Chapter 7

Calcium Binding to the Purple Membrane
Figure 7.1: Summary of simulation 1 (series A) with calcium ions placed in the Schiff base region (P1). Top: Stereoscopic image (cross-eye) of positions of calcium ions over time and a comparison of structures from the simulations with crystal structures 1QHJ[56] (grey), 1AP9[80] (green) and 2AT9[30] (blue). Numbers indicate residues which are highlighted. 1. Asp85, 2. Asp212, 3. Tyr57, 4. Arg82, 5. Tyr83, 6. Glu194 and 7. Glu204. The calcium ion has a well defined position. The presence of the ion causes D85 (1) and D212 (2) to move closer together than observed in the crystal structures. On average, Tyr57 (3) is moved outward with respect to the orientation in the crystal structures. Arg82 (4) takes an outward position, whereas Tyr83 (5) is moved down. Glu194 (6) moved away from the proton release group and Glu204 (7). Bottom: Stereoscopic image (cross eyed) of the coordination of the calcium ion at P1 in simulation 1. Amino acid ligands are labelled in magenta, whereas water molecules, originally present as crystal bound water, are labelled in blue, with arbitrary numbering.
Figure 7.2: Summary of simulation 2 (series A) with a calcium ion placed near the proton release group (P2, Glu194/Glu204). Top: Stereoscopic image (cross-eye) of positions of calcium ions over time and comparison of structures from the simulations with crystal structures 1QHJ[56] (grey), 1AP9[80] (green) and 2AT9[30] (blue). Numbers indicate residues which are highlighted in the text. 1. Asp85, 2. Asp212, 3. Tyr57, 4. Arg82, 5. Tyr83, 6. Glu194 and 7. Glu204. Calcium ion placed at P1 (Schiff base region). The calcium ion has a well defined position. Asp85 (1) moves towards the Schiff base proton. Asp212 (2) adopts a conformation in agreement with that observed in the crystal structures. Tyr57 (3) is tilted and turned towards Arg82 (4), which moved inward with respect to the crystal structures. Tyr83 (5) reoriented slightly. Glu194 (6) and Glu204 (7), which form the proton release group, adopt a configuration in close agreement with that observed in structure 1QHJ. Bottom: Stereoscopic image (cross eyed) of the coordination of the calcium ion at P2 in simulation 3. Amino acid ligands are labelled in magenta, whereas water molecules, originally present as crystal bound water, are labelled in blue, with arbitrary numbering.
1 Introduction

Bacteriorhodopsin (bR) is a light-sensitive receptor found in certain species of halophilic (salt-loving) archaebacteria. These archaebacteria are typically found in salt lakes or brine pools where the concentration of salt is around 3.3 M or approximately 200 g l\(^{-1}\). Bacteriorhodopsin absorbs green light (500 – 650 nm, maximum absorbance 568 nm), giving rise to an intense purple colour which can cause the salt lakes and pools where the archaebacteria are found to appear purple.

Bacteriorhodopsin is localized in specific patches on the cell surface which are called the purple membrane (PM). The PM is a two-dimensional crystal with an almost perfect hexagonal lattice and a high protein to lipid ratio. The only protein found in the purple membrane is bacteriorhodopsin and the lipid composition of the purple membrane is distinct from the rest of the bacterial membrane.

Bacteriorhodopsin is a member of the superfamily of seven transmembrane helical (7TM) receptors. This family also includes the G-protein coupled receptors, which contains many important (potential) targets for pharmaceutical intervention. For this reason and the ease with which large quantities can be obtained, bacteriorhodopsin has been used as a model for the understanding of sensing and signalling by 7TM receptors.

Functional bacteriorhodopsin consists of a protein part, bacterioopsin, and a covalently linked chromophore, retinal. The linkage is established through the formation of a Schiff base between retinal and Lys216. On exposure to green light the chromophore is excited and isomerizes from an all-trans form to a 13,14-cis configuration. This initiates a series of deprotonation – protonation events, resulting in the effective translocation of a proton from the intracellular matrix to the exterior of the cell. The action of light on bacteriorhodopsin can cause the interior of the cell to become more than 10,000 times more alkaline than the surrounding environment. The proton gradient generated is then used to drive ATP-synthesis.

The purple membrane and bacteriorhodopsin were first isolated in 1970 by Oesterhelt and Stoeckenius[1] and has been the focus of intense study since, with more than 5800 publications to May 2006 (ISI Web of Knowledge). The amino acid sequence or primary structure of bacteriorhodopsin was determined in 1979 by Ovchinnikov et al., who stated that “… it will not be long before we shall be in a position to comprehend within the limits of our present day science the way whereby this magical molecule utilizes light for its functioning.”[2] More than 25 years later, many factors that effect the photo-induced proton translocation by bR remain to be resolved. For example, it is still not certain if bacteriorhodopsin is an outward proton pump[3, 4] or an inward hydroxide pump[5-8].
Another long standing question in regard to bacteriorhodopsin and the purple membrane is the location and role of divalent cations. Acidification\cite{9, 10} of the PM results in a shift in the absorption spectrum $\lambda_{max}$ of bR from 570 nm (purple) to 620 nm (blue). This shift coincides with a loss of proton pumping functionality, which has been ascribed to the protonation of Asp85\cite{11-13}, the key residue in the first of a series of proton transfer steps. It is well established that under physiological conditions, a number of calcium and magnesium ions are bound to the PM \cite{10, 14, 15}. Removal of these cations by chelation\cite{10} or by deionization\cite{10} will result in an increase of the $pK_a$ of Asp85 by about 3 pH units\cite{10}.

Several studies have suggested that the effect of cations is the result of non-specific binding to the lipids of the PM competing with associated protons and thus raising the $pK_a$ of the lipid head groups (Gouy-Chapman effect)\cite{16-19}. The resulting change in the concentration of protons at the membrane surface would then account for the deprotonation of Asp85. Furthermore, results obtained by Váró \textit{et al.}\cite{19} led to the suggestion that cations bind non-specifically and with equal affinity to both the extracellular and intracellular membranes, although it has been argued that this finding may have been the result of rapid equilibration of proton concentrations\cite{20} on both sides of the membrane, preventing the measurement of differences in the cytoplasmic and extracellular surface pH\cite{21}.

Most of the available data suggests, however, that divalent cations bind to specific locations in or around bacteriorhodopsin. Several groups have suggested that a location near Asp85 in the retinal pocket of bR is the critical cation binding site\cite{11, 22, 23}. The possibility of a binding site in this region is supported by a number of studies, including recent XANES results\cite{24-26} and would be consistent with results obtained from one- and two-photon spectroscopy\cite{27}. Nevertheless, there is also considerable evidence against binding at such a location\cite{28, 29}. In particular, in none of the high-resolution crystal structures has it been possible to definitely identify a cation bound in the vicinity of the chromophore, although some density is observed in the structure solved by Mitsuoka \textit{et al.} 2AT9\cite{30}. While this suggests against a binding pocket in this region, the crystallization conditions may have resulted in the loss of the associated cations.

