Abstract

The kinetic Monte Carlo Reaction Path Following (*kMCRPF*) technique [1] is developed to find the lowest energy anionic pathway along the curvilinear pores of four bacterial ClC Cl^- structures [2,3]. We determined the lowest energy curvilinear pathway, identified anion-coordinating amino acids and calculated the electrostatic potential energy profiles. We find all four crystallographic structures correspond to closed states: in StClC WT and EcClC WT, E148 and S107 occlude both sides of the crystal binding site; both the EcClC E148A and EcClC E148Q mutants are blocked at S107. We examine how residues E148, R147, E111 and E113 affect anion permeation by calculating electrostatic potential energy profiles. Neutralizing E148 by lowering pH or mutation to E148A creates a deep electrostatic trap near 4 Å (the external binding site). This suggests a reasonable anion conduction mechanism: neutralize E148, displace the side chain of E148 from the pore pathway to relieve the steric barrier , then trap the anion at the E148 locus, and finally either deprotonate E148 and block the pore (pore closure) or bring a second Cl^- into the pore to promote anion flow (pore conductance). Neutralization of the charged conserved R147 produces an electrostatic barrier at the periplasmic pore exit, suggesting that positive charge at R147 is important for anion conduction. Neutralizing E111 and/or E113 affects energetics in the cytoplasmic mouth; energy wells appear at -6 Å (the internal binding site) and at -11 Å. This suggests the charge state of E111 and/or E113 electrostatically controls anion conductance and occupancy of the internal binding site.

Introduction

The original crystal structures [2,3] permit theoretical study of chloride permeation, and determination of the bacterial systems' curvilinear ionic pathways. Even though the bacterial systems are transporters, not channels [4,5] the prokaryotes share signature sequence identities with their eukaryotic relatives; consequently such atomic level analyses might help in understanding ion transport in eukaryotes and ClC channel function generally. Despite a wealth of experimental mutagenesis data, major issues need to be resolved at the atomic level: What is the exact chloride pathway? Which amino acids coordinate the translocating ion and what are their roles? What is the effect of charged amino acids located within the pore mouths on the anion translocation? Identification of the role of pore lining amino acids is important for understanding both conduction and gating, as mutations of charged residues believed to be chloride coordinating affect both these properties simultaneously. Recent experiments [6] suggest that the electrostatic effects produced by the charged residues located in the inner pore mouth of ClC-0 play a crucial role in controlling channel conductance and occupancy of the internal Cl^- binding site.

We provide a detailed description of chloride coordination in its translocation through the pore and determine coordinates for the curvilinear pore through the bacterial proteins. To get an intuitive feeling for the nature of this path, we examine the effect that E111, E113, E148 and R147 amino acids have on ion coordination by calculating electrostatic potential profiles. These demonstrate well-articulated electrostatic barriers and wells for Cl^- translocation. The wells define positions (possible binding sites) that may be preferentially occupied by Cl^-.

Computational Model

Simulations are performed using our *MCICP (Monte Carlo Ion Channel Proteins)* code [1] with the following assumptions (**Figure 1**):

- Two subunits of the ClC Cl^- protein are immersed in a low dielectric ($\varepsilon = 1$) membrane slab. The protein mouths of subunit A were filled by explicit water molecules (~1,500-2,000) located in a cylinder of 25 Å radius, centered on the central binding site of subunit A. Bulk water regions are treated as continua with $\varepsilon = 80$. The reaction field is treated by the method of images; van der Waals and electrostatic interactions are computed with no cutoff.
- We use partial charges and van der Waals parameters of the CHARMM22 all-hydrogen force field. The four crystallographic X-ray structures of the bacterial ClC Cl^- channels [2,3] (StClC (pdb entry 1KPL) and three EcClC (pdb entries 1OTS, 1OTT (E148A mutant) and 1OTU (E148Q mutant)) channels) were used in the simulations. During simulations of chloride translocation, the ClC Cl^- protein is treated as a rigid body.
- The *kMCRPF* technique [1] was used to determine the coordinating residues and lowest energy paths, delineating putative pathways through the curved ClC pores. Preferential sampling techniques were incorporated to move the anion and nearby water molecules more frequently than those further away.

Calculating the Anion Interaction Energy

Determination of the ion-protein interaction energy along the curved paths is not straightforward, as a priori coordinates for the low energy paths are unknown. To overcome the steep energy barriers found along the ClC pathways we combine a constrained Metropolis Monte Carlo (MMC) method with the kinetic Monte Carlo (kMC) approach, developing a bootstrap technique for determining reaction coordinates: *kMCRPF* [1]. The MMC method permits moves to states of higher energy with a Boltzmann probability. The smaller the energy difference the greater the probability of accepting an uphill move. Using this property we constrain the anionic Z coordinate (the obvious choice for reaction coordinate) and allow only its unidirectional increment (constrained MMC). All other monitored degrees of freedom (e.g., anionic X and Y coordinates, positions and orientations of explicit waters) are unconstrained. The traditional kMC method is adapted to generate the random anionic jumps to one of the nearest neighbor sites. The jump step is not fixed in advance; it is sampled randomly and corresponds to accepted configurations with a Boltzmann probability. Thus, evolution proceeds via small anionic jumps toward the nearest lowest energy uphill or downhill states (kMC technique) and jumps are thermally activated (constrained MMC). The result is a new *kMCRPF* method [1], with transition state search on the fly. The reaction coordinate evolves slowly relative to the other degrees of freedom. For accepted configurations many MC trials are used to relax the anion, fixing the new value of the reaction coordinate. The *kMCRPF* technique allows the anion to overcome steep electrostatic and even steric barriers in following the lowest energy path.

