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Abstract

Motivation. In this paper we analyze the electrostatic interactions in the open and closed states of the KcsA channel. We focus on the stability of the KWKWK…K sequence already proposed as a model for the permeation mechanism of the KcsA transmembrane channel. The fact that the channel can accommodate more than one ion in the pore is crucial to explain the high diffusion rate of K^+ ions observed. We also address the question of the accuracy of the force fields by comparing them to *ab initio* calculations.

Method. On the basis of molecular dynamics calculations, we determine the electrostatic potential due to the protein embedded in its membrane using either AMBER6 force fields or quantum calculations conducted with GAUSSIAN 03 at the HF 6–31G(d) level.

Conclusions. Both force field and quantum approaches show that water molecules located in the KWKWK…K sequence play a structuring role for the ions in the filter. When compared to the closed state situation, the open state conformation of the protein promotes very different behaviors of K^+ and water motions in the hydrophobic cavity and in the external mouth of the channel.

Keywords. KcsA channel; *ab initio*; molecular dynamics; electrostatics.

Abbreviations and notations

K_n, Potassium ion located in the nth site of the pore	HF, Hartree–Fock
W_n, Water molecule located in the nth site of the pore	DFT, Density functional theory
MD, Molecular dynamics	QM/MM, Quantum mechanics/molecular mechanics
BD, Brownian dynamics	

1 INTRODUCTION

Research in ion channels has long had a strong experimental tradition with relatively little input from theory [1,2]. The reason for this imbalance between theory and experiment is usually attributed to the complexity of both the biological processes and the underlying molecular structures involved in their realization. Rapid advances at two fronts, namely determination of the tertiary structure of macromolecules from X–ray diffraction and NMR techniques and an exponential

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increase in computational power allowing large–scale simulations of biological processes now give hope that theoretical and experimental studies might progress in a complementary way.

Studies of ionic transmembrane protein channels witness these complementarities. Indeed, the publication of the crystal structure of the KcsA channel at 3.2 Å resolution [3] and its refinement at 2.0 Å [4,5] has given rise to a large effervescence in this field of research. The locations of the binding sites of K^+ ions and water molecules in or near the selectivity filter were unambiguously observed. Besides, a large number of molecular dynamics (MD) or brownian dynamics (BD) simulations [6–17] have provided an opportunity to relate these atomic resolution structures of the potassium channel to their various physiological properties (selectivity, gating, permeation...). A remaining problem for modeling purposes is that the crystal structure of KcsA corresponds to a closed state of the channel and of course the knowledge of the open state conformation is indispensable to calculate the ionic conductance which will be compared to experimental measurements. Open state structures were proposed either on the basis of homology with other protein pores, MthK and KvAP [18–21], or by artificially dilating the pore by the use of repulsive spheres or cylinders with variable diameters [22–24]. Nevertheless, no real relaxed (meta)stable open states were reached in such approaches.

Moreover, two linked practical additional problems arise from MD or BD. They first concern simulation time and secondly the accuracy of force fields used to describe molecular interactions. In MD simulations, integration over femtosecond time steps is usually required, *e.g.* to describe correctly the rotational motions of water molecules. Hence, with current computational power, only a few nanoseconds of production runs can be generated in a reasonable CPU time. This prevents to be able to do correct statistics on the K^+ diffusion motions through KcsA which occur typically every 30 ns. BD allows studies over much longer times but solvent has been up to now treated as a continuum. This latter assumption is surely dramatic in confined systems and reveals the second problem. Indeed, simulation studies of a number of channel models with cylindrical shapes have shown that the average dynamical properties of water molecules through the protein pores differ substantially from those in bulk water, exhibiting in particular decreased translational and rotational mobilities [25–29]. One reason is that the confinement changes the dielectric constant of the solvent which, in turn, influences the effective electrostatic field within the liquid [30]. A tremendous amount of work has been deployed for the development of force fields into biomolecular applications since the early 1980's, resulting in simulation packages such as AMBER [31], CHARMM [32] or GROMOS [33]. The K^+ force field has been parameterized from simulations in water and it is therefore questionable if this force field is able to describe accurately K^+ interactions with a protein.