In recent years, a number of binding sites on the extracellular side of bR have been proposed. Among the sites suggested are a position directly associated with the proton release group, formed by Glu194 and Glu204 and a site in the vicinity of Glu9, which is connected to the proton release group by a network of water molecules\cite{31}. Others have however proposed that the most likely binding sites are on the protein – lipid interface, rather than in the interior of the protein. EPR results suggest that a high-affinity binding site was located at a distance of 9.8 ± 0.7 Å of Glu74\cite{21}. This finding is consistent with results obtained from $^{13}$C NMR\cite{32}, which pointed to a location at the protein – lipid interface in the region between helices F and G, close to Ala196.

Here we present the results of molecular dynamics simulations designed to shed light on the possible positions and functional roles of divalent cations within the PM and bR. Over the past decade many molecular dynamics simulation and quantum mechanical studies of bacteriorhodopsin have been performed. Most have focused on the role of internal water molecules\cite{33-35}, retinal isomerization\cite{36} and the initial stages in proton translocation\cite{37-39}. The models used for these studies have included isolated bR molecules\cite{40}, single bacteriorhodopsin molecules in an argon slab\cite{41} or in a DMPC bilayer\cite{42}, bacteriorhodopsin trimers immersed in a bilayer of POPC lipids\cite{33, 34} and a single bR molecule in a disc of DPPC lipids, surrounded by truncated lipoproteins\cite{43}. To date, only one study has been performed of a near complete representation of the purple membrane\cite{35}.

For the current work, the primary aim of which is to investigate calcium binding to the PM, the use of a complete model, including the characteristic lipids is essential. For this reason a new model of the purple membrane was constructed. This system resembles that used by Baudry \textit{et al.}\cite{35}.
Figure 7.3: Summary of simulation 3 (series A) with calcium ions placed in the Schiff base region (P1) and the proton release group (P2). Stereoscopic image (cross-eye) using a setup similar to Figure 7.1. The calcium ion placed at P1 has a well defined position, whereas the calcium ion placed at P2 has a less defined position and was seen escaping to the solvent in two out of nine cases. A third ion initially placed in the interior of bacteriorhodopsin near Glu9 escaped within one nanosecond of simulation in all nine cases. The presence of the calcium ion at P1 causes D85 (1) and D212 (2) to move closer together than observed in the crystal structures. Asp212 is seen in direct contact with the Schiff base proton. The orientation of Tyr57 (3) is in agreement with the orientation observed in crystal structures 1HQJ and 1AP9, but not with that observed in crystal structure 2AT9. Arg82 adopted an outward configuration and interacted directly with Glu9 for some of the time. Tyr83 (5) also adopted an upward configuration. Glu194 (6) bent away from the cation, whereas Glu204 (7) retained its coordination.

Figure 7.4: Summary of simulation 4 (series A) with calcium ions placed in the Schiff base region (P1) and the proton release group (P2). Stereoscopic image (cross-eye) using a setup similar to Figure 7.1. The calcium ion placed at P1 has a well defined position, whereas the calcium ion placed at P2 has a less defined position although it remained coordinated during the whole simulation in all nine cases. A third ion initially placed in the interior of bacteriorhodopsin near Glu9 escaped within one nanosecond of simulation in all nine cases. The presence of the calcium ion at P1 causes D85 (1) and D212 (2) to move closer together than observed in the crystal structures. Asp212 is seen in direct contact with the Schiff base proton. The orientation of Tyr57 (3) is in agreement with the orientation observed in crystal structures 1HQJ. Arg82 adopted an outward configuration and interacted directly with Glu9 for some of the time. Tyr83 (5) also adopted an upward configuration. Glu194 (6) and Glu204 (7) moved upward together, but retained their coordination of the calcium ion at P2.
Figure 7.5: Calcium densities over time. Stereoscopic images showing averaged densities over time of calcium ions distributed in the solvent corresponding to the extracellular side of the membrane. Densities are shown volume rendered in yellow. A more intense colour indicates a higher occupancy. Blue circles are used to highlight two binding sites on the bacteriorhodopsin – lipid interface. A. Three calcium ions per unit bacteriorhodopsin. B. Ten calcium ions per unit bacteriorhodopsin. C. Ten calcium ions per unit bacteriorhodopsin. In addition, a calcium ion was placed in the Schiff base region in the interior of bacteriorhodopsin.
The most notable difference concerns the nature and position of the lipids, which we base as much as possible on experimental data in the present model[44-46]. Secondly, because the use of periodic boundary conditions can potentially lead to artefacts in small systems, especially in the case of a semi-crystalline system such as the purple membrane, the system used in this study consisted of three unit cells. In this way no trimer is in direct contact with any of its periodic images.

Two questions are addressed in this study, regarding the binding sites of divalent cations (calcium). First we assess whether calcium ions placed at several putative binding sites are stable in simulation. The sites include the Schiff base region, as proposed by Jonas and Ebrey[11], Stuart et al.[22], Pardo et al.[23] and Sepulcre et al.[24], the proton release group, as suggested by Sanz et al.[31] and a site in the interior of bR near Glu9 as proposed by the same group. Second we investigate whether calcium ions placed in the extracellular solvent bind to specific locations in bacteriorhodopsin, at the protein–lipid interface or bind to the PM in a non-specific manner.

2 Results

Two series of simulations were performed to investigate the binding of divalent cations to bR and the PM. These are summarized in Table 7.1. The first series comprised simulations in which calcium ions were introduced into the interior of bacteriorhodopsin at putative binding sites. These sites are termed P1 for the location in the Schiff base region in between the two aspartate residues (Asp85 and Asp212), P2 for a position at the proposed proton release group (Glu194, Glu204) and P3 for a position near Glu9 close to the extracellular surface. Four simulations were performed, with different initial configurations. In simulation 1 calcium ions were placed at P1 in each of the bacteriorhodopsin molecules in the system and the proton release group was protonated. In simulation 2 a single calcium ion was placed near the proton release group (P2) which was not protonated. In simulation 3 calcium ions were placed at all three putative binding sites and in this simulation the proton release group was protonated. In simulation 4 calcium ions were placed at all three putative binding sites and in this simulation the proton release group was not protonated.