Conduction Pathway and Chloride-Coordinating Amino Acids

A Cl^- ion was placed at the central crystallographic site in subunit A of the four original structures and simulations carried out with the *kMCRPF* technique [1]. Explicit water molecules were equilibrated via standard MMC. The ionic pathway was followed by moving away from the binding site and towards both cytoplasm and periplasm, thus determining the lowest energy pathways and the corresponding anion interaction energy profiles.

Figure 2 illustrates the anion conduction pathway (green curve) through pore A in the StClC wild-type (WT) structure. The pore length is about 16 Å. Pore lining α-helices and residues are shown. In the cytoplasmic region the pore is lined by helices R and D. At the extracellular entrance and in mid-membrane the ion pathway is coordinated by helices F and N. The water molecules are illustrated in the cavities approaching the pore from the cytoplasmic (~ -6 Å) and extracellular (~ 10 Å) sides. In the cytoplasm the pore entrance is formed by I448, S107, P110, F348 and Y445. In all four crystal structures (StClC, EcClC and its two mutants) the side chain of S107 sterically restricts ion entrance into the pore from the cytoplasmic region. At the central binding site the anion (green sphere) is coordinated by backbone HNs of I356 and F357 (helix N) and by hydroxyl HOs of Y445 and S107. In the periplasm the pore entrance is formed by G146, R147, E148, F190 and A358. The side chain of R147 is easily accessible to water molecules. Then the E148 side chain, which forms bonds with numerous protein hydrogens, blocks the pathway in both WT StClC and WT EcClC crystal structures.

Figure 3 illustrates residues on the anion's pathway in the cytoplasmic part of pore A in StClC. At ~ -6 Å (the internal binding site [3]) the anion abuts the intracellular water molecules. Side chains of P110, I448 and F348 (Fig. 3a) coordinate its entrance into the pore. The aryl ring of F348 points toward the pore pathway, aligned roughly perpendicular to the ion trajectory, permitting favorable anion interaction with an aryl hydrogen and shielding the chloride from the ring's π-electrons. On the other side of the pore (Fig. 3a) the anion is coordinated by backbone HNs from the end of helix D (G108 and S107). In the StClC and EcClC WT crystal structures and both EcClC mutants, S107 sterically restricts ion access to the central binding site (Fig. 3b). The steric block is due to OG and CB of S107 and OH and CE2 of Y445. The residues adjacent to S107 are glycines; the side chains of S107 and Y445 may both be easily moved, thus opening the pore. At the central binding site (Fig. 3c) the anion is coordinated by backbone HNs of I356 and F357 and hydroxyl HOs of Y445 and S107 [2]. Backbone HNs of I109 and G149 also contribute to the anion coordination.

Figure 4 illustrates residues on the pathway in the extracellular part of pore A in StClC. At the entrance (~10 Å) the anion is coordinated by the side chain of R147 and by backbone HNs from G315, G316 and F317 (Fig. 4a). This site is accessible to water molecules and the anion also interacts with three or four waters (see Fig. 2). The E148 side chain belonging to helix F forms hydrogen bonds with its own polypeptide loop (helix F) and residues belonging to another polypeptide chain (helix N) (Fig. 4b). Thus, the E148 oxygens, which form bonds with many protein hydrogens, sterically block the pathway.

Pore Size

Figure 5 illustrates the effective radius of pore A along the pathway in WT StClC. The pore radius was determined using our *MCICP* code [1] by squeezing a variable radius sphere along the pathway. At Z ~ 6 Å the extracellular pore is narrowest, ~ 0.46 Å, forming the constriction region leading to the binding site from the periplasmic pore entrance. Here the E148 oxygens block the pore. At the binding site (Z ~ 0 Å) the pore is not constricted (pore radius ~ 1.88 Å and Cl^- fits perfectly). On the cytoplasmic side the constriction region is located at Z ~ -2.5 Å. Here the pore narrows again; its radius drops to ~ 0.92 Å due to the S107 and Y445 side chains which prohibit anion escape from the binding site and access to the cytoplasmic side of the pore. At about Z ~ -6 Å the pore again opens out and its radius increases to ~ 2 Å. These results clearly demonstrate that the crystal structure has captured the bacterial system in a closed state, with access forbidden from either side of the central binding site.