Further problems may issue from the neglect of polarization effects. *Ab initio* quantum calculations allow these effects to be taken into account. It was recently shown [34], using density

functional theory (DFT), that polarization effects play a significant role in the mechanism which regulates K^+ binding and permeation. *Ab initio* Hartree–Fock (HF) [35] and DFT [36] calculations have been performed recently on KcsA potassium channel. The electrostatic potential and K^+ binding energies were computed and compared to force field results. Polarization was shown to be very important for the K^+ nearby atoms. Both HF and DFT methods produce good estimations of K^+ binding energies, but while HF data reveal a decrease of about 30% of the K^+ ion net charge, DFT results suggest abnormally large polarization effects leading to an almost complete loss of the K^+ charge [36]. Moreover, M. Green and coworkers [37–39] studied the gating mechanism of the KcsA channel with *ab initio* calculations. They noticed that the water molecules located in the gate region, namely near glutamate 118 residues, have a special behavior, with the occurrence of a charge transfer involved in the asymmetric bridging between three of the four glutamates and a charged H_5O_2 group. Such charge transfer processes cannot be modeled correctly using classical MD simulations.

In this paper, we propose to analyze the behavior of the sequence KWKWK…K where K and W define potassium ion and water molecule, respectively, currently considered as a reasonable empirical model for membrane permeation. We first address the question of the accuracy of usual force fields by comparing the electrostatic potential experienced by a K^+ ion keeping its original charge and moving through the filter region, given either by the force field of AMBER or quantum calculations conducted with GAUSSIAN 03 [40] at the HF/6–31G(d) level. Then we study the behavior of K^+ ions and water molecules in the filter and filter mouth and in the cavity in both closed and open states of the pore, which we have built using usual and targeted molecular dynamics, respectively. In Section 2, we give an overview of the methods, while Section 3 will especially outline the structuring role of water in the selectivity filter and cavity of KcsA.

2 METHODS

Our starting models for the closed and open conformations of the KcsA channel were based on the experimental structures determined by X–ray experiments at 100 K [3–5] and by site–directed labeling and EPR spectroscopy [41–43]. Figure 1 shows a backbone representation of the protein. Three main regions appear in this structure. The narrowest extracellular oriented part of the pore, where are lining the backbone carbonyl oxygens as well as hydroxyl oxygens of the TVGYG sequence, acts as a selectivity filter (F) for the K^+ ions. It extends over about 12 Å with a mean radius of 1.4 Å. At the membrane center, the size of the pore increases to form a 6 Å radius hydrophobic cavity (C), which can contain up to 40 water molecules. The gate (G) is a thin hydrophobic pore formed by the inner M_2 helix bundle, which closes the channel with a small radius of about 1.6 Å on the intracellular side.
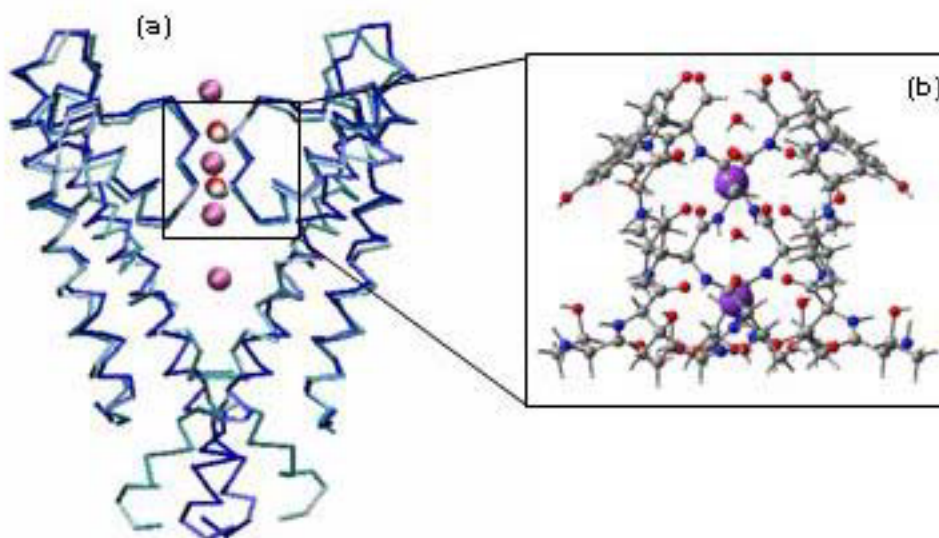
Figure 1. (a) Backbone representation of the KcsA protein channel into the two studied forms (open in green and closed in blue). For clarity only two over the four monomers have been drawn. The sequence $K_0W_1K_2W_3K_4…K_{cav}$ is given with the potassium ions in purple, oxygen atoms in red and hydrogens in light gray. (b) Part of the channel named selectivity filter which has been considered in *ab initio* calculations to test the potential energy used in AMBER6 MD simulations. 304 atoms have been included, with terminal groups completed as explained in the text. Carbons are in dark gray.

