} ions to halorhodopsin causes their binding to a member of the trimer, thereby inducing an alternation in the conformation of the other members. This proposal lends support to previous studies concerning the apo membrane of bacteriorhodopsin, demonstrating that binding of retinal to one monomer affects its binding to the other trimer members.[50,51]

The binding of a large number of Mn^{2+} ion equivalents to phR and, probably, the formation of clusters are prevented either by the oxidation of the bacterioruberin chromophore by potassium persulfate or by the formation of an apo membrane by cleavage of the protonated Schiff base linkage and the creation of a retinal oxime. The cluster of Mn^{2+} ions may involve the bacterioruberin chromophore and may also contain oxygen atoms from protein residues, like carboxylates and/or the hydroxyl groups of bacterioruberin. The oxidation reaction, which usually proceeds via a radical mechanism, oxidizes the bacterioruberin's double bonds [which is evident in the formation of a blue-shifted (∼300 nm) absorption maximum of the product] but may also react with the tertiary hydroxyl groups, thereby preventing their participation in cluster formation. Another possible scenerio is that altering the protein's conformation, triggered by the bacterioruberin structural modification, prevents cluster formation. Formation of the apo membrane also prevents the assembly of the ion clusters. Because it is reasonable to assume that the retinal chromophore does not directly contribute to the cluster structure, it is conceivable that formation of the retinal oxime induces protein conformational alterations that may prevent cluster assembly. In this regard, we note that formation of the retinal oxime alters bacterioruberin absorption (data not shown) in spite of the large distance between the two chromophores.

Binding of Other Cations. In addition to our Mn^{2+} binding studies, we investigated the binding of other cations, e.g., Ca^{2+} and Na^+, to phR. We found that Ca^{2+} cations do bind to the protein but more weakly than Mn^{2+} ions, as deduced from competition studies. Na^+ ion binding appears to be even weaker, like that observed for their binding to BR, whereby

DOI: 10.1021/acs.biochem.5b00039
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divalent cations exhibit far greater binding affinity than monovalent cation.

The competition experiments we performed indicate that Ca^{2+} ions can compete effectively with the first 2 equiv of Mn^{2+} ions, and especially the first equivalent. However, incubation with a greater number of equivalents of Mn^{2+} ions results in marginal displacement by Ca^{2+} ions. It is, therefore, plausible that both ions share the same strong binding sites (2 equiv). The Ca^{2+} ions do not exhibit the second region of positive cooperativity in the binding curve. The much weaker binding of the Ca^{2+} cations can be linked to their inability to form clusters, as opposed to Mn^{2+} ions.[42,43] Extensive work was conducted to try to identify the location of cation binding sites on other retinal proteins. Several studies of bacteriorhodopsin have suggested that at least one of the cations' binding sites is located in the retinal binding pocket.[24,30−32] In contrast, studies of Asp85 reprotonation as a function of cation size have proposed that the color-controlling cation binding site is positioned in an exposed location on or close to the membrane surface.[36] The latter conclusion is based on the observation that the large cation $Fe(OP)_3^{2+}$ competes effectively and can replace the Ca^{2+} ion. In contrast to the results concerning bacteriorhodopsin, our findings regarding phR from competition studies with $Fe(OP)_3^{2+}$, which is believed to be capable of binding only to the protein surface, indicate that this cation fails to displace the bound Mn^{2+} ions from the tight binding region of halorhodopsin. This observation suggests that the 2 equiv of Mn^{2+} characterized by high-affinity binding sites is not located on or close to the protein surface. However, $Fe(OP)_3^{2+}$ does compete with Mn^{2+} in the region of positive cooperativity, suggesting that these binding sites are positioned on or near the protein surface. Nevertheless, we cannot completely exclude the possibility that $Fe(OP)_3^{2+}$ binds to another binding site in the vicinity of the protein surface, thereby affecting the Mn^{2+} binding site and weakening manganese ion binding. Further studies are needed to locate this cation's binding sites.