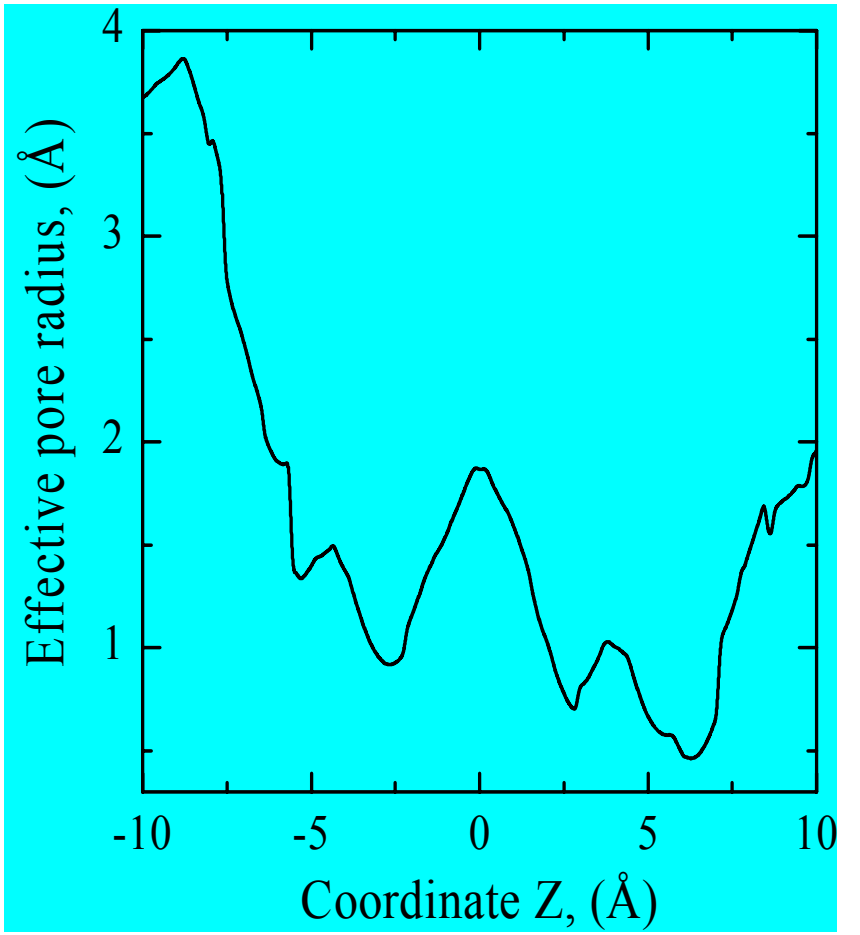

Figure 5. The effective pore radius as a function of Z along subunit A's Cl^- pathway in WT StClC.

Anionic Electrostatic Potential Energy Profiles

As the pore is sterically constricted, the total non-bonded interaction energy has no meaning. However, the electrostatic potential energy profile along the path may provide the clues as to the energy barriers and wells in the open pore. Our rationale is that the blocking residues must slightly rearrange themselves [2,3] for the pore to open and the anion to move unimpeded. These local processes most likely do not require global structural alterations.

Figure 6 illustrates electrostatic potential energy profiles along the pathway in pore A of the EcClC WT structure. In the cytoplasmic and extracellular mouths the anion is solvated by the water molecules. In the pore region there are large electrostatic barriers at E148 and S107 sites (*black trace*, Z ~ ± 4 Å). These barriers are somewhat overestimated as the anion passes very close to the glutamate and serine oxygens (the pore is constricted). Here we focus on the charge states of E148 and R147. Mutating E148A [3] or neutralizing E148 by lowering pH [7] creates a deep electrostatic trap. The barrier near 4 Å is converted into a deep well (*red trace*). For the E148A or E148Q mutants the behavior of the electrical potential energy profile is very close to that of neutralized E148 with R147 in its native state. The energy minimum at Z ~ 4 Å is then located precisely at the position previously occupied by the oxygens from E148 (the external binding site [3]). The anion's energy is less than in the periplasmic mouth, a favorable condition for anion entrance into the pore from the periplasm. When R147 is also neutralized by proton transfer to E148 (the OE2(E148)-HH12(R147) distance is 6.4 Å) an energy barrier appears at ~ 10 Å (*green trace*). This illustrates the importance of the R147 charge, which may facilitate smooth anionic flow from the periplasmic mouth into the pore.

Figure 7 illustrates how putative changes in the charge state of conserved acidic residues (E111 and E113), located close to the cytoplasmic pore entrance, alter the electrostatic potential energy profile. We focus on WT EcClC, introducing mutations S107G and E148A to artificially create an open permeation pathway. The S107G mutation has reduced the electrostatic barrier at the S107 site (compare the *red trace* of Fig. 6 with the *black trace* of Fig. 7). E111 and E113 residues influence the energy in the region from Z ~ -15 to Z ~ 5 Å. Neutralizing E111 impacts the electrostatic barrier at the S107 site (*red trace*). The internal binding site at Z ~ -6 Å becomes pronounced. The energy drops to the level of that in the cytoplasmic mouth. Experiments show that the highly conserved E111 (E127 in ClC-0) electrostatically controls anion conductance and occupancy of the internal binding site in the cytoplasmic pore [6]. Neutralizing E113 has the same effect as neutralizing E111. The electrostatic influence of E113 is even more pronounced in the range from Z ~ 0 to Z ~ 5 Å than that of E111 (*blue trace*) because E113 is nearer the central binding site. In the bacterial systems E113 (helix D) forms a bridge with helix R (residue S446). The side chain of Y445 is also coordinated by one of the carboxylate oxygens from E113. In eukaryotic channels this bridge is stronger and is polarity reversed, E113(D) and S446(R) being strictly replaced by LYS and ASP respectively (in ClC-0, K129(D) and D513(R)). When both E111 and E113 are neutralized the charge effect nearly doubles (*green trace*). The absence of negative charge on the E111 and E113 side chains leads to a deep energy well in the region from Z ~ -15 to Z ~ 10 Å, which would favor anion entry into the pore from both the periplasm and cytoplasm. The center of this energy well is somewhat shifted towards the cytoplasm.

