Experimental and Theoretical Characterization of the High-Affinity Cation-Binding Site of the Purple Membrane

Leonardo Pardo,* Francesc Sepulcre, Josep Cladera, Mireia Duñach, Amílcar Labarta, Javier Tejada, and Esteve Padrós

*Laboratori de Medicina Computacional, Unitat de Bioestadística, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona; †Unitat de Biofísica, Departament de Bioquímica i de Biologia Molecular, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona; and ‡Departament de Física Fonamental, Facultat de Física, Universitat de Barcelona, 08028 Barcelona, Spain

ABSTRACT Binding of Mn$^{2+}$ or Mg$^{2+}$ to the high-affinity site of the purple membrane from Halobacterium salinarium has been studied by superconducting quantum interference device magnetometry or by ab initio quantum mechanical calculations, respectively. The binding of Mn$^{2+}$ cation, in a low-spin state, to the high-affinity site occurs through a major octahedral local symmetry character with a minor rhombic distortion and a coordination number of six. A molecular model of this binding site in the Schiff base vicinity is proposed. In this model, a Mg$^{2+}$ cation interacts with one oxygen atom of the side chain of Asp$^{85}$, with both oxygen atoms of Asp$^{212}$ and with three water molecules. One of these water molecules is hydrogen bonded to both the nitrogen of the protonated Schiff base and the Asp$^{85}$ oxygen. It could serve as a shuttle for the Schiff base proton to move to Asp$^{85}$ in the L-M transition.

INTRODUCTION

The purple membrane (PM) from Halobacterium salinarium is a specialized part of the cellular membrane that translocates protons under light absorption (Oesterhelt and Stoeckenius, 1973). It contains a unique transmembrane protein, bacteriorhodopsin (BR), which is formed of an apoprotein of $M_r$ 26,000 and a retinal molecule bound to the protein through a protonated Schiff base. Native purple membrane ($\lambda_{\text{max}}$ 568 nm, light adapted) contains five bound cations (one Ca$^{2+}$ and four Mg$^{2+}$) per bacteriorhodopsin molecule (Kimura et al., 1984; Chang et al., 1985). Acidification of a PM suspension gives rise to a blue form absorbing at $\sim$600 nm, which is due to the protonation of Asp$^{85}$, the Schiff base counterion (Subramaniam et al., 1990; Jonas and Ebrey, 1991; Metz et al., 1992). Upon deionization, the apparent pK of the purple to blue transition in water suspension increases by $\sim$2.5 pH units, as compared to the native membrane. The deionized membrane can be fully regenerated by adding a wide variety of cations (Kimura et al., 1984; Chang et al., 1985; Ariki and Lanyi, 1986). The blue membrane has an altered photocycle, and it is unable to translocate protons (Mowery et al., 1979; Chang et al., 1985). On the other hand, a relationship between the retinal pocket and some of the divalent cation-binding sites has been shown (Duñach et al., 1986; Sepulcre and Padrós, 1992).

The binding of the Mn$^{2+}$ cations to the blue membrane at pH 5 was determined, by spin-labeling methods, to consist of a high-affinity site (affinity constant 26 $\mu$M$^{-1}$), three sites of 2 $\mu$M$^{-1}$, and one site of 0.6 $\mu$M$^{-1}$ (Duñach et al., 1987). Similar values were found at pH 5 for Ca$^{2+}$ binding, with a rapid-filtration technique (Duñach et al., 1988b). Other workers reported, by using potentiometric techniques, the presence of only two medium-affinity sites (2.4 $\mu$M$^{-1}$ and 0.4 $\mu$M$^{-1}$, respectively) plus four low-affinity sites at pH 4.3 (Zhang et al., 1992). In addition, extended x-ray absorption fine structure (EXAFS) studies provided evidence for a tetragonal coordination of Mn$^{2+}$ with six oxygen atoms located in the protein molecule (Sepulcre et al., 1996).

The magnetic susceptibility technique provides an independent means of corroborating our previous EXAFS results (Sepulcre et al., 1996). In the present work, we collected magnetic susceptibility data obtained by superconducting quantum interference device (SQUID) magnetometry from the blue membrane substituted with one Mn$^{2+}$ cation occupying the high-affinity site. In the scope of the crystal field theory, this study allows us to deduce both the local symmetry and the electronic structure of Mn$^{2+}$ bound to this site. A possible structure for the high-affinity cation-binding site in bacteriorhodopsin is proposed; its feasibility is tested by quantum mechanical calculations.

