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Exploring the Gating Mechanism in the ClC Chloride Channel *via* Metadynamics

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Computer simulations have been used to probe the gating mechanism in the *Salmonella* serovar typhimurium chloride channel (st-ClC). Specifically, the recently developed metadynamics methodology has been exploited to construct free energy surfaces as a function of the positions of either one or two chloride ions inside the pore, the position and protonation state of the key E148 residue, and the number of water molecules coordinating the translocating ions. The present calculations confirm the multi-ion mechanism in which an ion-push-ion effect lowers the main barriers to chloride ion translocation. When a second anion is taken into account, the barrier for chloride passage through the E148 narrow region is computed to be 6 kcal/mol in the wild-type channel, irrespective of the protonation state of the E148 residue, which is shown to only affect the entrance barrier. In the E148A mutant, this barrier is much lower, amounting to 3 kcal/mol. The metadynamics calculations reported herein also demonstrate that before reaching the periplasmic solution, chloride ions have to overcome an additional barrier arising from two different effects, namely the rearrangement of their solvation shell and a flip in the backbone angles of the residues E148 and G149, which reside at the end of the αF helix.

Keywords: chloride translocation; ClC channel; gating; molecular dynamics; metadynamics

Introduction

ClC chloride channels are expressed in a wide range of organisms, and play a role in various biological functions. In eukarya they regulate transepithelial transport of ions, the electrical excitability of the plasma membrane in skeletal muscle cell and acidification of the stomach and intracellular vesicles.[1,2] In bacteria, ClC channels are probably involved in acid shock resistance[3] and act as a pump, exchanging two Cl^- anions for one proton.[4] In the last decade many salient properties of the ClC-type channels have been clarified. Among these is the unique manner in which the conduction is turned on or off, a process defined as "fast gating". Electrophysiological studies have shown that the fast gating is voltage-dependent and the opening of the channel is facilitated by chloride ions in the extracellular solution,[5–8] and possibly by low external pH.[7–9] At physiological pH the fast gating is activated by the permeating anion in what is seemingly a coupled conduction and gating mechanism.[5,10] Moreover the observation of an anomalous mole fraction in a mixed solution of chloride and nitrate anions suggested a multi-ion conduction process.[5]

The recent publication of the crystal structure of a bacterial ClC chloride channel in closed[11] and open form[10] revealed a complex geometry of the channel and suggested possible gating mechanisms. The structure is homodimeric, with each monomer having 18 α-helices arranged in an antiparallel architecture that forms an intracellular and extracellular vestibule separated by a narrow and substantially curved pore approximately 15 Å long. In the closed structure the pore was found blocked by a glutamate (E148). This evidence, taken together with the open nature of the two mutants (E148A and E148Q), provides the link between the

Abbreviations used: WT, wild-type; st-ClC, *Salmonella* serovar typhimurium chloride channel; FES, free energy surface; CV, collective variables.

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glutamate residue E148 and the gating mechanism. Additional support is provided by the fact that, at low external pH, both the mutant and the wild-type channel are more conductive[8,9] However, a recent crystallographic study has shown that pH has no effect on the structure and ion binding properties of Ec-ClC.[12] In the mutant structures, where the channel is in the open state, three chloride ions are inside the channel: one close to the intracellular entrance to the pore (S_{int}), one at the filter binding site (S_{cen}), and one in place of the E148 (S_{ext}). Understanding the permeation mechanism of bacterial ClC channels at an atomistic level is of great importance in its own right, since they are one of the few currently available types of ion channel structures, and as such may thus serve as a prototype for understanding the analogous eukaryotic channels, given their extensive homology. Indeed, there is good evidence that both the ClC channels and the Ec-ClC transporters have conserved modes of ion binding.[13–15]

The availability of the crystal structure made possible the computational study of the conduction mechanism of the transporter.[16–21] These studies clarified many aspects of the mechanism. The favorable docking positions of the chloride inside the pore were studied in depth as well as the qualitative electrostatic effects of strongly conserved charged residues.[21,18] The multi-ion nature of the conduction process was verified,[21] a king-of-the-hill mechanism proposed[16] in which two *Cl* ions compete for a favorable position at the pore center and free energy profiles evaluated.[16,17,20] Despite such studies, questions still remain either unanswered or only partially answered.