Conclusions

- The X-ray structures of the bacterial ClC Cl- channels [2,3] correspond to closed states. The anion is locked in the central binding site. In addition to E148 the side chain of S107 sterically impedes ionic passage from the binding site into the cytoplasm in all four structures. In both the EcClC E148A and EcClC E148Q mutants there is a small steric barrier near E148. It suggests that the anion fits snugly the whole length of the pore and that flexibility of the anion-coordinating side chains crucially influences permeation.
- When the E148 is mutated or neutralized, it creates an electrostatic trap, binding the anion near mid-membrane. This suggests the possibility of an electrostatic mechanism [8,9] for controlling anion flow through the periplasmic pore: neutralize E148, displace the side chain of E148 from the pore pathway to relieve the steric barrier, then trap the anion at mid-membrane, and finally either deprotonate E148 and block the pore (pore closure) or bring a second Cl^- into the pore to promote anion flow (pore conductance). Side chain displacement may arise by competition for the binding site between the oxygens of neutralized E148 and the anion moving down the electrostatic energy gradient.
- The charged residues in the cytoplasmic mouth influence Cl^- permeation by an electrostatic mechanism [6]. The replacement of E111 and/or E113 by a neutral residues decreases the energy barrier, which should increase conductance. Occupancy of the internal Cl^- binding site is also affected by a negative charge at position 111. The absence of negative charge on the E111 and E113 side chains favors anion entry into the protein pore from both the periplasmic and cytoplasmic mouths.

Acknowledgements

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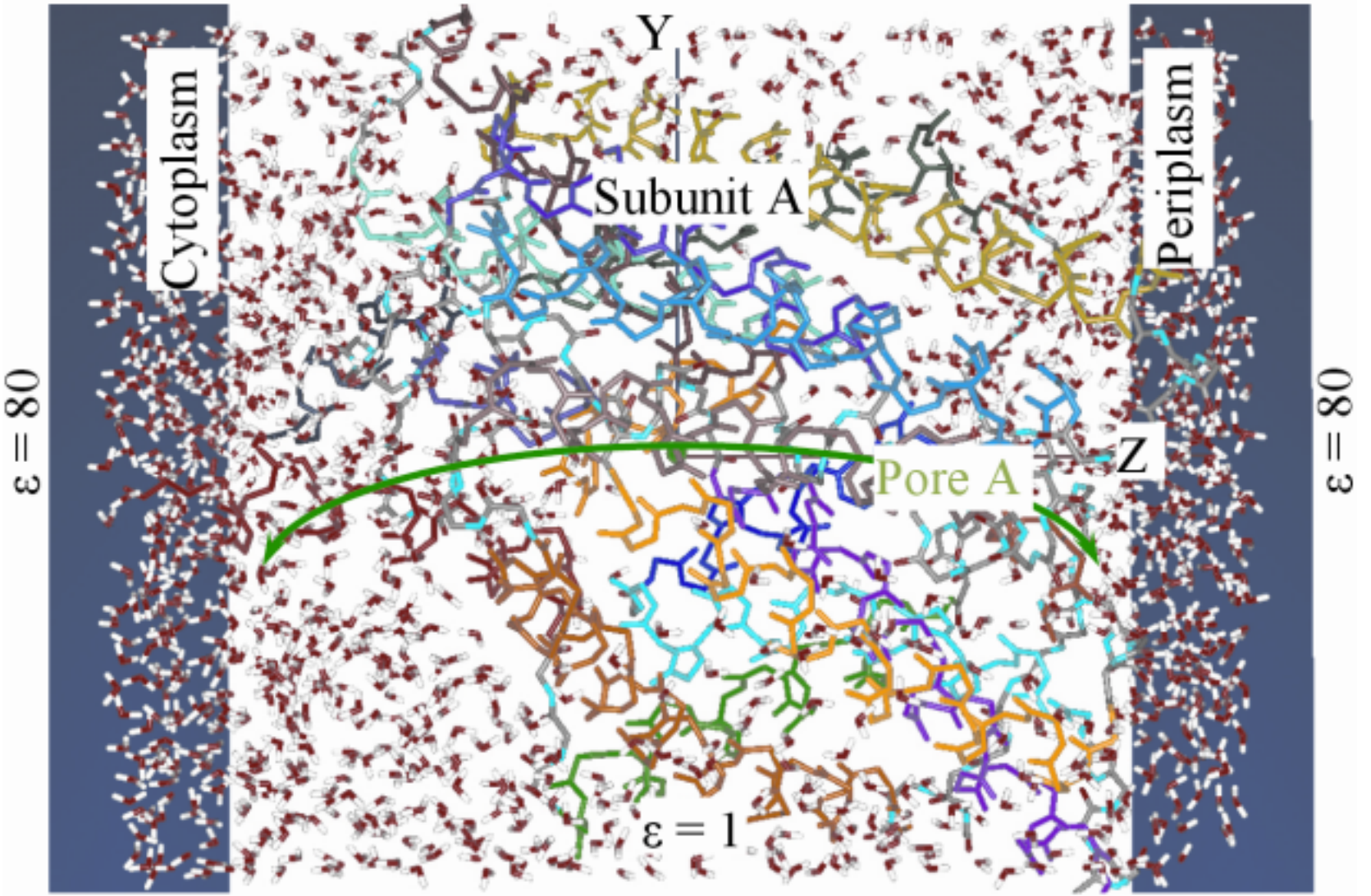

Figure 1. Molecular representation of the pore in ClC Cl^- subunit A. View from within the membrane along the X-axis connecting the anions in the central binding sites in subunits A and B. The bulk regions are continua with dielectric constant ε = 80, a membrane slab is a low dielectric medium (ε = 1). The image planes are dark blue panels.