The monovalent Na^+ ions have low binding affinity for phR and cannot compete with Mn^{2+} ions at low concentrations. However, titration of the deionized protein with $MnCl_2$ in the presence of relatively high concentrations of Na^+ ions (1 mM NaCl) changes the binding behavior of the Mn^{2+} ions and eliminates the positive cooperative effect. We, therefore, propose that at high NaCl concentrations, deionized phR adopts a conformation that prevents Mn^{2+} ions from associating with the high-affinity binding sites. The weak binding of Na^+ to phR resembles the behavior of BR, in which divalent cations bind more strongly than monovalent cations. In the case of bacteriorhodopsin, it was suggested that the cations bind to the membrane surface and control the apparent pK_a of Asp85, thereby bringing about the purple-to-blue transition through surface potential.[33−35] Free (Guy Chapman) or bound metal cations on the membrane surface compete with protons and, thus, determine the local proton concentration around the membrane. Therefore, a lower concentration of divalent cations relative to that of monovalent cations is needed to induce the same effect. This explanation can hold true also for halorhodopsin. Another possibility is associated with the higher affinity of divalent cations for specific binding sites, at least for the two strong binding sites of halorhodopsin.

Effect on Absorption Maxima. It is well-established that chloride ions bind to phR in a manner much stronger than that of sulfate ions, affecting the absorption maximum of halorhodopsin.[23,52,53] $MnCl_2$ and $MnSO_4$ exhibit highly similar binding behaviors for the first 2 equiv, as detected by the EPR measurements described above. These results suggest that binding of Mn^{2+} to the tight binding region does not depend on the nature of the anion. $MnSO_4$ binding is considerably weakened for the additional binding sites, and unlike $MnCl_2$, it lacks the positive cooperativity effect. Kanada et al.[23] have proposed that in the phR KM-1 mutant, the Cl^- ions occupy site I close to the retinal protonated Schiff base (Figure 2A), site II, located close the one terminal end of bacterioruberin bound to the interprotomer crevice (Figure 2B), and two additional sites located between the loops of each subunit. A different study conversely advocated that SO_4^{2-} can bind to only site II.[23] However, our observation of small changes in the retinal absorption band even in the case of $MnSO_4$ points either to Mn^{2+} binding altering the protein conformation such that sulfate ions can bind to some extent also close to the retinal binding site or to this conformational change itself inducing the absorption change. As discussed earlier, chloride ions have a greater number of binding sites and stronger affinity for phR compared to that of sulfate. Because $MnSO_4$ lacks the other $MnCl_2$ binding regions, it appears that the $MnCl_2$ occupancy of these sites arises from a synergistic effect exhibited by the chloride ions in the binding process.

Our absorption measurements indicate that binding of the first 2 equiv of Mn^{2+} ions to deionized phR, as well as 7 equiv (the cooperative region), does not induce a major change in the absorption spectra. Higher concentrations of chloride ions are needed to blue shift the absorption. Hence, the first 7 equiv of Mn^{2+} ions appears to bind strongly, as confirmed by EPR spectroscopy, but most Cl^- anions do not occupy the binding site close to the retinal protonated Schiff base. Upon binding of up to 30 equiv of Mn^{2+}, the absorption of the retinal band changes significantly. We, therefore, conclude that prior to binding to site I, the Mn^{2+} and Cl^- ions first occupy other binding sites that are not located near the retinal protonated Schiff base. The synergetic effect between cation and anion binding suggests that while the Cl^- anion does bind to the protein, its binding to site I requires a high concentration. Moreover, the change in the bacterioruberin absorption band intensity following the addition of the cations suggests that their binding affects the protein's conformation such that the bacterioruberin senses a shift in the protein environment and/or modifies its conformation. Moreover, it is clearly evident that association with the different binding sites influences RN absorption in a distinct manner (Figure S9 of the Supporting Information), suggesting that occupancy of each binding site induces a specific protein conformational change and, as a result, RN senses a different environment, reflected in its absorption.