The second series of simulations was performed to investigate calcium binding to the purple membrane from solution. The system was expanded and a water free layer (vacuum) introduced to separate the intracellular and extracellular matrices. Calcium ions were randomly positioned in the solvent layer corresponding to the extracellular volume. Three simulations were performed with different concentrations of calcium ions. In simulation 5 three calcium ions per molecule of bacteriorhodopsin were added (total 27). In simulation 6 10 calcium ions per molecule of bacteriorhodopsin were added (total 90). Finally, in simulation 7 10 calcium ions per molecule of bacteriorhodopsin were again added to the solvent and in addition a calcium ion was placed at P1 (total 99). This was done to investigate the effect of a divalent cation in the interior on the distribution of calcium ions at the surface. Chloride counterions were added to ensure that all systems were overall neutral.

The results obtained from the simulations were compared to the available crystal structures of bacteriorhodopsin in the ground state (BR state, crystal structures with PDB IDs 1AP9[47], 1AT9[48], 1BRR[49], 1BRX[50], 1C3W[5], 1FBB[51], 1IW6[52], 1KGB[53], 1M0L[54], 1PY6[55], 1QHJ[56], 1QM8[57], 1XJJ[58], 2AT9[30] and 2BRD[59]) and to other available experimental data. In addition, the bond valence contributions of ligating oxygen atoms were determined from the calcium–oxygen distances according to the method of Brown and Shannon[60]. This was done to investigate the interactions between the calcium ions and the protein. Note that in molecular dynamics, the bond valence sum results from interaction terms defined in the force field used, rather than from the physical principles underlying such interactions in reality.
2.1 System stability

The combined simulations provide the equivalent of approximately 1.4 µs sampling with regards to a single bacteriorhodopsin molecule. The system was found to be stable in all simulations. The equilibration times for the potential energy, volume and density were comparable for all simulations performed and were found to be on the order of one nanosecond. The lattice vectors remained stable during the whole simulation, although the volume of the unit cell decreased from an initial value of 730 nm$^3$ and plateaued at a value of 688 nm$^3$ in 1 ns (-5.8 %). The backbone RMSD was lowest in simulation 2, in which it was observed to level off after 2 ns to an average value of 0.15 nm. In the other simulations, which contained a calcium ion in the Schiff base region, the backbone RMSD values were slightly higher with average values from 0.17 nm for the simulation with a calcium ion in the Schiff base region to 0.2 nm for the simulations with calcium ions placed at P1, P2 and P3.

2.2 Binding of divalent ions: placement of calcium at putative binding sites

The results of the simulations in which calcium ions were placed at the putative binding sites, P1, P2 and P3, are summarized in Tables 7.2 and 7.3 and Figures 7.1-7.4. Table 7.2 presents the distances between the calcium ions and a number of selected atoms of key residues, flanking the putative binding sites. Table 7.3 gives the results from a bond valence analysis (see Methodology and Appendix A). Figures 7.1-7.4 show the distributions of calcium ions inside bacteriorhodopsin of simulations 1-4, together with an overlay of several crystal structures to compare the local geometry regarding a number of key residues (notably Tyr57, Arg82, Asp85, Glu194, Glu204, Asp 212 and Lys216). In Figures 7.1 and 7.2 the distributions of oxygen atoms around the calcium ions over the time of the simulation 1 and 2 are also shown.

<table>
<thead>
<tr>
<th>Series</th>
<th>System</th>
<th>Description</th>
<th>Remarks</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>Calcium ion positioned at P1</td>
<td>E194 protonated</td>
<td>20 ns</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Calcium ion positioned at P2</td>
<td>E194 deprotonated</td>
<td>20 ns</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Calcium ions positioned at P1, P2 and P3</td>
<td>E194 protonated</td>
<td>20 ns</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Calcium ions positioned at P1, P2 and P3</td>
<td>E194 deprotonated</td>
<td>20 ns</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>3 calcium ions per unit bR in extracellular solvent</td>
<td></td>
<td>25 ns</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10 calcium ions per unit bR in extracellular solvent</td>
<td></td>
<td>25 ns</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10 calcium ions per unit bR in extracellular solvent</td>
<td>Calcium ion placed at P1</td>
<td>25 ns</td>
</tr>
</tbody>
</table>
2.2.1 Calcium positioned in the Schiff base region

Considering simulations 1, 3 and 4 together, there are 27 BR molecules in which a calcium ion was positioned at P1, each with a different set of initial velocities. Together these simulations provide the equivalent of approximately 0.5 µs sampling time. The results of simulation 1 are summarized in Figure 7.1A, which shows all positions the calcium ion occupied, relative to the protein, and in Figure 7.1B, which shows the positions of coordinating oxygen atoms. Summaries for simulations 3 and 4 are given in Figures 7.3 and 7.4, respectively.

In simulation 1 the ion placed in between Asp85 and Asp212 quickly repositioned during unrestrained MD. For the remainder of the simulation time, the fluctuations around the average position were small (see Figure 7.1A). The calcium ion established a primarily eight, although sometimes nine fold, coordination (see Figure 7.1B). Calcium coordination primarily involved the surrounding aspartic acids (Asp85 and Asp212), the hydroxyl group of Tyr57 and on average three water molecules, which were originally present as crystal bound water. From Table 7.4 it can be seen that the average valence sum of the calcium ion at P1 in simulation 1 was 1.89 (± 0.11). The average valence sum contribution of the amino acid side chains was 0.93. Similar results were obtained for the calcium ion placed at P1 in simulations 3 and 4.

For each set of simulations the most representative structure from the trajectories of the individual bacteriorhodopsin molecules was obtained by constructing a distance matrix, involving selected atoms of a number of key residues given in Table 7.2 and selecting the frame for which the total sum of the squared deviations against the average distance matrix was lowest. For each of the simulations, this structure is shown in orange in Figures 7.1-7.4. When the structure obtained from simulation 1 using this method with the available crystal structures, several differences could be noted. These differences are indicated in purple in Figure 7.1A. First, in comparison to the majority of the crystal structures, Asp85 (1) and Asp212 (2) are oriented more towards the calcium ion in the simulation. Tyr57 (3), in contrast, projects more outward. The side chain of Arg82 (4) is also pushed outward to a more external location, with the guanidinium group interacting directly with the proton release group. In addition to these changes, note that Tyr83 (5) and Glu194 (6) also deviate from the crystal structures, both being oriented more towards the retinal binding pocket in the simulation.