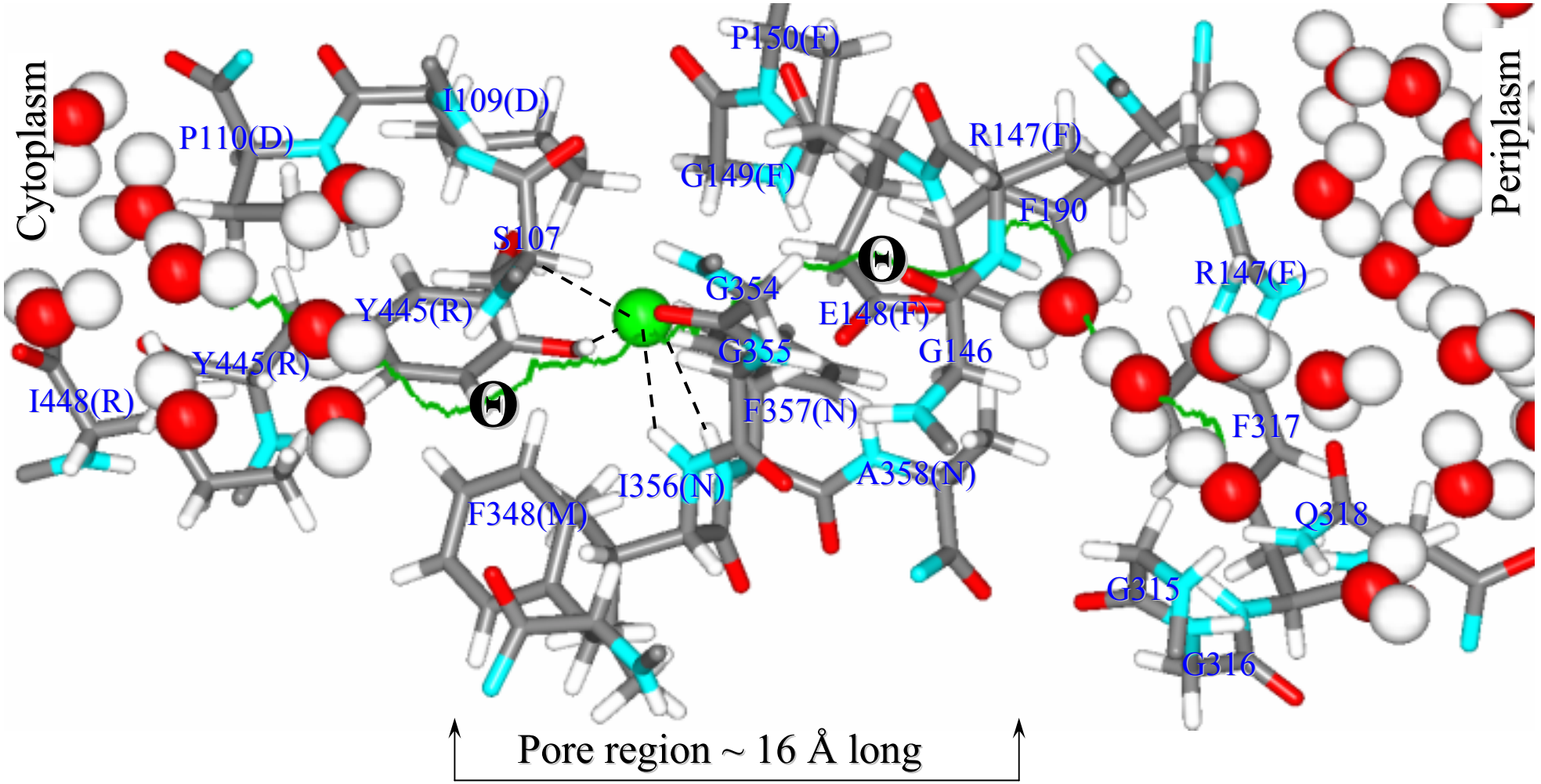

Figure 2. Anion pathway along pore A in StClC. The Cl^- trajectory is represented by the green curve. The anion in the central binding site is illustrated as a green sphere. The symbol **Θ** indicates trajectory sites where the pore is sterically blocked by S107 and E148 side chains. Important residues lining the pore are labeled. The α-helices to which they belong are indicated in parentheses.