The pore axis, at the filter level, contains both K^+ ions and water molecules. In agreement with experimental observations [5], six of the seven ionic sites elucidated, namely 2 extracellular sites S_{EXT} and S_0, and 5 sites S_1 to S_{cav} inside the filter and in the cavity, are filled by an alternate sequence of K^+ ions and water molecules. The experimental based structure $K_0W_1K_2W_3K_4…K_{cav}$ (numbering corresponding to sites), that we have considered here, is shown in Figure 1a. It is to notice that when the K^+ ions are in positions S_1 and S_3, they are stable on nanosecond time scale only if one additional ion is present inside the cavity [17]. The different aqueous regions of the membrane protein (extra and intracellular sides, cavity) have been hydrated respecting local densities.

All MD calculations were carried out using the AMBER6 suite of programs. The system was built from a cubic box of 79 Å side, which contains the protein embedded in an octane slab mimicking the hydrophobic part of the lipid bilayer and two water slabs representing the extra and intracellular media. Additional Cl^- and Na^+ ions have been included in the water slabs (150 mM ion concentration), in order to be consistent with the experimental ionic strength. For the details of the method, see Ref. [44]. The closed structure of the protein was relaxed during about 3.6 ns in (N,V,T) then (N,P,T) ensembles [44], till showing a global, very small, root mean square deviation (rmsd) of 1.3 Å. The open structure was obtained from the closed conformation, performing a targeted MD simulation directed toward the known final positions (especially the C_α's of the M_2 helices at the gate region) revealed by the EPR experiments [41–43]. The analysis of the gating

process (see animation given as supplementary material) shows a zipper–like aperture of the channel from the terminal residues located at the innermost part of the M_2 helices toward the extracellular side [45]. Only this part of the M2 helices undergoes large motions in the opening mechanism, while the filter region of the channel remains stable. To support the average conformation that we obtained at the end of the open state MD run, we decided to release the preceding constraints applied during forced opening in a supplementary simulation of 1.5 ns, thus checking that this state remained effectively stable [45].

In a preliminary step, we have conducted quantum calculations on some chosen conformations of the filter obtained from the classical MD simulations in order to test the accuracy of the interaction potentials used in AMBER6. Since, at present, it is difficult to calculate the entire protein electronic structure from first–principles methods, we have restricted our analysis to the filter region, notably in a configuration constituting a reasonable average representation of the K^+/protein interactions [35]. The geometry shown in Figure 1a corresponds to such a typical snapshot observed in the course of the MD simulation of the closed conformation. As the filter conformation is not significantly changed in the open state we have thus considered that the quantum calculations apply to both states.

We chose to more specifically look at S_0 to S_4 sites, and therefore truncated KcsA structure at the level of the four Tyr78 residues, where the peptide bonds with Gly79 were cut and ended with a hydrogen (–CHO), and near the cavity below the four Thr74, –NH of the amide bonds completed to NH_2. The sequence portion considered was thus (Thr74–Thr75–Val76–Gly77–Tyr78)$_4$ (Figure 1b). No lipidic or water environment constitutive of the MD construct was considered (except of course W_1 and W_3), and the total number of atoms was 304. The electrostatic potential energy surfaces were computed using GAUSSIAN 03 [40] at Hartree–Fock/6–31G(d) level of theory, without any further energy minimization, in order to be strictly consistent with the MD snapshot picture.

