CHIN. PHYS. LETT. Vol. 31, No. 6 (2014) 068701

Ion Binding Energies Determining Functional Transport of ClC Proteins *

YU Tao(于涛)[1**], GUO Xu(郭旭)[1], ZOU Xian-Wu(邹宪武)[2], SANG Jian-Ping(桑建平)[1,2**]

[1]*Department of Physics, Jianghan University, Wuhan 430056*
[2]*Department of Physics, Wuhan University, Wuhan 430072*

(Received 30 December 2013)

The ClC-type proteins, a large family of chloride transport proteins ubiquitously expressed in biological organisms, have been extensively studied for decades. Biological function of ClC proteins can be reflected by analyzing the binding situation of Cl^- ions. We investigate ion binding properties of ClC-ec1 protein with the atomic molecular dynamics simulation approach. The calculated electrostatic binding energy results indicate that Cl^- at the central binding site S_{cen} has more binding stability than the internal binding site S_{int}. Quantitative comparison between the latest experimental heat release data isothermal titration calorimetry (ITC) and our calculated results demonstrates that chloride ions prefer to bind at S_{cen} than S_{int} in the wild-type ClC-ec1 structure and prefer to bind at S_{ext} and S_{cen} than S_{int} in mutant E148A/E148Q structures. Even though the chloride ions make less contribution to heat release when binding to S_{int} and are relatively unstable in the Cl^- pathway, they are still part contributors for the Cl^- functional transport. This work provides a guide rule to estimate the importance of Cl^- at the binding sites and how chloride ions have influences on the function of ClC proteins.

PACS: 87.10.Tf, 87.14.ep, 87.16.Vy DOI: 10.1088/0256-307X/31/6/068701

The ClC-type membrane proteins, which transport Cl^- ions across the cell membrane, are widespread in the biological world. These proteins are associated with a variety of physiological and cellular processes such as membrane action potential regulation, muscle excitability, renal intravascular transport, and cell flexibility.[1−4] Recently, researchers have found that there are two types of mechanisms for the ions transporting across membranes: channels for selective permeation of Cl^- ions and Cl^-/H^+ transporters for exchange of two Cl^- ions in one direction with one proton in the opposite direction.[2,3] There are nine ClC protein members in mammalian species in which four are identified as channels and five are transporters.[4,5] Several genetic diseases in humans such as Bartter's syndrome, myotonia congenita, Dent's disease, and osteopetrosis are associated with functional defection of these mutant ClC proteins.[6]

Despite differences in biological function, the three-dimensional structures of ClC protein family members are believed to have a high degree of similarity. Among these ClC channel proteins, ClC-ec1 protein which belongs to *Escherichia coli* is the most widely investigated. The ClC-ec1 protein is finally encoded by the *eriC* and *mriT* genes, and has been shown to be involved in the extreme acid-resistance response that allows benign and pathogenic bacteria to survive in highly acidic environments, such as the stomach.[4,7] Since this protein is readily over expressed, purified, and reconstituted,[8,9] and moreover its high-resolution crystal structure is obtained by using x-ray diffraction,[10,11] it provides a more suitable platform for experimental and theoretical research. According to the experimental x-ray structure, there is a narrow selectivity filter region inside the wild-type transporter with two anion binding sites: S_{cen} and S_{int}. Meanwhile the negative gating glutamate residue E148 occupies the third binding site S_{ext} near the extracellular entrance of the selectivity filter. The Cl^- binding sites in ClC-ec1 crystal structure indicate the existence of a curved ion transport pathway connecting the intracellular and extracellular solutions.[11] Recently, Accardi and Miller have investigated the ion transporting properties in ClC-ec1 protein systematically. Their experimental results indicate that ClC-ec1 transports its substrate ions with a strict, obligatory stoichiometry of 2 Cl^- for 1 H^+, and they move in opposite directions.[12] At the same time, investigators have developed many theoretical and computational approaches based on the ClC-ec1 crystal structure, and obtained many valuable molecular details about the ion transit pathway and possible ion transport mechanism.[13−20] Although several mechanistic hot spots have been identified, the detailed influence of Cl^- binding to the ClC-ec1 protein with structural stability has not been provided. Recently, Picollo *et al* reported the measurements on the thermodynamic parameters for chloride ions binding to the wild-type ClC-ec1 channel and its mutants by using isothermal titration calorimetry (ITC) approach.[21,22] They found that the thermodynamics of synergistic Cl^- binding to ClC-ec1 induces protonation of cru-

*Supported by the National Natural Science Foundation of China under Grant Nos 11304123 and 31270761, China Scholarship Council Foundation, and Scientific Research Foundation of Jianghan University under Grant No 2013016.