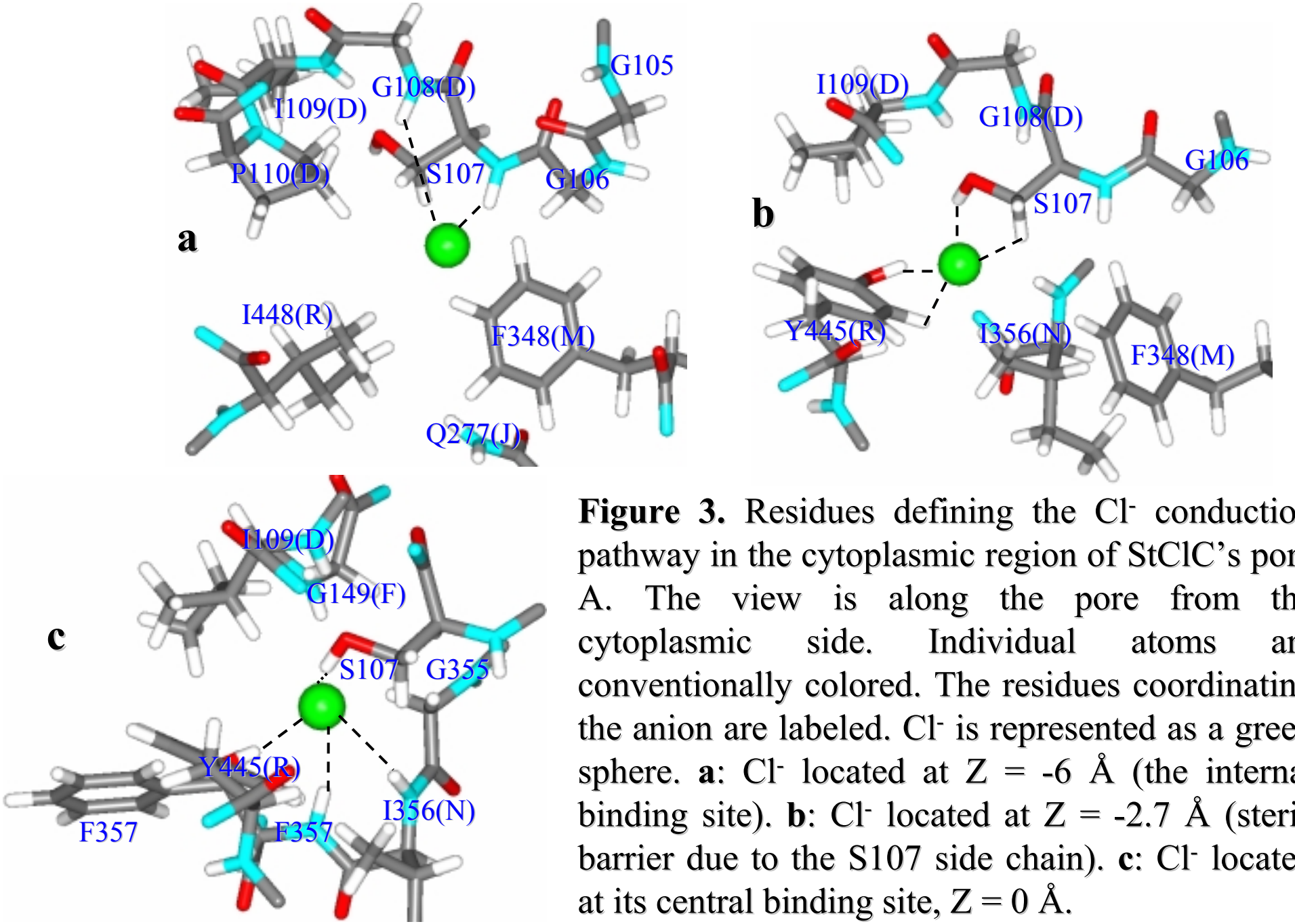

Figure 3. Residues defining the Cl^- conduction pathway in the cytoplasmic region of StClC's pore A. The view is along the pore from the cytoplasmic side. Individual atoms are conventionally colored. The residues coordinating the anion are labeled. Cl^- is represented as a green sphere. **a**: Cl^- located at Z = -6 Å (the internal binding site). **b**: Cl^- located at Z = -2.7 Å (steric barrier due to the S107 side chain). **c**: Cl^- located at its central binding site, Z = 0 Å.