3 RESULTS AND DISCUSSION

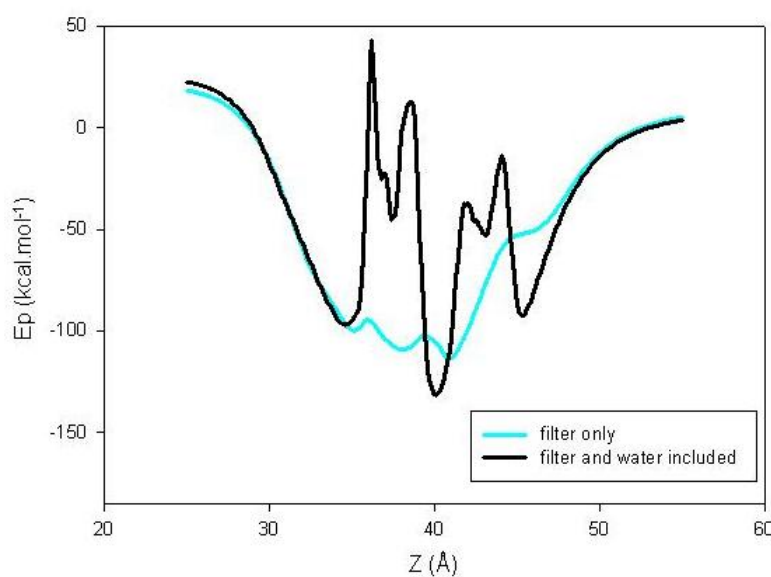### 3.1 Potential Energy Experienced by a K^+ Ion through the Filter

Figures 2 and 3 display the minimized potential energy curves experienced by a K^+ ion that moves adiabatically in the filter when water molecules are present or on the contrary missing.

In Figure 2, the curves are issued from *ab initio* calculations. The filter behaves as a strong trap for the ion, with a deep structured well (about -138 kcal mol^{-1}). Four minima can be observed at the bottom of the well when the filter is free of water molecules, two of which correspond to well–defined secondary wells, while the other two rather appear as shoulders with energy values around -115 and -92 kcal mol^{-1}. When water molecules are located at their preferential sites defined in experiments (S_1 and S_3), only three wells are clearly enhanced and they can be identified as the S_0,

S_2 and S_4 sites. It can be noticed that the location of these three wells is not changed with respect to the corresponding sites in the filter with no water molecules (only a slight shift). The barriers which occur between these three wells correspond to strong repulsive interactions when K^+ virtually moves across the filter without having the possibility to avoid the water molecules. This is consistent with the fact that the diffusion of K^+ and water should be simultaneous in the filter. The main feature deduced from Figure 2 is the reinforcement of the wells already created by the filter alone, when water molecules are present in their S_1 and S_3 sites.
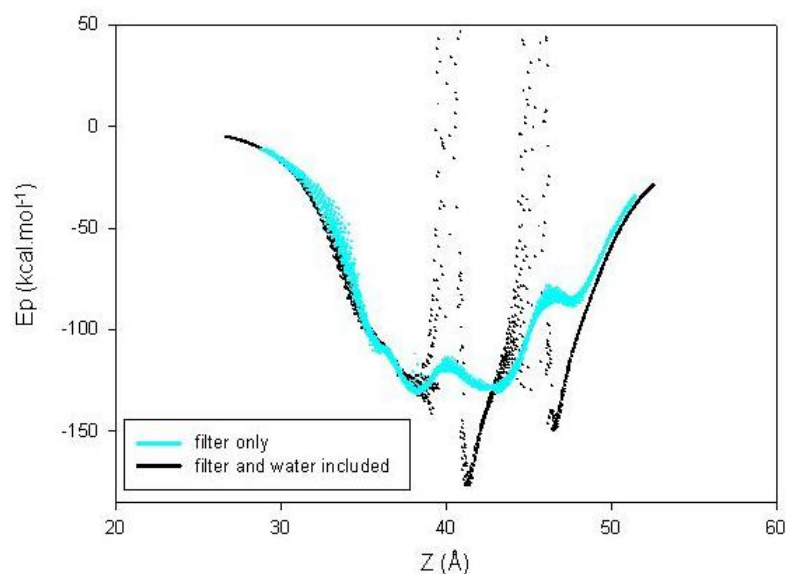

Figure 2. *Ab initio* calculation of the potential energy (kcal mol^{-1}) experienced by a K^+ ion of charge +1 moving adiabatically in the filter either empty or filled with 2 water molecules. The position of the K^+ is along the z axis and minimized with respect to the directions x and y.