<table>
<thead>
<tr>
<th>Residue: Atom</th>
<th>Calcium ion (simulation:position)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:P1</td>
</tr>
<tr>
<td>E9: OE*</td>
<td>1.39</td>
</tr>
<tr>
<td>Y57: OH</td>
<td>0.37</td>
</tr>
<tr>
<td>R82: NZ</td>
<td>0.88</td>
</tr>
<tr>
<td>D85: OD*</td>
<td>0.31</td>
</tr>
<tr>
<td>Y157: OH</td>
<td>0.43</td>
</tr>
<tr>
<td>E194: OE*</td>
<td>1.07</td>
</tr>
<tr>
<td>E204: OE*</td>
<td>1.12</td>
</tr>
<tr>
<td>D212: OD*</td>
<td>0.29</td>
</tr>
<tr>
<td>K216: NZ</td>
<td>0.47</td>
</tr>
<tr>
<td>K216: HZ</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Table 7.2: Average distances and standard deviations between calcium ions and selected atoms. All units are in nm. Distances are given in boldface and standard deviations are given in smaller normal font.
When a calcium ion was placed near the proton release group (P2), Tyr57 was found to maintain a position closer to that observed in the crystal structures (see Figures 7.3 and 7.4). However, the guanidinium group of Arg82 (4) in this case oriented toward E9, rather than toward the proton release group. In addition, Tyr83 (5) reorients, such that the hydroxyl group interacts directly with Glu194.

2.2.2 Calcium positioned in between Glu194/Glu204

Considering simulations 2, 3 and 4 together, there are 27 BR molecules in which a calcium ion was placed near the proton release group at P2, each with a different set of initial velocities. In simulation 3 Glu194 was protonated, while in the remainder of the simulations this residue was deprotonated. In simulations 3 and 4 calcium ions were also present at P1 and P3.

Figure 7.2 shows a summary of simulation 2 in which single calcium ions were positioned in between the proton release groups. The distribution of the calcium ion with respect to the protein, shown in Figure 7.2A, suggests that the ion occupies a well defined region. The representative configuration obtained from this simulation, shown in orange, closely resembles the configuration observed in most of the crystal structures of ground state bacteriorhodopsin (see Figure 7.2A). The most notable differences are the reorientation of Asp85 towards the Schiff base proton (1) and the more inward position of Arg82 (4). Note the reorientation of Asp85 was accompanied by the removal of a water molecule, suggested to be positioned in between Asp85 and the Schiff base in the crystal structures (not shown).

The average distances and fluctuations between the calcium ion and a number of residues in simulation 2 are given in Table 7.3. The valence sum contributions of the surrounding oxygen atoms are given in Table 7.4. From these tables the configuration established in the simulation appears well defined. The coordination, shown in Figure 7.2B, involved the two glutamate residues, which contribute 0.32 and 0.34 to the total valence sum of the calcium ion. Furthermore, three water molecules were coordinated by the calcium ion, with a valence sum contribution of 0.97 (± 0.27). The total valence sum for the calcium ion in this simulation was 1.90 (± 0.11) on average.

The results from simulation 3 are summarized in Figure 7.3. In this simulation, two out of the nine calcium ions placed at P2 escaped to the bulk solvent. A further two repositioned towards Glu9. Only four out of nine ions remained close to the initial position. Furthermore, their presence resulted

<table>
<thead>
<tr>
<th>Residue: Atom</th>
<th>Calcium ion (simulation:position)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y57: OH</td>
<td>0.11 0.12 0.10 0.12 0.04 0.07</td>
</tr>
<tr>
<td>D85: OD*</td>
<td>0.31 0.14 0.33 0.31 0.39 0.07</td>
</tr>
<tr>
<td>Y185: OH</td>
<td>0.04 0.08 0.01 0.01 0.04 0.08</td>
</tr>
<tr>
<td>E194: OE*</td>
<td>0.32 0.07 0.00 0.03 0.25 0.19</td>
</tr>
<tr>
<td>E204: OE*</td>
<td>0.34 0.06 0.26 0.19 0.38 0.07</td>
</tr>
<tr>
<td>D212: OD*</td>
<td>0.33 0.11 0.34 0.09 0.35 0.10</td>
</tr>
<tr>
<td>HOH*: O</td>
<td>0.93 0.14 0.97 0.27 0.98 1.78</td>
</tr>
</tbody>
</table>

Table 7.3: Average valence sum contributions and standard deviations of ligands. Valences are given in boldface and standard deviations are given in smaller normal font.
in a distortion of the local geometry. The apparent instability of the calcium ion positioned at P2 in simulation 3 is reflected in the low average valence sum contribution (0.26) of the glutamic acids Glu194 and Glu204 shown in Table 7.4 and in the high valence sum contribution of water oxygen atoms (1.78).

In simulation 4 a calcium ion is placed in the Schiff base region in addition to the configuration used in simulation 2. The results of this simulation are summarized in Figure 7.4, which shows that the presence of a calcium ion at P1 results in a more diffuse distribution of calcium at P2. In particular, Glu194 binds less to the calcium ion. This is reflected in the lower average valence sum contribution and the larger standard deviation thereof, given in Table 7.4. However, a comparison of the structure obtained from the simulation with the crystal structures (Figure 7.4) revealed that both Glu194 (6) and Glu204 (7) are displaced. As mentioned before, the presence of a calcium ion at P1 also caused displacement of Arg82 (4) and Tyr83 (3).

2.2.3 Calcium positioned near Glu9

Considering simulations 3 and 4 together, there are 18 BR molecules in which a calcium ion was placed near Glu9, each with a different set of initial velocities. In all cases the calcium ion moves away from the proposed position during energy minimization and escapes into the solvent during the initial stages of unrestrained MD.

2.3 Calcium adsorption from solution

To investigate the adsorption of calcium ions to the purple membrane from solution, three simulations were performed in which calcium ions were distributed randomly in the solvent corresponding to the extracellular side of the membrane. The aim of these simulations was to identify the presence, if any, of specific binding sites on the exterior of bacteriorhodopsin. The results of these simulations are shown in Figure 7.5. In this figure the purple membrane is shown using cartoon representation for bacteriorhodopsin and stick representation for the lipids, together with the densities of calcium over time in yellow. Calcium densities were determined by binning the simulation unit cell and calculating the relative occupancy of each cell. Using the translational and rotational symmetry of the purple membrane, the densities were averaged over all nine molecules bacteriorhodopsin.

With a concentration of three calcium ions per unit bacteriorhodopsin (simulation 5) three preferred binding sites were identified (Figure 7.5A). Two of these sites were located on the bacteriorhodopsin – lipid interface, one in close proximity of helices A and B, the other near helix F. These sites are indicated in Figure 7.5A by blue circles. The third site primarily involved the head groups of the PGP-Me lipids.

Using a higher concentration of calcium ions (ten per unit bacteriorhodopsin, simulation 6), the two sites on the bacteriorhodopsin – lipid interface were again identified (Figure 7.5B, blue circles). Two other binding sites on the protein – lipid interface are also observed, both near helix F. The other calcium ions bound to the purple membrane lipids.