Our EPR measurements indicate that binding of Mn^{2+} to phR is markedly affected by the mode in which the addition is performed, whether gradually or all at once. This phenomenon is further supported by the absorption measurements showing that the gradual addition of Mn^{2+} ions affects the retinal band intensity in a more significant manner. It is conceivable that a higher concentration of Mn^{2+} ions promotes their attachment to weaker binding sites due to a kinetic effect, thereby inducing an alternation to the protein's conformation that eliminates the strong cation binding sites.

pH Dependence and Significance of the Chromophore in the Binding Process. We observed a strong positive correlation between the pH and the strength of binding of Mn^{2+} ion to phR. This dependence can be linked to the

DOI: 10.1021/acs.biochem.5b00039
Biochemistry 2015, 54, 3164−3172

contribution of aspartate residues, which are deprotonated at higher pH, thereby promoting Mn^{2+} ion binding. It appears that the retinal and bacterioruberin chromophores also influence the binding process. Our EPR measurements for bleached phR lacking the retinal−protein protonated Schiff base linkage and for phR with oxidized bacterioruberin revealed a drastic reduction in the level of Mn^{2+} binding in both cases. These results highlight the vitality of maintaining the intact structure of both chromophores for strong Mn^{2+} binding. It was previously shown regarding BR that formation of the apo membrane by eliminating the protein−retinal protonated Schiff base covalent linkage alters the protein's conformation and eliminates part of the cations' strong binding affinity for the protein.[26] Our results indicate that producing the apo membrane of halorhodopsin by way of a reaction with hydroxylamine also affects the absorption of the bacterioruberin (data not shown). It is, therefore, conceivable that the protein undergoes conformational alterations that may prevent cations from binding. While we have yet to identify the location of the cation binding sites, the fact that oxidizing the bacterioruberin weakens the cation's binding affinity may suggest that bacterioruberin is involved in Mn^{2+} binding. The oxidation reaction significantly perturbed the conjugated polyene by oxidation of double bonds, thereby inducing a conformational change in phR and/or RN. It is likewise possible that the hydroxyl groups of RN are, too, involved in cation binding and that the oxidation with persulfate that proceeds via a radical mechanism may also react with the hydroxyl groups.

CONCLUSION

Our studies reveal that divalent cations do indeed bind to halorhodopsin. The biological function of this protein requires chloride anion binding in the vicinity of the retinal protonated Schiff base (site I). However, our studies indicate that the first 2 equiv of cations binds before site I is occupied by a chloride anion. This may suggest that binding of the first 2 equiv is a prerequisite for chloride binding to site I. The binding of a cation may alter the protein conformation in such a way that allows for chloride binding. For example, Arg123 (Figure 2A), which is crucial for chloride binding, may alter its location following cation binding. Moreover, it is known that light absorption prompts the chloride anion in site I to change its location.[17,54,55] The observation raises the question of whether divalent cations are affected, too. These issues, as well as the need to identify the exact location of the cation binding sites, should be the subject of future studies.

The special cooperative binding of Mn^{2+} cations and the relatively large number of equivalents that bind with high affinity have led us to suggest the formation of manganese clusters. We cannot determine at present the size of the clusters and their location within the protein, but it is plausible that bacterioruberin is involved in the creation of these clusters.

ASSOCIATED CONTENT

Supporting Information

More data related to competition studies with different Ca^{2+}, $[Fe(OP)_3]^{2+}$, and Na^+ ions and the difference in absorption spectra of deionized halorhodopsin as a result of titration with different cations, absorption changes due to the presence of sulfate ions, and comparative data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00039.

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Funding

We thank the Kimmelman Center for Biomolecular Structure and Assembly for support. M.S. holds the Katzir-Makineni Professorial Chair in Chemistry.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

BR, bacteriorhodopsin; hR, halorhodopsin; SR, sensory rhodopsin; phR, halorhodopsin from *N. pharaonis* mutant; RN, bacterioruberin; EPR, electron paramagnetic resonance; UV−vis, ultraviolet−visible; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Arg, arginine; Asn, asparagine; Lys, lysine; Ser, serine; Thr, threonine

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