Figure 3. Potential energy (kcal mol^{-1}) experienced by a K^+ ion moving along the channel axis z in the filter empty or filled by water molecules, calculated with AMBER6.

Figure 3 displays the same curves calculated with AMBER6 program. The comparison with Figure 2 shows a very good agreement since, for the filter free of water molecules, the four secondary minima observed in the deep global well are found back, reproducing nicely the same shape and locations of the two minima in the central part of the well and one shoulder on each side. When water molecules are included, the shape and locations of the three wells observed in *ab initio* results, S_0, S_2 and S_4, are very similar to those obtained with AMBER6, leading to the conclusion that, for this part of the protein, force field calculations can quite accurately describe the energy experienced by the K^+ ions in the filter. As a consequence, the sequence $K_0W_1K_2W_3K_4$ corresponding to the presence of three K^+ ions in the sites S_0, S_2 and S_4 separated by two water molecules W_1 and W_3 in the filter appears to be a reasonable picture of the reality. Since a complete *ab initio* study of the protein including the filter and the cavity would have been too time consuming, we will entirely base ourselves in the following on the results obtained from AMBER6.
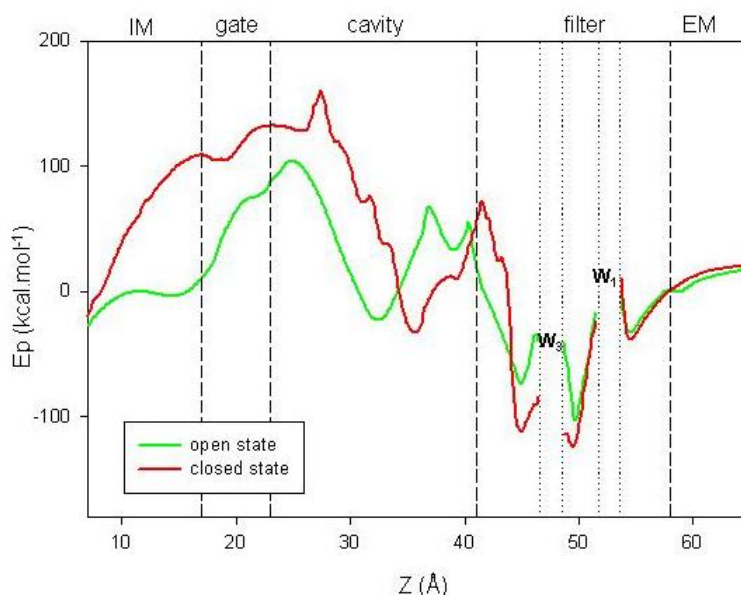
Figure 4. Potential energy curves (kcal mol^{-1}) experienced by a K^+ ion moving throughout the closed and open structures of the pore which contains water molecules. The various regions of the protein are given at the top of the figure. EM and IM mean extracellular and intracellular media, respectively. The disruptions in the curves in the filter region (dotted lines) correspond to unphysical high barriers experienced by the K^+ ion when it moves too close from water molecules in sites S_1 and S_3.

The electrostatic energy experienced by a K^+ ion moving adiabatically throughout the channel from the extra to the intracellular medium is drawn in Figure 4. There are only minor differences for the filter and external part of the pore in the shapes of the energy curves corresponding to the closed and open states of the protein. Let us only notice deeper wells for the S_2 and S_4 sites in the closed conformation, which are consistent with the intuitive idea of a more hindered diffusion in this state. By contrast, these curves display striking differences in the cavity and the gate regions. The site S_{cav} appears to be shifted by about 5 Å towards the gate and the well is slightly higher in the open state.

The shift of S_{cav} will have important consequences on the K_{cav} behavior in the cavity, as explained in Section 3.2. Another difference can be seen regarding the barrier height which hampers the diffusion of the K^+ ion from the cavity to the gate and back. This barrier is decreased by about 30 kcal mol^{-1} in the open state, but it remains still relatively high.