**Corresponding authors. Email: jianping.sang@gmail.com; tyu@jhun.edu.cn

© 2014 Chinese Physical Society and IOP Publishing Ltd

cial glutamate and determines the stoichiometry of ion transport. Since chloride ions bind to S_{int} so weakly and can easily be unnoticed by using the ITC approach, they suggested that heat release results are probably associated with Cl^- binding to S_{cen} for the wild-type ClC-ec1 structure, meanwhile S_{ext}, and S_{cen} for mutant E148A/E148Q structures. In this Letter, we analyze the Cl^- binding properties of ClC-ec1 channel protein by using the all-atom molecular dynamics (MD) simulation. Magnitude of the calculated electrostatic binding energy between Cl^- and protein represents the binding stability of Cl^-. Moreover, we attempt to find some potential relationships between our calculated results and the experimental results by using ITC approach.

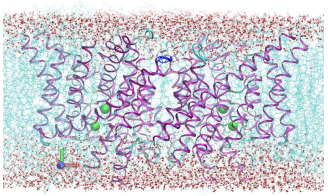

Fig. 1. (Color online) Topological structure of the initial ClC-ec1 protein-membrane-water system. Chains A and B are represented by purple helixes. Green spheres represent Cl^-.

In order to run normal MD simulation, a protein-membrane-water system, which is close to the actual solution environment, should be established. Firstly, the initial crystal structure of wild-type ClC-ec1 protein was taken from PDB database (PDBid: 1OTS). Missed hydrogen atoms were added by using PSFGEN tool of visual soft VMD.[23] In the crystal structure, the central and internal binding sites (represented by S_{cen} and S_{int}) were occupied by chloride ions. Then, chains A and B of ClC-ec1 protein were selected, and embedded in an explicit POPE membrane with size of $120\text{Å} \times 120\text{Å} \times 40\,\text{Å}$. Finally, a TIP3 water layer with thickness of 10 Å was added on both top and bottom of the membrane by using VMD. Meanwhile 57 Cl^- and 47 Na^+ ions were mingled in water in order to make the solution concentration 300 mM and total charge of the system zero. The whole system consisted of 103,936 atoms, and 13,590 atoms belonged to ClC-ec1 protein. The atomic charges and atomic radius parameter were obtained from CHARMM27 force field.[24] In this way, the initial protein-membrane-water system was constructed (see Fig. 1), but this whole structure was not in the thermodynamic equilibrated state. The established ClC-ec1 protein system is shown in Fig. 1. Two binding chloride ions in the pathway can also be seen from Fig. 1.

To equilibrate the whole system, energy minimization process and MD equilibrated simulation were used for making the initial protein-membrane-water structure equilibrate. The whole process was carried out by using soft NAMD following standard MD procedure.[25] Firstly, the initial structure was compacted at a lower energy state by minimization for 5000 steps. Then, the whole system was equilibrated by MD simulation under a constant NPT condition. In order to minimize the interspaces between protein and membrane interface, NAMD was run for 1 ns by fixing all the protein atoms of ClC-ec1 and freeing all the membrane atoms and water molecules. Lastly, NAMD simulation was then performed for 27.5 ns by freeing all the atoms of the system. The periodic boundary condition was applied in all dimensions. Evaluation of long-range electrostatics was handled by using the PME (Particle Mesh Ewald) algorithm. During the equilibrated MD simulation, the temperature of the system was maintained at 298.15 K and pressure was retained at 1 atm. All simulation tasks were performed on our local Linux GPU clusters.