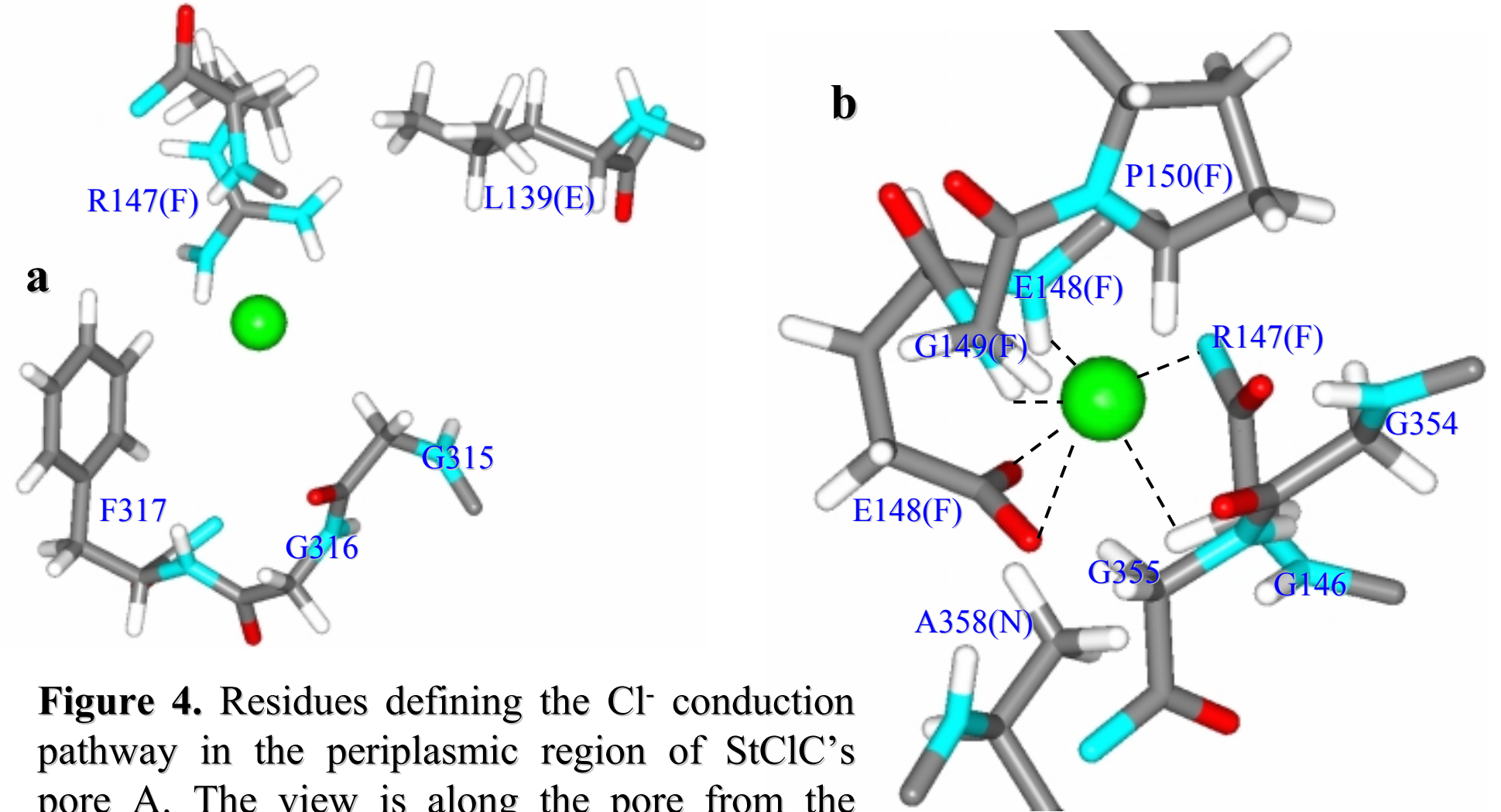

Figure 4. Residues defining the Cl^- conduction pathway in the periplasmic region of StClC's pore A. The view is along the pore from the cytoplasmic side. **a**: Cl^- located at Z = 10 Å (the entrance from the periplasm). **b**: Cl^- located at Z = 4.8 Å. The E148 side chain sterically blocks the pore.