3.2 Behavior of the Sequence KWKWK…K *vs.* Simulation Time

The trajectories of the three ions K_0, K_2 and K_4 in the filter and of K_{cav} in the cavity have been studied from MD simulations during 3.6 ns for the closed state of the channel (Figure 5a) and 1.5 ns for the relaxed open state (Figure 5b), following targeted MD opening.
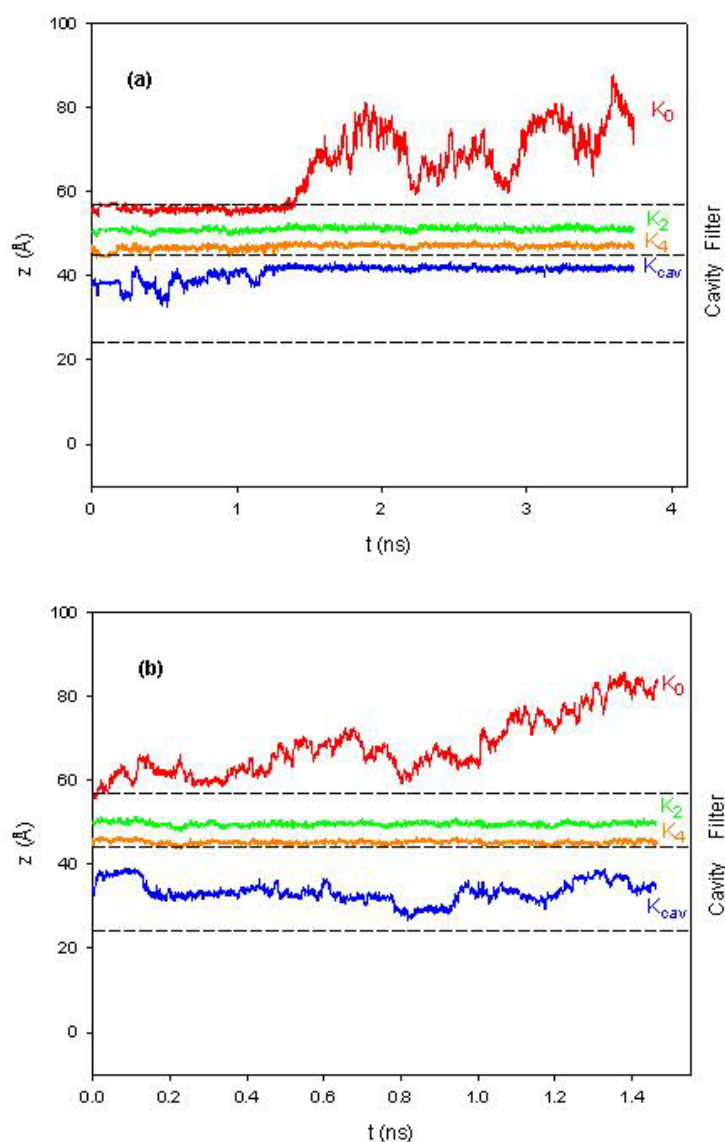
Figure 5. (a) Position along the channel axis z of the potassium ions in the sequence KWKWK…K *vs.* simulation time for the closed state of the channel. The boundaries of the filter and cavity are indicated (dashed horizontal lines). (b) Same as (a) but for the open state during relaxation time.

In the closed state, we see that the two ions K_2 and K_4 remain very stable in their respective sites S_2 and S_4 over the MD run, as already shown in Figure 3. The position of the K^+_{cav} ion in the cavity fluctuates during the first nanosecond of simulation, being in average close to the center of C, and then stabilizes at the upper side of the cavity, nearby the filter part for the remaining time of the run. This position is consistent with the well found in the cavity for the closed state of the pore. We can see a very slight movement upwards of the K_2 and K_4 ions when K_{cav} reaches definitively this upper side ($\approx$ 1.2 ns). The third ion K_0 at the outer–mouth of the filter, which remained very stable during this time, suddenly moves and leaves its initial site S_0 to reach the extracellular part of the membrane. No stable site for this fully hydrated K_0 in this extracellular region is observed and rather large fluctuations of its position occur over about 20 Å along the z direction (parallel to the pore axis), nevertheless indicating that this ion remains globally confined at the filter mouth. The fact that K_0 moves again closer from this mouth between the 2nd and 3rd ns of simulation, depends on the mutual motions of water molecules around it, and this could only be accounted for by examining the minimized trajectory along x and y (not given here). The tendency to an unexpected departure of K_0 observed here, may eventually be correlated with the fact that K_{cav} has reached its optimal position in the cavity.