In Figure 7.5C, the results from simulation 7 are shown, in which a calcium ion was placed in the Schiff base region (P1) and a concentration of 10 calcium ions per unit bacteriorhodopsin was added to the extracellular solvent layer. The calcium ion at P1 gives rise to an intense colouring in the core of each molecule bacteriorhodopsin. The two sites observed in simulations 5 and 6, located at the protein – lipid interface, were again identified (Figure 7.5C, blue circles). However, as can
be seen, the colouring is less intense, indicating decreased binding. In fact, comparing the calcium
densities from this simulation with those observed in simulation 6, it can be seen that the presence
of a calcium ion in the core of bacteriorhodopsin leads to less binding at the protein – lipid interface
and more diffuse distribution of calcium ions over the lipid head groups.

3 Discussion

3.1 Stability of calcium ions positioned at putative binding sites

3.1.1 The Schiff base region

When a calcium ion is not present in the Schiff base region (simulation 2), the configuration of
residues in this region in the simulation is in good agreement with that found in the majority of the
high resolution crystal structures. Only Arg82 is found to adopt a more inward position, interacting
directly with Asp85, whereas in most of the crystal structures the guanidinium group of Arg82
is oriented toward Asp212. Note that in simulation 2 a calcium ion was present at P2. This might
have caused the inward orientation of Arg82. However, a similar effect was previously observed by
Kandt et al.[34] who performed a simulation of bacteriorhodopsin in which the proton release group
was protonated with no cations present.
The placement of a calcium ion at P1 within the Schiff base region does not result in instabilities or
a distortion of the overall protein conformation. However, when comparing the resulting structures
to the high-resolution crystal structures available, notable differences were observed. For instance
Asp85 and Asp212 are pulled inward, towards the calcium ion, and Tyr57 is tilted, with the hydroxyl
group directed toward the ion. In addition, the side chain of Arg82 moves to a more external location,
with the guanidinium group interacting with the proton release group.
The configuration obtained from simulation 1 in which a calcium ion was placed in the Schiff base
region shows some similarities with structure 1AP9[47]. Specifically, the position of the side chain
of Asp212 is in agreement with that structure, and the orientation of Asp85 is similar, though in
the simulations Asp85 lies closer to the calcium ion. Moreover, the tilt of the phenyl group of Tyr57
observed in the crystal structure is also reproduced, although there the ring takes a more inward
position. Note in addition that Arg82 is found in a more outward configuration in 1AP9 than in
the other crystal structures, although not as exposed as in the simulation. Interestingly, it has been
argued previously that the structure obtained by Pebay-Peyroula et al. requires inclusion of a cation
in the Schiff base region to be consistent with one and two-photon spectroscopic data[27]. The
present results support this hypothesis.
Taken together, the results demonstrate that the Schiff base region could accommodate a calcium
ion, without causing distortion of the overall structure of bacteriorhodopsin. In particular, we find
that the surrounding oxygen ligands can adopt a suitable configuration to coordinate a calcium
ion. At the same time, the placement of a calcium ion in the Schiff base region results in a distortion
of the local geometry. As mentioned earlier, the fact that no calcium ion is present in this location
might be a result of the crystallization condition, leading to the loss of calcium ions or possibly to
the exchange of divalent with monovalent cations.
3.1.2 The proton release group

The simulations suggest that the proton release group can function as a calcium binding site. Notably, the similarity between the results obtained from simulation 3 and the majority of the crystal structures suggest that a calcium ion could be accommodated in the region of the proton release group. As mentioned above, in this simulation Arg82 adopts a more inward position. In addition, during the simulation a direct interaction was established between Asp85 and the Schiff base proton, whereas in the crystal structures it was suggested that these two groups were bridged by a water molecule. The loss of the bridging water molecule has been previously observed by Kandt et al.[34].

The simultaneous placement of calcium ions in the Schiff base region and the proton release group destabilized bacteriorhodopsin. Thus either the Schiff base region or the proton release group could accommodate a calcium ion, but not both. Note, when a calcium ion is positioned in between Glu194 and Glu204, the results of the simulations are consistent with the majority of the available crystal structures. This is not the case when a calcium ion is placed in the Schiff base region. Thus we consider it more likely that the proton release group functions as a cation binding site.

3.1.3 In the vicinity of Glu9

The proposed location in the interior of bacteriorhodopsin near Glu9 is unlikely to be a calcium binding site. In the simulations, a calcium ion placed in this region was not stable. Note, it is possible that the inclusion of polarization terms in the force field might enhance the stability of calcium at this site. However, polarization effects alone cannot account for the rapid loss observed in the simulations and the probability of calcium binding at this position is low.

3.2 Binding of calcium ions from solution

The simulations in which calcium was placed in solution suggest the presence of two primary binding sites per molecule bacteriorhodopsin. One of the sites corresponds to the site proposed near Ala196 at the protein – lipid interface in between helices F and G[21, 32]. The other site is located at the interface near helices A and B, close to Glu9. Glu9 has also been suggested to coordinate a calcium ion at the interior of bacteriorhodopsin[31], but this proposal is not supported by our simulations. Note that the suggestion that Glu9 is involved in calcium binding comes in part from the finding that this residue plays a role in maintaining the hexagonal lattice structure of the purple membrane. Interestingly, the glycolipids (S-TGA-1) which lie adjacent to the helices A and B[61] are also known to play an important role in the formation of the purple membrane and stability of the lattice[62]. Given that many carbohydrates chelate calcium ions[63], a calcium ion at the indicated position may play a role in interaction between S-TGA-1 and bacteriorhodopsin and in that way contribute to the stability of the purple membrane. Note also that interactions between the sugar moieties of S-TGA-1 would be consistent with the results obtained by EXAFS, which suggested that there were no interactions between calcium ions and either phosphate or sulfate[64].

These results suggest that calcium binds specifically, not non-specifically according to the Guoy-Chapman effect. If the adsorption was to be explained by non-specific binding, the distribution of calcium would be more diffuse, especially given the averaging over the nine different bacteriorhodopsin units in the system.
The presence of a calcium ion in the Schiff base region (simulation 7) causes the binding of calcium ions to the protein – lipid interface to decrease. This suggests that the calcium ion in the core of bacteriorhodopsin has a significant effect on the surface electrostatic potential of the protein. Vice versa, this suggests that the environment formed by the lipids and potentially by bound cations will influence the electrostatic and chemical properties inside bacteriorhodopsin. Therefore, it is important to regard the effect of the purple membrane lipids in studies assessing properties of bacteriorhodopsin, as well as the placement of possible cations, in particular in molecular dynamics simulations.

4 Conclusions

In total seven simulations of 20 to 25 ns have been performed using a complete model of the purple membrane containing three bacteriorhodopsin trimers. This is the most extensive set of simulations on bacteriorhodopsin to date, with a total sampling time equivalent to approximately 1.4 µs of a single bacteriorhodopsin molecule.