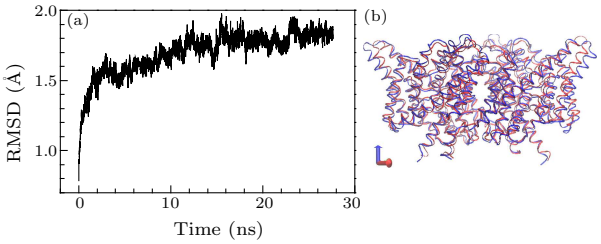

Fig. 2. (Color online) Structural variation of the wild-type ClC-ec1 protein in the MD simulation. (a) The root-mean-square deviation (RMSD) of protein backbone changes with MD time. (b) Comparison between the initial structure (red helix) and the equilibrium structure (blue helix). The equilibrium structure is achieved by an energy minimization for 5000 steps, and followed by molecular dynamics simulation for 27.5 ns. The final RMSD of the protein backbone is about 1.8 Å.

Compared to the experimental structure, each part of ClC-ec1 protein has undergone the corresponding changes in the MD simulation process. Through energy minimization and thermodynamic equilibrating MD simulation, the final equilibrated wild-type ClC-ec1 protein-membrane-water system was obtained. Figure 2(a) plots the backbone root-mean-square deviation (RMSD) of the ClC-ec1 protein as a function of MD simulation time. These RMSD values indicate that the whole system reaches the equilibrium after 17 ns. In order to compare differences of the ClC-ec1 protein structure before and after the MD simulations, Fig. 2(b) shows these conformational changes between the initial structure and equilibrated conformation for a wild-type structure. The whole structure has undergone slight variation during the MD simulations, and the center coordinates change in X, Y, and Z directions are 0.03 Å, 0.11 Å, and 0.02 Å, respectively. Moreover, the chloride ion at the internal binding site S_{int} may finally move into the intracellular solvent during the MD simulation for the wild-type

ClC-ec1 structure, therefore the relatively stable site near the intracellular entrance to the pore is taken as the internal binding site of the chloride ion in the MD configuration. Because equilibrated MD structure represents the thermodynamic state in nearly real solutions, it is reliable in theoretical analysis.

Transport rate of Cl^- across the membrane is closely related to the biological function of ClC proteins. During this process, binding properties at binding sites of Cl^- are critical for the transport rate. In order to describe binding properties of the Cl^- ion in ClC-ec1 channel protein, we calculate the electrostatic binding energies of Cl^- ions at the binding sites by using Eq. (3). When calculating the Cl^- binding energies, we select at least 10 MD snapshot trajectories, and calculate the average value of these 10 structures. The RMSD values of these selected trajectories are around the overall average RMSD value and the space time between two selected MD snapshots is more than 500 ps. Taking the wild-type (WT) structure as an example, the electrostatic binding energies for central binding site S_{cen} and internal binding site S_{int} are $-8.1(0.4)$ and $-2.5(0.3)$ kal/mol, respectively. These calculated results indicate that Cl^- at the central binding site S_{cen} has more binding stability than the internal binding site S_{int}. Because the Cl^- of the internal binding site locates at the intracellular entrance, and moves into the intracellular solution easily in our MD simulation, the Cl^- of the internal binding site is less favorable than the central binding site. The mutant E148A/E148Q structures correspond to open state of ClC-ec1 protein, so there are three binding sites: S_{ext}, S_{cen} and S_{int}. The Cl^- electrostatic binding energy data of these three binding sites are summarized in Table 1. Chloride ions at external and central binding sites are also more stable than those at the internal binding site.

Table 1. The Cl^- electrostatic binding energies of different binding sites for the wild-type ClC-ec1 protein and its E148A/E148Q mutant structures. The unit of energy is kcal/mol.