MATERIALS AND METHODS

Membrane preparation

The purple membrane was isolated from the Halobacterium salinarium strain S9 as described in Oesterhelt and Stoeckenius (1974). Deionized samples were prepared by passing membrane suspensions through a cation exchange column (Dowex 50W). After addition of enough MnCl$_2$ to fill the high-affinity site (Duñach et al., 1987), the pH of the sample was adjusted to pH 5 with small amounts of concentrated NaOH. Correct binding of cations was controlled by observing the blue shift of the visible absorption spectrum (Duñach et al., 1987). Five milligrams of the partially regenerated membrane was lyophilized for magnetic susceptibility measurements.
SQUID magnetometry

Magnetic susceptibility measurements were carried out by using a SQUID magnetometer working in a temperature range between 2 K and 310 K, and with an applied magnetic field, \( H \), of 5 kOe. Experimental error of temperature measurements were less than 0.1 K, whereas the estimated error for each \( \chi(T) \) point was below 5%. The diamagnetic correction, due to both the cylindrical plastic boat and the membrane, was achieved by recording the thermal dependence of the susceptibility under different values of the applied magnetic field ranging from 2 kOe to 15 kOe.

Near room temperature, the temperature dependence of the susceptibility can be expressed as

\[
\chi(T) = \frac{C}{T} + \chi_d
\]

where \( C \) is the Curie constant and \( \chi_d \) is the diamagnetic susceptibility due to the container and membrane diamagnetic atoms. Therefore, \( \chi(T) \cdot T = C + \frac{\chi_d}{T} \cdot T \), and \( \chi(T) \cdot T \) has a linear dependence on \( T \), where \( \chi_d \) is the corresponding slope. We verified this linearity with different values of \( H \) and by using the preceding equation, we evaluated the average \( \chi_d \) value.

Susceptibility calculations

The energetically lowest lying multielectron terms of the Mn\(^{2+}\) cation have been obtained, in the scope of a single-point crystal-field model, from the diagonalization of the Hamiltonian,

\[
H_o = H_{ee} + H_{cf}
\]

on the basis of the 3d\(^5\) configuration. In this Hamiltonian, \( H_o \) corresponds to the Coulomb repulsion between electrons, and \( H_{cf} \) accounts for the crystal field potential, which for the position of the Mn\(^{2+}\) cations in the purple membrane is assumed to have \( C_{2v} \) symmetry (tetragonal with rhombic distortion) and can be expanded in terms of the \( V_{ee} \) to the Coulomb repulsion between electrons, and \( H_x \) to the container and membrane diamagnetic atoms. Therefore, \( \chi(T) \cdot T = C + \chi_d \cdot T \), and \( \chi(T) \cdot T \) has a linear dependence on \( T \), where \( \chi_d \) is the corresponding slope. We verified this linearity with different values of \( H \), and by using the preceding equation, we evaluated the average \( \chi_d \) value.

Geometries and energetics

All of the quantum mechanical calculations were performed by ab initio methods in the GAUSSIAN-94 system of programs (Frisch et al., 1995).

The structure optimizations of (Mg\(^{2+}\) \cdot 2H\(_2\)O), (Mg\(^{2+}\) \cdot 3H\(_2\)O), and Mg\(^{2+}\) complexes were performed with the 3–21G* basis set. Energy calculations of the interaction between the cation and the protein model, \( E_{int} \), were performed with the 6–31 + G* basis set at the level of Restricted Hartree-Fock (RHF). Solvation energies, \( E_{sol} \), of isolated (Mg\(^{2+}\) \cdot 2H\(_2\)O) and (Mg\(^{2+}\) \cdot 3H\(_2\)O) were calculated with a polarized continuum model (Miertus et al., 1981; Miertus and Tomasi, 1982), as implemented in GAUSSIAN-94. The enthalpy of formation of the complex between the cation and the protein model was calculated as \( \Delta H_f = E_{\text{int}} - E_{\text{sol}} \).