This work suggests that the Schiff base region could accommodate a calcium ion and that the resulting structure would be roughly in line with one of the models tested and judged plausible by Kusnetzow et al.[27] and the crystal structure proposed by Pebay-Peyroula et al.[47]. However, this structure differs significantly from the majority of crystal structures of ground state bacteriorhodopsin. A more likely candidate for a specific calcium binding site is the proton release group. Placing a calcium ion in this location resulted in a structure which closely resembles the majority of the crystal structures.

Although it is not possible to give a conclusive answer with respect to the possible binding of a calcium ion in the Schiff base region or the proton release group based on these simulations, the present results argue against binding at both sites simultaneously. They also do not support the possibility of a binding site near Glu9 in the interior of bacteriorhodopsin.

The adsorption of calcium to the purple membrane from solution suggests two specific binding sites at the protein – lipid interface on the extracellular side. The simulations do not support the proposal that calcium ions bind non-specifically to the lipid head groups. However, the lipids do play a role when the binding primarily takes place at the interface. It would be interesting to further investigate a possible role of the glycolipids S-TGA-1 in this respect.

The simulations have allowed us to rationalize some of the experimental data and while it is not possible to provide conclusive evidence in favour of specific binding sites, the results do show that binding to the purple membrane is unlikely to occur in a non-specific manner and indicate that two of the previously suggested sites in the interior of bacteriorhodopsin are consistent with the present data.
5 Methods

5.1 The simulation model

5.1.1 Construction of the model

The construction of the model largely followed the approach taken by Baudry et al.[35]. The primary improvements over this model relate to the type and placement of the lipids to narrow the gap between the simulation model and the real purple membrane. The basic hexagonal unit cell, shown in Figure 7.6, containing a bacteriorhodopsin trimer and its associated lipids was constructed using information from three different crystal structures, namely:

1. 1BRR, a bR trimer, resolved to 2.9 Å resolution[49]
2. 1QHJ, a bR monomer, resolved to 1.9 Å resolution[56]
3. 1C3W, a bR monomer, resolved to 1.55 Å resolution [5]

The bacteriorhodopsin trimer (1BRR) was taken as the basis for the system. The three bR units of this structure have certain structural differences. The missing side chain atoms (residues 3, 225, 227, 229, 230 and 232 of chain A, residues 3, 4, 227 and 232 of chain B and residues 1, 227, 229, 230 and 232 of chain C) were modelled using the SwissPDB Viewer. The first two residues of the N-termini of chains A and B are not resolved. These were modelled based on the configuration of these residues in chain C. The N-terminal glutamine group was modelled as pyroglutamic acid. C-termini were not resolved for any of the chains and residues after Glu232 were omitted from the system.

The protonation states of titratable residues were chosen according to normal protonation states at pH 7.0 except for Asp96, Asp115 and Glu194 which were protonated, in accordance with the expected protonation states for the initial ground state of bacteriorhodopsin. However, in the experiments in which a calcium ion was placed in between the proton release group, the two glutamate residues (Glu194 and Glu204) were deprotonated, with the exception of one of the simulations.

5.1.1 Lipid constitution

Several studies have attempted to resolve the constituents of the purple membrane and their relative concentrations[44-46]. The studies agree on the main constituents being PGP(-Me), S-TGA-1 (sulphated triglycosyl diphytanylglycerol) and Squalene, though the numbers of each of these per unit of bacteriorhodopsin differ. With regard to the glycolipid S-TGA-1 information from neutron diffraction experiments supports the suggestion of two of these molecules per unit bR and suggests their positions with respect to the protein[61]. Based on this information six molecules of S-TGA-1 were added to the initial system. S-TGA-1 is a glycolipid containing three sugar moieties linked to an archeol group. These sugar groups are, in sequence from the archeol: 1,2-α-D-glucose, 1,6-α-D-mannose and 1-β-D-galactose-3-sulfate. The head groups of the intra-trimer glycolipids were taken from structure 1BRR, where these were resolved. The positions of the glycolipids in the inter-trimer space correspond to the diphytanoyl chain numbered 501 in structure 1QHJ. The head group of an intra-trimer (resolved) S-TGA-1 unit was modelled onto that lipid part in such a way that there were no van der Waals contacts with neighbouring lipids or the protein.
The S-TGA-1 molecules account for two of the lipids associated with each unit as found in the crystal structures. The other phytanoyl lipids resolved in the crystal structures were all chosen to be PGP-Me. A total of seven PGP-Me molecules per unit bR were added to the system, six of which according to the positions of the diphytanoyl-glycerol tails (502-507) resolved in the structure 1QHJ. Three additional PGP-Me moieties (one for each BR unit) were added in the intra-trimer region on the intracellular side.

The PGP-Me head group was modelled using the PRODRG server[65] to obtain a reasonable geometry. The head groups on each of the diphytanoyl-glycerol chains was modelled so as to maximize the space between neighbouring head groups, to avoid introducing bad van der Waals contacts.

The third lipid constituent was squalene, which is a hexamer of isoprene. The conformation of squalene was taken from structure 1C3W, where it was found to lie in an S-like conformation close to the side of bacteriorhodopsin. Side chain methyl groups were modelled to complete the molecule.

5.1.2 Bound waters

A network of water molecules within bacteriorhodopsin, which is believed to be involved in the proton translocation, is resolved in several of the crystal structures. The positions of the crystal waters from structure 1C3W[5] also coincide well with the positions found based on free energy perturbation calculations[35]. For this reason the crystal waters as they were resolved in structure 1C3W were incorporated in the models.

5.1.3 Solvent

The archaea in which the purple membrane is found are extremely halophilic and thrive at salt concentrations of around 3.3M. These high concentrations of salt affect the electronic properties as well as the stability and dynamics of the protein and membrane. Therefore it was chosen to mimic the physiologic environment by addition of corresponding amounts of sodium chloride. The only exception was in the simulations where calcium ions were distributed in the solvent (see below).

5.1.4 Lattice vectors

The purple membrane is a two dimensional crystalline system with a hexagonal lattice structure. The simulation system consisted of one hexagonal unit cell of the purple membrane. The lattice vector perpendicular to the membrane plane was chosen such that two adjacent layers were kept from directly interacting and that enough solvent was present to allow for bulk like behaviour in the middle region (7.6 nm).