Structures	Electrostatic binding energies (kcal/mol)		
	S_{ext}	S_{cen}	S_{int}
WT		$-8.1(0.4)$	$-2.5(0.3)$
E148A	$-8.3(0.5)$	$-9.6(0.5)$	$-3.1(0.3)$
E148Q	$-8.5(0.4)$	$-9.0(0.5)$	$-3.0(0.4)$

Recently, Picollo *et al* reported the measurements on the thermodynamic parameters for chloride ions binding to the wild-type ClC-ec1 protein and its mutant structures by using the isothermal titration calorimetry (ITC) approach.[21,22] In the structure of ClC-ec1 protein, Cl^- binding to the central binding site is crucial for Cl^-/H^+ coupling transport cross the membrane. This synergistic substrate binding property of ClC-ec1 protein induces protonation of the crucial gated glutamate acid, and determines the stoichiometry of ionic transport. There are two Cl^- binding sites (S_{cen} and S_{int}) for the wild-type ClC-ec1 structure and three Cl^- binding sites (S_{ext}, S_{cen} and S_{int}) for the E148A/E148Q mutant structures. Since chloride ions bond to S_{int} is so weak that it is relatively difficult to detect using a conventional experimental approach, Picollo *et al* suggested that heat release results of Cl^- binding to ClC-ec1 protein are primarily associated with the S_{cen} site for the wild-type structure, meanwhile S_{ext} and S_{cen} sites for mutant E148A/E148Q structures.

Table 2. Comparisons between the experimental heat releases results measured by Picollo *et al*[21,22] and the calculated electrostatic binding energies based on the MD simulations. The second column lists the average electrostatic binding energies if only considering chloride ions bound to S_{cen} in wild-type structure of ClC-ec1 protein and to S_{ext}+S_{cen} in E148A/E148Q structures, respectively. The third column lists the average electrostatic binding energies if considering the chloride ions bound to S_{cen}+S_{int} in wild-type structure and to S_{ext}+S_{cen}+S_{int} in E148A/E148Q structures, respectively. The unit of energy is kcal/mol.

Structures	Average binding energies (kcal/mol)		
	$\langle S_{ext}+S_{cen}\rangle$	$\langle S_{ext}+S_{cen}+S_{int}\rangle$	ITC[21,22]
WT	$-7.1(0.4)$	$-5.3(0.5)$	$-4.3(0.1)$
E148A	$-8.6(0.5)$	$-7.0(0.5)$	$-6.7(0.1)$
E148Q	$-8.4(0.4)$	$-6.8(0.5)$	$-5.7(0.1)$

In order to verify the experimental results of Picollo *et al*, we have completed the corresponding calculations. Table 2 lists the comparison between the heat release results measured by Picollo *et al* and our calculated electrostatic binding energy based on the MD simulation. The second column of Table 2 lists the average electrostatic binding energies as chloride ions bind to S_{cen} (WT) and S_{ext}+S_{cen} (E148A/E148Q mutants), respectively. The third column lists the corresponding values as chloride ions bind to S_{cen}+S_{int} (WT) and S_{ext}+S_{cen}+S_{int} (E148A/E148Q mutants), respectively. It can be seen from Table 2 that all three sets of data show the same tendency: when the wild-type ClC-ec1 protein mutates to E148A/E148Q mutants, the binding sites' energies decrease and become larger in absolute values.[21,22] Going a step further, quantitative comparison between the heat release results and our calculated average electrostatic binding energies indicate that chloride ions binding to S_{int} may be more possible to appear in the WT and E148A/E148Q mutant structures. When the chloride ions bind to S_{cen}+S_{int} (WT) and S_{ext}+S_{cen}+S_{int} (E148A/E148Q mutants), we can explain the heat release experiments as follows. In both the wild-type ClC-ec1 and E148A/E148Q mutant structures, the central binding site S_{cen} and external binding site S_{ext} are stable and the internal binding site S_{int} is less stable. The binding energy at S_{int} is so weak (from -2.5 to -3.0 kcal/mol) that heat release associated with Cl^- binding to S_{int} is small and it is relatively diffi-

cult to detect using the ITC approach. Therefore, the measured heat release results are approximately the average effect of the Cl^- binding energies. According to the above analysis, the internal Cl^- binding site S_{int} for WT and E148A/E148Q mutants should be considered. That is to say, the chloride ions bound to S_{int} make less contribution to heat release and they are easily unnoticed by ITC, however they are still part of the heat release contributors.