The model of BR sites employed in the calculation of \( E_{\text{int}} \) comprised the \( C_{3v} \) and the side chains of Asp\(^{85}\), Asp\(^{212}\), and Lys\(^{216}\) Schiff base. The retinal chromophore bound to Lys\(^{216}\) via a protonated Schiff base was replaced with a \( \equiv \text{CH}_2 \) group. During the energy optimization of the system, the position of the atoms \( C_{12} \) of Asp\(^{85}\) and Asp\(^{212}\), and \( C_{6}, C_{b}, C_{c}, C_{p}, N_{c}, \) and \( C_{13} \) of Lys\(^{216}\) Schiff base were kept fixed at the positions originally determined by electron microscopy (Henderson et al., 1990).

RESULTS

Magnetic susceptibility experiments

The results of the magnetic susceptibility measurements are given in Fig. 2. These data have been fitted to the theoretically calculated magnetic susceptibility, using as adjustable parameters the values of the 3d\(^1\) splittings, \( \xi_i \) (\( i = 1, 2, 3 \)) and \( D \), and the spin-orbit coupling constant \( \lambda \), which can be expressed as a function of the free ion spin-orbit coupling constant \( \lambda_{\text{f}} = 300 \text{ cm}^{-1} \) and a fit parameter taking into account the covalency degree of the binding of the Mn\(^{2+}\) cations with its ligands (\( \lambda = \lambda_{\text{f}} \xi \)). Table 1 summarizes the results of the fitting procedure, compared with the experimental data. The resulting energy diagram of the low-lying multielectron states of Mn\(^{2+}\) cation is shown in Fig. 3.

The values obtained for the crystal-field parameters \( \xi_i \) and \( D \) can be correlated with the local structure of the Mn\(^{2+}\) site. The \( \xi_3 \) value gives the energy of the antibonding single electron orbital \( d_{x^2-y^2} \) referred to the \( d_{xy} \) orbital. The high value found for \( \xi_3 \) suits well the major tetragonal character of the local symmetry around the Mn\(^{2+}\) location site. This suggests a strong interaction between the Mn\(^{2+}\) ion and the ligands lying in the \( xy \) plane. \( \xi_2 \) is the energy of the antibonding \( 3d_{z^2} \) orbital referred to the \( 3d_{xy} \) orbital. This value is much lower than \( \xi_3 \) (see Table 1). This indicates that the interaction between the Mn\(^{2+}\) and the ligands lying in the \( z \) direction is different between them or is different from the other ligands of the \( xy \) plane. In addition, the low value obtained for the \( D \) parameter indicates a minor rhombic local distortion around the Mn\(^{2+}\) site in the \( xy \) plane.

Comparison of our results with those previously published for heme systems and Mn\(^{2+}\)-phthalocyanine complexes (Thomanek et al., 1977; Labarta et al., 1984, 1985) show that the \( ^2E \) low-spin state appears as the ground term in the present case. This is a consequence of a higher value of the crystal field intensity as it is characterized by the \( \xi_3 \) parameter. Therefore, it is reasonable to assume that the interactions between the Mn\(^{2+}\) cation and the ligands, indicated by \( \xi/\lambda \) ratios (where \( \xi \) is the effective neighbor charge and \( \lambda \) is the distance between this neighbor charge and the cation) are higher in our case than in the heme systems or in the Mn\(^{2+}\)-phthalocyanine complex.
It should be highlighted that these results are in good agreement with EXAFS data, which demonstrated that Mn$^{2+}$ in the high-affinity binding site presents a distorted tetrahedric symmetry with a coordination number of 6. A location of this site within the protein and not in the lipid phase was also suggested (Sepulcre et al., 1996). The independence of the two techniques used reinforces the conclusions obtained. Thus, having corroborated the metal coordination and geometry, we proceeded toward finding a suitable molecular environment for the cation.

In the following, we take as equivalent a binding site occupied indistinctly by Ca$^{2+}$, Mn$^{2+}$, or Mg$^{2+}$.

Several previous results can aid in defining a probable location for the cation-binding site. Although indirect effects could also account for the observed events, it is generally thought that a cation site near the retinal Schiff base is necessary to explain 1) the well-known effect of cation

![Figure 2](image2.png)

**Figure 2.** Plot of $P^{-1}$ values as a function of temperature. The continuous line corresponds to the least-squares fit of $P^{-1}$ to the experimental values, using as adjustable parameters the values of the 3d$^4$ splittings and $D$, and the spin-orbit coupling constant $\delta$.