The hexagonal unit cell thus obtained was used as the basis of an enlarged system, consisting of three unit cells, containing a total of nine bacteriorhodopsin molecules (Figure 7.7). Multiple unit cells were simulated to reduce the effect of the periodic boundary conditions and to allow greater sampling and improve statistics. This model, comprising of three original unit cells, was the basis for all simulations performed. For some of the experiments, adaptations to this base model were made to fit specific needs.
Figure 7.6: Hexagonal unit cell of the Purple Membrane. The hexagonal unit cell shown contains three molecules bacteriorhodopsin, six molecules S-TGA-1, twenty-one molecules PGP-Me and three molecules squalene. This unit cell was used as the basis for all simulations. A. Side view. B. Top view.

Figure 7.7: Simulation system consisting of three basic unit cells. A. The simulation unit cell, consisting of three hexagonal unit cells, containing three bacteriorhodopsin trimers with associated lipids. B. View on the infinite simulation system formed by tesselating the simulation unit cell according to the appropriate lattice vectors. These lattice vectors are shown as white arrows. Note that the enlarged simulation unit cell can be mapped to a hexagonal unit cell with exactly three times the volume of the original hexagonal unit cell. This hexagonal prism is shown in white. The figure also demonstrates that in the enlarged system no bacteriorhodopsin trimer is in direct contact with its own periodic image. For example, the darkest coloured trimer is only surrounded by the lighter coloured ones. In this way artefacts due to the use of periodic boundary conditions on a small crystalline system are reduced.
5.1.5 Force field parameters

The protein was described using the GROMOS96 43a2 united atom force field[66, 67]. Parameters for retinal and the Schiff base Lys216 were taken from Kandt et al.[34]. Parameters for the lipid tails, including squalene, were based on parameters for branched lipids. For the lipid tails two extra atom types were added to the force field with improved parameters for protein-lipid interactions (Jakob Wohlert, personal communication). Parameters for the PGP-Me head groups were based on parameters for phospholipids. Parameters for the carbohydrate moieties of S-TGA-1 were based on the GROMOS96 45a4 force field[68]. The building blocks for 1,4-α-D-glucose and 1,4-β-D-glucose from this force field were adapted to form the corresponding 1,2-α-D-glucose, 1,6-α-D-mannose and 1,3-β-D-galactose.

This model is the most complete model of the purple membrane to date. Each unit cell contains just over 17k atoms. The total nonameric model contains about 52k atoms. Note the current fully periodic system is computationally much more efficient than simulations performed of a single monomer or trimer of bR in a POPC bilayer.

5.2 Simulations

5.2.1 General

Simulations were performed using the Gromacs package for molecular simulations[69-71]. Water was treated explicitly using the Simple Point Charge (SPC) model[72]. Non bonded interactions were evaluated using a twin range cut off of 0.9 and 1.4 nm. Interactions within the shorter range cut off were evaluated at every step whereas interactions within the longer range cut off were evaluated every 10 steps. To correct for the neglect of electrostatic interactions beyond the longer range cut off, a Reaction Field (RF) correction[73] was used with \( \varepsilon_{RF} = 78.0 \). In all simulations the system was kept at a constant temperature of 300K by applying a Berendsen thermostat[74]. Protein and solvent were independently coupled to the heat bath with a coupling time of 0.1 ps. The pressure was weakly coupled to a reference pressure of 1 bar using an anisotropic Berendsen barostat[74], with a coupling time of 1.0 ps and a compressibility of \( 4.6 \times 10^{-5} \) bar\(^{-1} \). The time step used for integration of the equations of motion was 0.002 ps. The bond lengths and angle of the water molecules were constrained using the SETTLE algorithm[75]. Bond lengths within the protein were constrained using the SHAKE algorithm[76].

5.2.2 Calcium ions positioned at putative binding sites

The first simulation system (1, see Table 7.1) was generated from the original system by placing a calcium ion in the interior of each bacteriorhodopsin unit near the Schiff base (P1), in between Asp85 and Asp212 in such a way that the overlap with the surrounding atoms was minimal. The system was energy minimized using a steepest descend algorithm and subsequently a short simulation (10 ps) was performed in which the positions of heavy atoms were restrained to the reference positions. After a short equilibration time (10 ps) the system was used for a production run of 20 ns.

For the second simulation system (2) the proton release group Glu194/Glu204 was deprotonated and a single calcium ion was placed in between these two residues for each unit bacteriorhodopsin in such a way that the overlap with the surrounding atoms was minimal. The system was energy
minimized using a steepest descend algorithm and subsequently a short simulation (10 ps) was performed in which the positions of heavy atoms were restrained to the reference positions. After a short equilibration time (10 ps) the system was used for a production run of 20 ns.

The third simulation system (3) was built from the original system by the addition of three calcium ions per unit bacteriorhodopsin near Asp85 (P1), Glu194/Glu204 (P2) and Glu9 (P3) in such a way that the overlap with the surrounding atoms was minimal. The system was energy minimized using a steepest descend algorithm and subsequently a short simulation (10 ps) was performed in which the positions of heavy atoms were restrained to the reference positions. After a short equilibration time (10 ps) the system was used for a production run of 20 ns.

The fourth simulation system (4) was built from the original system in a way similar to simulation system 3, with the distinction that the proton release group was deprotonated. Again, the system was energy minimized using a steepest descend algorithm and subsequently a short simulation (10 ps) was performed in which the positions of heavy atoms were restrained to the reference positions. After a short equilibration time (10 ps) the system was used for a production run of 20 ns.

5.2.3 Calcium in the extracellular solvent

The simulations in which calcium ions were placed in solution to study the adsorption to the purple membrane and bacteriorhodopsin were started from a modified base system. To assure that binding could only occur on the extracellular side, a vacuum layer was introduced to separate the two sides of the purple membrane. To reduce the effect of solvent ordering at the vacuum – water interface, the thickness of both layers of solvent were increased by 1 nm. These simulations were performed at constant volume, rather than at constant pressure, in order to maintain the separation of the two solvent layers. A lower concentration of NaCl (0.2 M) was used in these experiments, to decrease competition between the monovalent Na⁺ and the divalent Ca²⁺.

For the first of these simulations (5) a total of 27 calcium ions were randomly distributed in the solvent layer corresponding to the extracellular side of bacteriorhodopsin. In this simulation the crystal bound water was removed. After energy minimization and a short (10 ps) simulation in which the heavy atoms were constrained to their initial positions, a short equilibration run (10 ps) was performed, followed by a production run of 25 ns.

The second of the simulations with calcium ions in solution (6) was set up in a similar way as 5, but instead of 27 calcium ions a total of 90 calcium ions were randomly distributed in the solvent layer corresponding to the extracellular side of bacteriorhodopsin. Again, after energy minimization and a short (10 ps) simulation in which the heavy atoms were constrained to their initial positions, a short equilibration run (10 ps) was performed, followed by a production run of 25 ns.