One relevant question is the role played by the residues R147 and E148 in the gating mechanism. It is known that the anion conductance is affected by the charge state of R147,[22,23] since mutations of the homologous residues in ClC-1 and ClC-0 were found to alter the anionic selectivity sequence and to increase the cation permeability or to render the channel dis-functional. In agreement with these experiments the Monte Carlo simulations of Miloshevsky and Jordan found that, when R147 is neutralized by proton transfer to E148, an energy barrier to the ion permeation appears. This result confirms the importance of the strictly conserved positive charge. They also found that a mutation of E148 (E148A and E148Q) as well as its neutralization, creates an electrostatic trap that blocks the anion at a mid-membrane position. Building on this, they propose that the displacement of the trapped anion is made possible by a new ion that comes from the periplasm and pushes the first Cl^-. The overall result is similar to the king-of-the-hill mechanism proposed by Cohen and Schulten,[16] in which a lone ion bound to the center of the ClC pore is pushed out by a second ion that takes its place. The difference between the two proposals is that in the latter case the protonation (and maybe deprotonation) of E148 plays a fundamental role.

The role of E148 protonation is also underlined in the study by Bostick and Berkowitz.[20] who calculated by molecular dynamics/umbrella-sampling the potential of mean-force of an anion penetrating the channel from the periplasm. Their potential of mean force is in agreement with the results of Miloshevsky and Jordan and predicts that the protonation of E148 dramatically lowers the barrier to the anion entrance and forms an electrostatic trap. Both of these studies predict a high permeation barrier for the wild-type (WT) channel, while in the study by Cohen and Schulten the barrier is predicted to be only a few kcal/mol. This difference could be due to the fact that in the latter case the channel was prepared in an open state and assumes a king-of-the-hill scenario by pushing simultaneously two ions.

None of the above mentioned studies addressed explicitly the role of the ion hydration/dehydration process, whose importance has been underlined among others by Roux *et al.*[21] and in a different channel by Warshel *et al.*[24] Moreover the role of possible conformational changes of residues in the pore region was not considered.

Accordingly, in the present study by using classical molecular dynamics (MD) and taking advantage of the recently developed metadynamics methodology[25–27] the movement of the chloride from the proximity of the cytoplasmic site to the external solution and *vice versa* has been probed in the Salmonella st-ClC channel and in its E148A mutant. Simulations have also been performed with two different protonation states for the E148 residue.

The present results confirm the previously postulated multi-ion conduction mechanism in which an ion-push-ion effect lowers the main barriers to chloride ion translocation. In the WT channel there are two barriers to the translocation of the Cl^-. The first is met between S_{cen} and S_{ext} and is 6 kcal/mol high in the presence of an ion-push-ion effect and the formation of a salt-bridge between E148 and R147. The latter plays a key role in the opening of the E148 gate that allows the translocation of the Cl^-. The second barrier is found between S_{ext} and the periplasmic solution both in the WT and in the E148A mutant and it is due to the need for rearrangement of the ion's solvation shell and a flip in the backbone of the residues E148 and G149.

Since St-ClC is a transporter exchanging two Cl^- anions for one proton, the effect of the protonation of E148 was studied in order to understand whether or not there is any coupling between H^+ and Cl^-. The present simulations indicate that protonation of E148 induces an asymmetry between the entry of the chloride from S_{ext} to S_{cen} and *vice versa*. Indeed the Cl^- enters the protonated pore without barrier, but once inside it is trapped by a strong electrostatic interaction. The mutation E148A has the effect of eliminating this barrier both for the ingoing and outgoing process (Figure 1).