![Figure 3](image3.png)

**Figure 3.** Electronic structure levels of Mn$^{2+}$ occupying the high-affinity site of purple membrane.
binding on the visible absorption maximum; 2) the change in number and affinities of the cation-binding sites by retinal removal (Chang et al., 1986; Dunaev et al., 1986; Zhang et al., 1992); and 3) the change in cation binding by Schiff base reduction or by isomerization to 9-cis, i.e., the pink membrane (Dunaev et al., 1988a). If the retinal absorption maximum is modulated primarily by the protonation state of Asp$^{85}$ and its distance to the Schiff base, a natural site for the cation would be near Asp$^{85}$. In addition, experiments with mutated BR demonstrated a strong influence of Asp$^{85}$ and Asp$^{212}$, especially the latter, on the binding affinity of Ca$^{2+}$ (Zhang et al., 1993). On the other hand, EXAFS results indicated a maximum of three carbon atoms forming the second shell of the Mn$^{2+}$ cation and excluded a participation of P or S atoms (Sepulcre et al., 1996).

Taking into account the above considerations, and the arrangement of the lateral chains near the Schiff base that arise from the structural model of Henderson et al. (1990), we have undertaken a theoretical analysis of the possible environment of a Mg$^{2+}$ cation near the Schiff base.

**A model of the binding site in the Schiff base environment**

As a working hypothesis, we can assume that Mg$^{2+}$ binds to BR through an octahedral coordination shell formed by the two carboxylic side chains of Asp$^{85}$ and Asp$^{212}$, located in the base of the pyramid, and two discrete water molecules located in the axis. To evaluate computationally the feasibility of this hypothesis, a molecular model consisting of (Mg$^{2+}$ - 2H$_2$O) and the side chains of Asp$^{85}$, Asp$^{212}$, and the Schiff base was energy optimized. During the optimization (see Fig. 4 A and Materials and Methods), the C$_n$ of the amino acids and the heavy atoms of the side chain of Lys$^{216}$ forming the Schiff base were kept fixed at the positions originally determined by electron microscopy (Henderson et al., 1990). For a buried cation in the interior regions of BR, it is clear that the cation must be desolvated. We considered first the contribution of solvation energies to the stabilization of the proposed complex. Results in Table 2 show the obtained values of $E_{\text{int}}$, $E_{\text{solv}}$ and $\Delta H_f$. As expected, $E_{\text{solv}}$ is very high: $-330.0$ kcal/mol for (Mg$^{2+}$ - 2H$_2$O). This energy is compensated for by the strong interaction with the highly polar sites on the protein model: $-403.1$ kcal/mol, resulting in a value of $\Delta H_f$ of $-73.1$ kcal/mol. The negative sign in $\Delta H_f$ indicates that the formation of the complex is favorable. It is important to clarify that the calculation of $\Delta H_f$ does not include the change in solvation energy of BR or its conformational change. However, given the large value of $\Delta H_f$ obtained in the formation of the complex, inclusion of these terms into $\Delta H_f$ is expected not to modify the obtained preference of the complex over the isolated ligands.

We can conclude that the binding of the divalent cation to the retinal pocket of BR, through the side chains of Asp$^{85}$ and Asp$^{212}$, is energetically feasible despite the presence of the positive charge of the Schiff base. Fig. 4 B presents a detailed view of the computed cation-binding site. Selected geometrical parameters of the optimized structure are shown in Table 3. The proposed interaction between the cation and the Asp residues is directly satisfied by the geometry constructed here. As can be seen in Fig. 4 B and Table 3, the Mg$^{2+}$ cation has an octahedral coordination shell formed in the base of the pyramid by the O$_a$ atoms of Asp$^{85}$ (Mg$^{2+}$ - O$_a$ distances of 2.08 and 2.23 Å), and the O$_w$ atoms of Asp$^{212}$ (Mg$^{2+}$ - O$_w$ distances of 2.23 and 2.36 Å); and at the vertex of the pyramid by two water molecules (W1 and W2; Mg$^{2+}$ - O$_w$ distances of 2.11 and 1.96 Å, respectively). The mean interatomic distance between Mg$^{2+}$ and O, obtained with ab initio structure optimization,
TABLE 2 Energy of interaction, energy of solvation, and enthalpy of formation of the complex between the cation and the protein model