During stabilization of the open conformation of the protein by totally releasing the constraint on the sequence (Figure 5b), the stability of the two K_2 and K_4 ions in sites S_2 and S_4 is not affected. On the contrary, the position of the cavity ion K_{cav} is strongly changed since K_{cav} occupies in average the core of the cavity and it can even move at the cavity bottom, with relatively large fluctuations over the run time. This position is consistent with the well found in the cavity for the open state of the pore. The behavior of K_0 is clearly less ambiguous than compared to the closed state. Indeed during the targeted simulation, it was constrained at its initial site S_0, but releasing totally this constraint clearly shows its quite intuitive tendency to escape from the closest limit of the filter mouth toward the extracellular region.

Figures 6a and 6b display the trajectories of the two water molecules W_1 and W_3 belonging to the filter sequence and those corresponding to a small subset of water molecules in the cavity, named W_{cav1}, W_{cav2} and W_{cav3}, in the closed and open states. In the closed state, the two water molecules of the sequence follow the same behavior as K_2 and K_4. They are stable in their respective sites S_1 and S_3 and only move very slightly within these sites. The three water molecules selected to illustrate the motions of water in the cavity remain nicely inside it, even though their positions can appreciably fluctuate.

While in the open state of the pore the water molecule W_3 remains strongly trapped in its site S_3, W_1 gets out of the filter after the first half nanosecond. This also corresponds to a longer escape of

the K_0 ion, which was probably retaining it up to this limit. W_1 then exhibits large fluctuations, with a trajectory slope which demonstrates a nearly definitive escape toward the extracellular region. It may be noticed that during the W_1 escape, other water molecules belonging to the extracellular region move to occupy the vacancy created by W_1 [46]. However, due to rapid orientational–translational motions of H_2O molecules, W_1 can move fast and comes close to the filter mouth again, as seen at around 1.2 ns in the simulation.
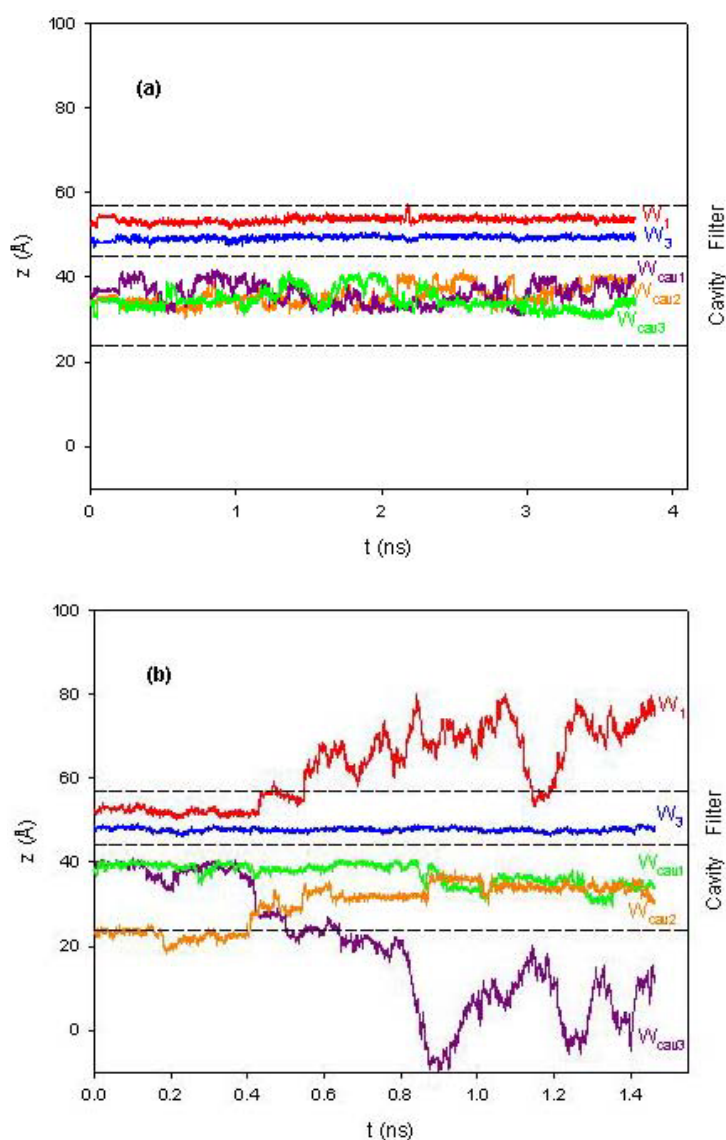
Figure 6. (a) Positions along the channel axis z of the 2 water molecules in the filter and some illustrative water molecules in the cavity (W_{cav1}, W_{cav2} and W_{cav3}) *vs*. simulation time for the closed state of the channel. (b) Same as (a) but for the open state.