The last simulation in this series (7) was performed on a system with a similar setup as 6, also with 90 calcium ions randomly placed in the solvent layer corresponding to the extracellular side of bacteriorhodopsin. However, in this simulation the original crystal bound water was retained and an additional calcium ion was placed in the Schiff base region (P1). After energy minimization and a short (10 ps) simulation in which the heavy atoms were constrained to their initial positions, a short equilibration run (10 ps) was performed, followed by a production run of 25 ns.
5.3 Analysis

5.3.1 Comparison with crystal structures

The simulation results for 1 – 4 were compared with the available crystal structures (PDB entries IDs 1AP9[47], 1AT9[48], 1BRR[49], 1BRX[50], 1C3W[51], 1FBB[51], 1IW6[52], 1KGB[53], 1M0L[54], 1PY6[55], 1QHJ[56], 1QM8[57], 1XJI[58], 2AT9[30] and 2BRD[59]). The comparison focused on the geometry of bacteriorhodopsin around P1, P2 and P3. For the comparison, from each simulation the most representative structure was extracted. This was done by first building a distance matrix involving selected atoms from a number of key residues possibly involved in calcium binding or otherwise related to the catalytic site (the Schiff base region). Distance matrices were built for each individual unit bacteriorhodopsin and each frame. For each simulation the total average distance matrix was determined. Subsequently for each distance matrix the sum of the squared deviations from the corresponding elements in the average matrix was determined and the frame with the lowest sum of squares was chosen as the most representative structure.

5.3.2 Bond valence sums

To investigate the coordination of the calcium ion and the interactions with its surroundings, the bond valence contributions were calculated for each oxygen atom lying within 0.5 nm of the calcium ion. From the individual contributions the bond valence sum was determined for each calcium ion. The bond valence contributions and sums were calculated according to the method of Brown and Shannon[60] and parameters for calcium – oxygen bonds given by Brown and Wu[77]. The relation between the bond valence and the calcium – oxygen distance is given by

$$ s = \left( \frac{r}{r_1} \right)^N $$

(7.1)

where $r$ is the distance between the two atoms corresponding to unit valence. The parameters used for $r_1$ and $N$ are 0.1909 nm and 5.4, respectively[77]. The background of the bond valence sum is given in Appendix 7A.

For the analysis of bond valences and valence sums a new program, g_valence, was written (Appendix A). This program calculates the minimal distances between a set of cations and a set of oxygen atoms, taking periodicity into account according to the general method given in Appendix C of Chapter 3. From the distances the time of contact between each cation – oxygen pair, and the bond valence contribution are derived. In addition the bond valence sums are calculated for each cation over time.

5.3.3 Calcium densities

To assess whether calcium ions in solution are adsorbed to the purple membrane or bacteriorhodopsin in a specific or a non-specific manner, the calcium densities in the simulation cell were determined over the length of the simulation. The densities were averaged over all three trimers in the simulation system, using all rotameric states. For this purpose each trajectory was copied three times. For each copied trajectory the distribution of calcium ions was determined around a different bacteriorhodopsin trimer. This was done using the routine to construct a molecular shaped box.
(Appendix B, Chapter 3), using the particular trimer as the generator and the calcium ions as the
group to rearrange. The distributions of calcium ions thus obtained were multiplied over all three
rotameric states and the total set of positions, equal to nine times the original number of calcium
ions, was used to determine the relative densities in the cell. The calculation
The densities were averaged over all nine copies of bacteriorhodopsin in a simulation cell and
visualized using Pymol[78] and POV-Ray[79].

6 Appendix A: The bond valence model in molecular
dynamics

The bond valence method traces back to the pioneering work of Linus Pauling, who in 1929 suggested
that the valence of an atom equals the sum of the bond strengths between that atom and the bonded
atoms. In terms of ionic interactions, this implies that the sum of the individual interactions between
a cation and its ligands equals the valence of the cation. This work has laid the basis for a number
of empirical methods for the investigation of atomic interactions, in particular interactions between
cations and coordinating ligands. One of the best known and most widely used models is the one
proposed in 1973 by Brown and Shannon[60], who give the relation between the bond length and
the bond valence as

\[ s = \left( \frac{r}{r_1} \right)^{-N} \]  \hspace{1cm} (7.1, repeated)

where \( s \) is the bond valence, \( r \) is the observed distance, and \( r_1 \) and \( N \) are constants specific to a given
combination of atom types. \( r_1 \) is the distance corresponding to unit valence. The total valence sum of
a given atom (cation) is given by the sum of the individual bond valences.

The values for the constant parameters are generally obtained from screening the crystal structures
available in the Cambridge Structural Database (CSD)[81] or from experimental methods such as
extended X-ray absorption fine structure (EXAFS) or X-ray absorption near-edge structure
(XANES). The parameters currently available cover a large range of atom pairs, although the most
interesting from a biological point of view are those involving cation – oxygen or cation – nitrogen
interactions.

The bond valence method provides a way to investigate protein – cation interactions. As such it has
been used in crystallography to identify cation binding sites. E.g. Nayal and DiCera have used the
valence sum in combination with a grid search to identify putative calcium binding sites in crystal
structures[82] and have used the method to identify potential sodium binding sites by screening
reported crystal bound water molecules[83]. In addition, Kombo et al have used the bond valence
to compare the interactions between sodium and the operator binding domain of the lambda
repressor protein in molecular dynamics simulations, illustrating the applicability of the method
in this discipline.

In this work, the valence sum method was used to investigate the interaction between calcium ions
and bacteriorhodopsin. To this purpose a new program, g_valence, was written for the calculation
of valence sums from the minimal distances between given sets of cations and oxygen atoms. This
program is built as a tool for the Gromacs package and shares the general features of this suite with
regards to the input file types.

The program uses two predefined (index) groups, corresponding to a cation type (most cation types
are possible) and an anion type (currently limited to oxygen). For each frame of the trajectory the
 Cartesian coordinates are transformed to box coordinates and the minimal distance for each pair of group 1 and group 2 is determined according to the method given in Appendix C, Chapter 3. From the distance the bond valence is calculated according to equation 7.2. In addition, it is inferred whether the two atoms are considered to be in contact, with regards to a predefined criterion (e.g. a distance less than 0.4 nm). For each atom in the first group the valence sum is calculated.

The program can be used in two modes. The first mode is for screening of contacts occurring at any time during the simulation and giving the general results for each atom in the first group. In a second pass, the information obtained in the screening mode can be used to obtain more verbose output with regards to those atoms which are in contact at any time. In this mode, the valences over time are recorded for each pair and the time series of each interaction in terms of the minimal distance, the valence and whether or not the atoms are in contact can be saved to a file. This allows a thorough investigation of the interactions between cations and (protein) ligands.

7 References