<table>
<thead>
<tr>
<th>Cation</th>
<th>(E_{\text{int}})</th>
<th>Protein</th>
<th>(E_{\text{solv}})</th>
<th>(E_{\text{f}})</th>
<th>(\Delta H_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\text{Mg}^{2+} \cdot 2\text{H}_2\text{O}))</td>
<td>-330.0</td>
<td>Asp(^5) \cdot Asp(^{212}) \cdot Lys(^{216})</td>
<td>-403.1</td>
<td>-73.1</td>
<td></td>
</tr>
<tr>
<td>\text{Mg}^{2+}</td>
<td></td>
<td>Asp(^{5})</td>
<td></td>
<td>-367.5</td>
<td></td>
</tr>
<tr>
<td>\text{Mg}^{2+}</td>
<td></td>
<td>Asp(^{212})</td>
<td></td>
<td>-351.1</td>
<td></td>
</tr>
<tr>
<td>((\text{Mg}^{2+} \cdot 3\text{H}_2\text{O}))</td>
<td>-292.1</td>
<td>Asp(^5) \cdot Asp(^{212}) \cdot Lys(^{216})</td>
<td>-351.9</td>
<td>-60.8</td>
<td></td>
</tr>
<tr>
<td>\text{Mg}^{2+}</td>
<td></td>
<td>Asp(^{5})</td>
<td></td>
<td>-317.0</td>
<td></td>
</tr>
<tr>
<td>\text{Mg}^{2+}</td>
<td></td>
<td>Asp(^{212})</td>
<td></td>
<td>-362.6</td>
<td></td>
</tr>
</tbody>
</table>

\(E_{\text{int}}\) Energy of interaction; \(E_{\text{solv}}\) energy of solvation; \(\Delta H_f\) enthalpy of formation. Values are in kcal/mol.

is in very good agreement with the experimental distance between \(\text{Mn}^{2+}\) and O, obtained with the EXAFS technique (Sepulcre et al., 1996; 2.16 versus 2.17 Å; see Table 3). In addition, the water molecule located in the upper vertex of the pyramid is hydrogen bonded to the protonated Schiff base nitrogen.

However, these results are not in good agreement with some experimental determinations. In particular, mutation of Asp\(^{85}\) to Asn decreases the affinity of BR for Ca\(^{2+}\) by about three times, whereas mutation of Asp\(^{212}\) to Asn decreases the affinity by 15 times (Zhang et al., 1993). This suggests that the cation is more tightly bound to Asp\(^{212}\) than to Asp\(^{85}\). The values of \(E_{\text{int}}\) obtained for the interaction between \(\text{Mg}^{2+}\) and both Asp residues, shown in Table 2, are not in agreement with this rank order of affinities. Thus the model structure depicted in Fig. 4 B cannot explain the different observed affinities of Asp\(^{85}\) and Asp\(^{212}\) for the cation.

A possibility for decreasing the energy of interaction of \(\text{Mg}^{2+}\) with Asp\(^{85}\) is the introduction of a new water molecule in the \(xy\) plane. The optimized geometry of the system is shown in Fig. 5. The \(\text{Mg}^{2+}\) cation has the O\(_5\) atoms of Asp\(^{212}\) (\(\text{Mg}^{2+} \cdot \cdot \cdot \text{O}_5\) distances of 2.24 and 2.09 Å), the O\(_{61}\) atom of Asp\(^{85}\) (\(\text{Mg}^{2+} \cdot \cdot \cdot \text{O}_6\) distance of 2.18 Å), and the oxygen atom of a water (W3) molecule (\(\text{Mg}^{2+} \cdot \cdot \cdot \text{O}_w\) distance of 2.02 Å), as equatorial ligands. The axis of the pyramid is formed by the other two water molecules (\(\text{Mg}^{2+} \cdot \cdot \cdot \text{O}_w\) distances of 2.20 and 1.99 Å). The average interatomic distance between \(\text{Mn}^{2+}\) and O is 2.12 Å (Table 3). In addition to the above interactions the system contains hydrogen bonds between the O\(_{62}\) atom of Asp\(^{85}\) and W2 and W3 (see Fig. 5 and Table 3). It is quite evident from the value of \(\Delta H_f\) in Table 2 that the binding of \(\text{Mg}^{2+} \cdot 3\text{H}_2\text{O}\) to BR (Asp\(^{85}\) \cdot Asp\(^{212}\) \cdot Lys\(^{216}\) Schiff base) remains favorable (−60.8 kcal/mol). Furthermore, the different coordination of \(\text{Mg}^{2+}\) in this model relative to the previous one shown in Fig. 4 B results in a predicted order of affinities between \(\text{Mg}^{2+}\) and Asp\(^{85}\) and Asp\(^{212}\), based on the energies of the interaction (see Table 2), that qualitatively reproduces the rank order of affinities found experimentally. It also agrees with having a maximum of 3 C atoms in the second coordination shell, as deduced from the EXAFS results (Sepulcre et al., 1996).