The behavior of the selected W_{cav} molecules in the open state (not necessarily the same molecules as in the closed state) is more intricate. W_{cav1} remains localized in the cavity during the complete run time, while W_{cav2}, which was initially at the lowest part of the cavity, clearly moves toward its center. W_{cav3} has a totally different behavior since, while it was initially well confined at

the cavity center during the first half nanosecond, it completely escapes from the cavity to definitively reach the intracellular region, with very large fluctuations. Let us note that the exit from or the entry in the cavity is always preceded by a plateau in the trajectory which could indicate a possible trapping at the cavity bottom (gate region) for $W_{cav.}$

The correlation between the positions of W_1, W_3 and K_2, K_4 in the filter are straightforward and they have been accurately demonstrated in a previous paper [44], on the basis of the calculations of their position correlation functions. Figures 5b and 6b show that the motions of K_0 and W_1 are also probably strongly correlated in the open state. Moreover, although this is less obvious in these figures, we will show in a forthcoming paper [46] that the motions of the K_{cav} ion and of the water molecules in the cavity are strongly dependent one of the others. In particular let us mention here the occurrence (*i*) of strongly correlated motions of the centers of mass of K_{cav} and the water molecules: H_2O's follow the displacements of K_{cav} inside the cavity from the filter inner–mouth to the gate, and (*ii*) of a strong dipole moment resulting from the orientation of the cavity water molecules, which can change its orientation along the channel axis depending on the location of K_{cav} in the cavity.

The present results obtained with MD of the sequence KWKWK...K show in a qualitative way that water has a structuring role in the filter. This is also true for the cavity where the K_{cav} ion experiences strongly changing electric fields due to large fluctuations of the H_2O centers of mass and axes [45], especially in the pore open state. Of course, a continuum description of the solvent would not allow to put in evidence such features. They also reveal a strong correlation between translations of the ions and water molecules (in fact also orientations of H_2O's) in the open state of the channel, which have to be considered for an accurate treatment of the diffusion mechanism of potassium ions in the KcsA channel.

A quantitative study of potassium ion transport throughout the membrane would however require a much more quantitative examination of the correlated motions of W and K in the diffusion mechanism. In particular, free energy differences [6] for transporting an ion from the extracellular medium to the filter and from the cavity to the filter should be determined using in principle QM/MM or more simply empirical valence bond methods [47], to precise the efficient solvation/desolvation mechanism of the potassium ions at the entrances of the filter. It would also be interesting in the future to get energy profiles using a Poisson–Boltzmann continuum model to describe polarization of the system in order to reach a better understanding of the role of water as for the selectivity of the filter. This could be done by removing water molecules in the cavity or in the filter to determine the relative electrostatic influence of the protein, the membrane and the water slabs in the structuring role of water.

4 CONCLUSIONS

The aim of this paper was to show how the stability of the sequence KWKWK…K, which is considered as being a good representation of the linear distribution of the potassium ions and water molecules in the pore, is modified by the pore opening. Based on previous MD simulations on the gating mechanism, and supported by potential energy calculations obtained from both force field and *ab initio* methods, the analysis of the positions of K and W species in the sequence points out several interesting features regarding the stability of K_2, W_3 and K_4 species in the filter, the correlated motions of K_0 and W_1 with respect to the gating mechanism of the pore. This demonstrates that diffusion in the pore requires a more accurate (at a discrete molecular level) description of the role of the various partners that is for instance not taken into account in current Brownian dynamics calculations. The MD on the configuration of K^+ and H_2O sequence that we investigated would merit to be complemented by a study of the sequence $K_1W_2K_3W_4$, *i.e.* by inverting the potassium and water sites, in order to simulate the diffusion process with one possible follow–up.

Supplementary Material

The gating mechanism which was simulated by targeted Molecular Dynamics is deposited as an animation file.

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