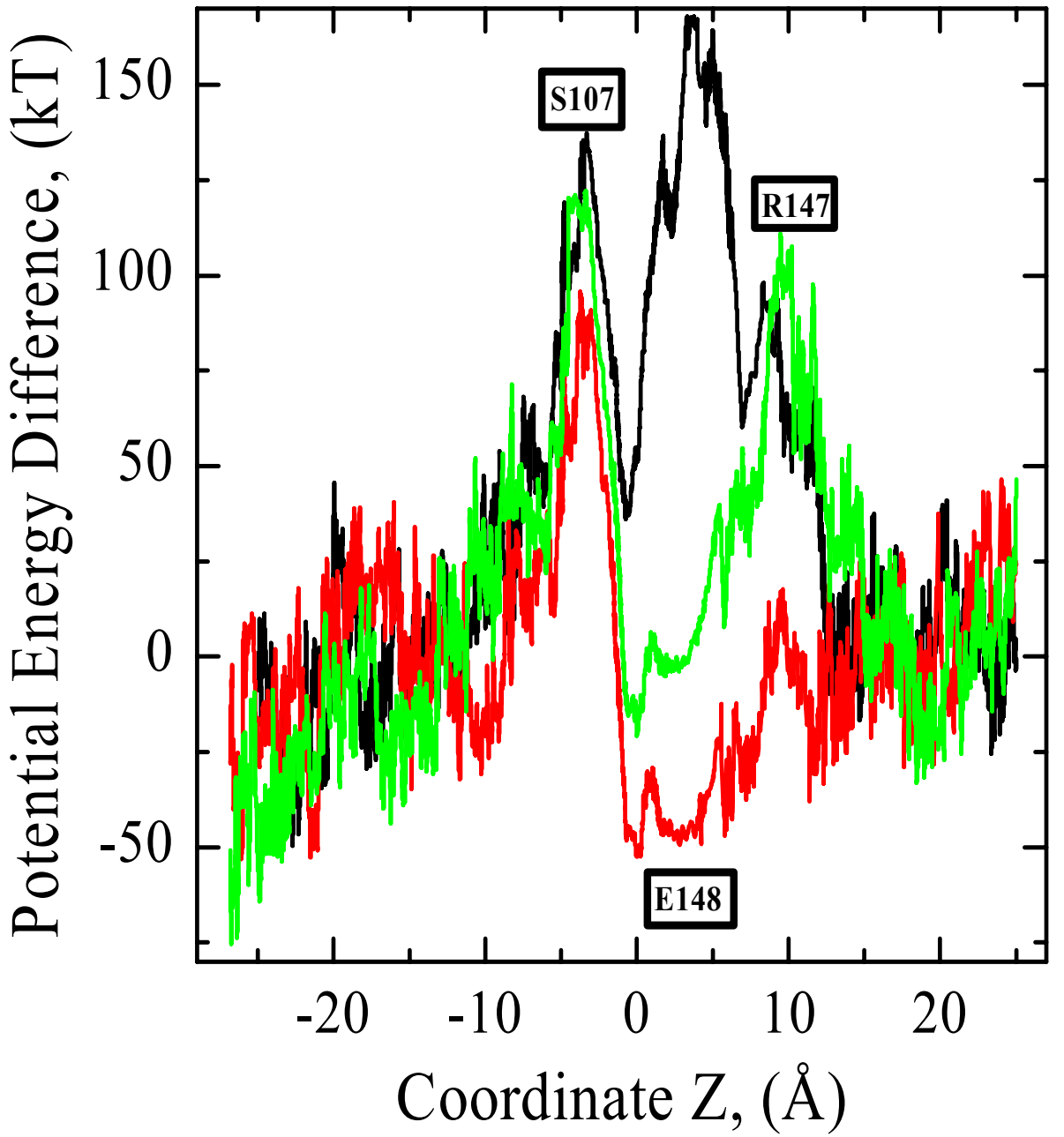

Figure 6. Effect of the charge state of selected charged residues on anionic electrostatic potential energy profiles in the periplasmic pore of WT EcClC. Charge mutations of E148 and R147, with the locations of S107, E148 and R147 labeled. The black trace is for the native state of WT EcClC (with both E148 and R147 charged). The red trace illustrates the effect of the mutation E148A or of neutralizing E148 by pH decrease. The green trace describes the case where R147 is also neutralized by proton transfer to E148.

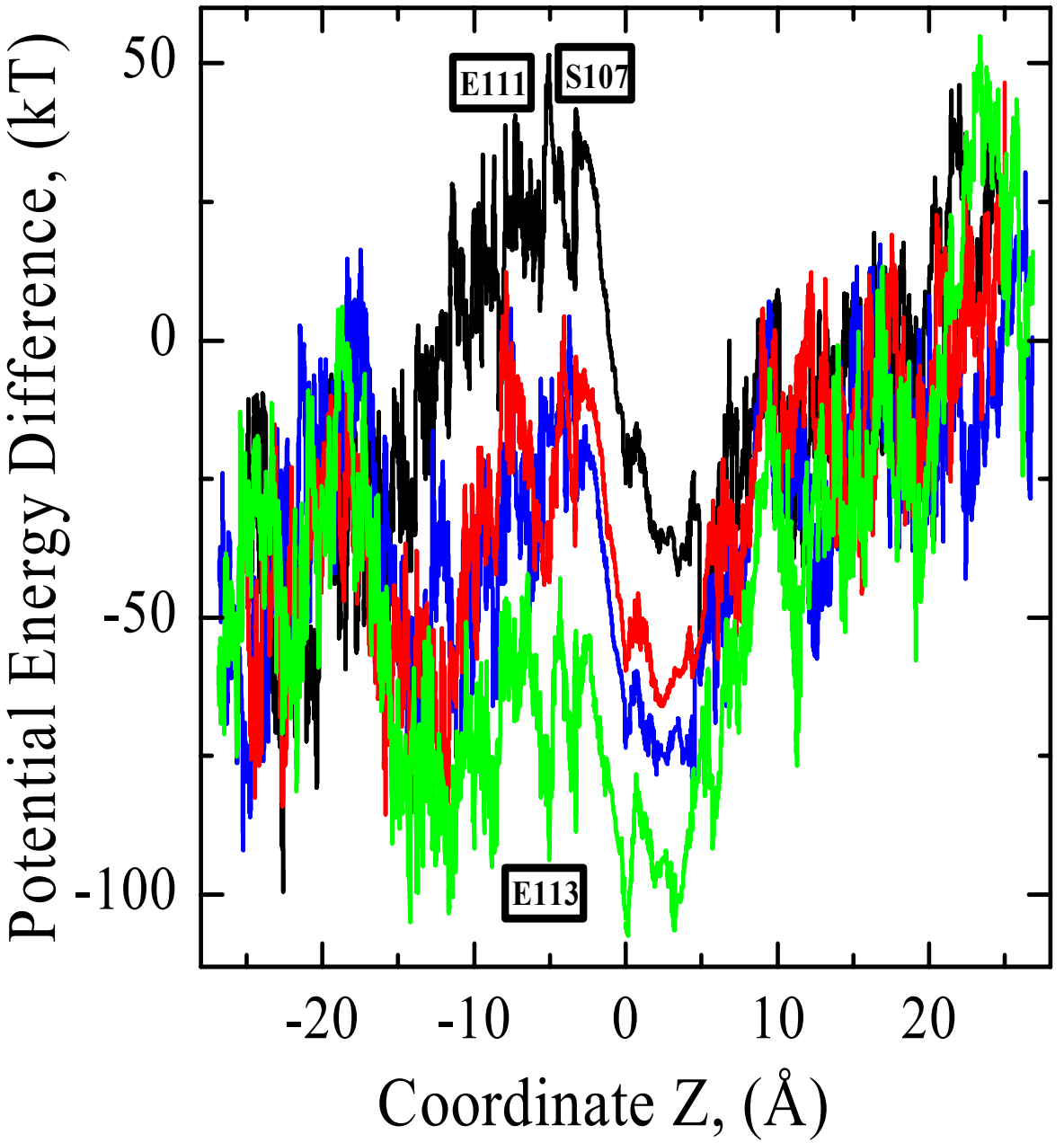

Figure 7. Effect of the charge state of selected charged residues on anionic electrostatic potential energy profiles in the cytoplasmic pore of WT EcClC. Charge mutations of E111 and E113, with the locations of S107, E111 and E113 labeled. The black trace is for the double mutation, S107G and E148A, introduced to artificially create an open permeation pathway. The other traces illustrate effects of further mutations. Red and blue traces demonstrate the effect that neutralizing E111 (red trace) or E113 (blue trace) has on the electrostatic energy profile in the cytoplasmic pore region. The green trace corresponds to the case with both E111 and E113 neutralized.