TABLE 3 Selected distances of the optimized molecular models consisting of (\(\text{Mg}^{2+} \cdot 2\text{H}_2\text{O}\)) or (\(\text{Mg}^{2+} \cdot 3\text{H}_2\text{O}\)) and the side chains of Asp\(^{85}\), Asp\(^{212}\), and Lys\(^{216}\) of BR

<table>
<thead>
<tr>
<th>Residue (atom)</th>
<th>Asp(^{85})</th>
<th>Asp(^{212})</th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>Mean</th>
<th>Exp</th>
</tr>
</thead>
</table>
| \((\text{Mg}^{2+} \cdot 2\text{H}_2\text{O}) \cdot \cdot \cdot \text{Asp}^{85} \cdot \text{Asp}^{212} \cdot \text{Lys}^{216}\) | \begin{align*} 
\text{Mg}^{2+} & \quad 2.08 \quad 2.23 \\
\text{Lys}^{216} (N_z) & \quad 2.23 \quad 2.36 \\
\text{Lys}^{216} (H_z) & \quad 2.11 \quad 1.96 \\
\end{align*} & \begin{align*} 
\text{Mg}^{2+} & \quad 2.18 \quad 2.24 \quad 2.09 \\
\text{Lys}^{216} (N_z) & \quad 2.20 \quad 1.99 \quad 2.02 \\
\text{Lys}^{216} (H_z) & \quad 2.68 \quad 1.79 \\
\text{W1 (O_w)} & \quad 2.41 \\
\text{W1 (H_w)} & \quad 1.60 \\
\text{Asp}^{85} (O_{62}) & \quad 2.77 \quad 2.63 \\
\end{align*} & | 2.17 |

Distances are in Å.
susceptibility experiments, the distance between Mn$^{2+}$ and O obtained with the EXAFS technique (Sepulcre et al., 1996), and the rank order of affinities between the cation, Asp$^{85}$, and Asp$^{212}$ determined by site-directed mutagenesis (Zhang et al., 1993). In addition, difference infrared spectroscopy experiments (Fischer et al., 1994) have detected the presence of a water molecule, located in the active site of BR, which is structurally active during the BR6K primary phototransition. The same authors postulated the possibility that this structurally active water molecule was located between Asp$^{85}$ and the protonated Schiff base. The most salient geometrical feature of the model proposed here (Fig. 5) is the presence of a water molecule (W1) hydrogen bonded to both the O$_{61}$ atom of Asp$^{85}$ and the oxygen atom of a water molecule (not labeled, located behind the Mg$^{2+}$ cation) as equatorial ligands. The axis of the pyramid is formed by the other two water molecules (W1 and W2).

FIGURE 5 Optimized geometry of the cation-binding site of bacteriorhodopsin. The Mg$^{2+}$ cation has the O$_{6}$ atoms of Asp$^{212}$, the O$_{61}$ atom of Asp$^{85}$, and the oxygen atom of a water molecule (not labeled, located behind the Mg$^{2+}$ cation) as equatorial ligands. The axis of the pyramid is formed by the other two water molecules (W1 and W2).
mented in Tan et al., 1996), which can only occupy a surface location. However, taking into account the suggested existence of several proton channels through which Asp85 can be protonated (Friedman et al., 1997), it is likely that cation binding can affect the state of protonation of Asp85 (and thus the purple-to-blue transition) in different ways: 1) by binding in the neighboring Schiff base; 2) by influencing the proton channels’ conductivity through changes in protein conformation or through changes in the pKₐ of key side chains; 3) by changing the proton concentration at the entrance of the channel or even at the membrane surface. The fact that it is possible to obtain the purple form of the deionized membrane by increasing the pH (pKₐ of ~5.4; Duñach et al., 1988a) gives support to the latter effect.

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