In the case of chloride ions binding to S_{cen}+S_{int} (WT) and S_{ext}+S_{cen}+S_{int} (E148A/E148Q mutants), slight differences also remained between the measured heat release and our calculated average electrostatic binding energy. The discrepancy may come from three aspects. First, it is obviously known that electrostatic binding energy of Cl^- is concentration dependent. The Cl^- concentration and solution pH gradient have significant influence on transport rate and staying time of the chloride ion. Heat release measurement is carried out under the condition of Cl^- concentration varying from 5 mM to 200 mM. The model used for calculating average binding energy in the present study is in the environment with constant Cl^- concentration of 300 mM. Second, in the calculation we have taken the average value of the electrostatic binding energy at S_{cen}+S_{int} (WT), S_{ext}+S_{cen}+S_{int} (E148A/E148Q mutants) as the equivalent of heat release. This implies that chloride ions bound to any binding site have an equal chance. However, chloride ions may prefer to bind to certain sites rather than bind to other sites in the wild-type ClC-ec1 protein and its mutants. Third, the experimental data of heat release were obtained by using the isothermal titration calorimetry to measure the energy differences with different Cl^- concentrations. Since Cl^- binding is likely to induce conformational change to ClC-ec1 and because the extent of the conformational change is likely to depend on the Cl^- concentration, the measured energy difference may consist of the Cl^- binding affinity (dominant) and a modicum of other contribution such as the conformational change effect. Therefore, deeper relationship between the calculated average electrostatic binding energies and the measured heat release data of Cl^- binding affinity needs further investigation.

In summary, we have analyzed the Cl^- binding properties of ClC-ec1 protein based on the all-atom MD simulation. According to the quantitative comparison between measured heat release data and our calculated average electrostatic binding energy, we suggest that perhaps chloride ions prefer to bind to S_{cen} than S_{int} in the wild-type ClC-ec1 structure. In the mutant E148A/E148Q structures, chloride ions prefer to bind to S_{ext} and S_{cen} than S_{int}. Even though the chloride ions bound to S_{int} make less contribution to heat release and they are relatively unstable in the Cl^- pathway, they are still part of the heat release contributors. Compared with electrostatic binding energies, the Cl^- binding free energies along the channel can provide more detailed information about Cl^- functional transport. A more rigorous free energy calculation over the MD trajectories of ClC-ec1 protein should be investigated in the future by using effective approaches such as thermodynamics integration approach, adaptive biasing forces (ABF) method, steered molecular dynamics simulation method, and so on.

Methods: The biological function of ClC proteins is mainly expressed as ionic transport through its pathway which is related to the binding property between Cl^- ion and protein. Therefore, the functional role of ClC proteins can be investigated by calculating binding energy of ions at the binding sites. Based on this theory, we calculate the electrostatic binding energy of the chloride ions (S_{cen}+S_{int} for the WT structure, S_{ext}+S_{cen}+S_{int} for E148A and E148Q mutant structures) in chains A and B. Before getting the electrostatic binding energy, electrostatic potential of the equilibrated protein-membrane-water systems must be calculated. The distribution of electrostatic potential Φ can be given by solving the linear Poisson–Boltzmann equation[26,27]

$$\nabla \cdot [\varepsilon(r)\nabla\Phi(r)] - \kappa^2(r)\Phi(r) = -4\pi \sum_i^N q_i \delta(r - r_i), \quad (1)$$

where q_i and r_i denote the charge and coordinate of the ith atom, N is the number of atoms in the whole system, $\kappa(r)$ is the Debye–Huckel ionic screening factor, $\varepsilon(r)$ denotes the position-dependent dielectric constant.

The above Poisson–Boltzmann equation was solved by soft APBS, in which a finite element method with discrete grid and the automatically-configured sequential computation method are used.[28] The APBS parameter settings are taken as follows: the number of grid points is $161 \times 161 \times 129$, the coarse grid region size is $130\,\text{Å} \times 130\,\text{Å} \times 110\,\text{Å}$ and the focusing fine grid region is $60\,\text{Å} \times 60\,\text{Å} \times 60\,\text{Å}$ with grid size $0.375\,\text{Å} \times 0.375\,\text{Å} \times 0.469\,\text{Å}$ in calculating Cl^- binding free energies. The dielectric constant ε is partitioned into different values in two regions: $\varepsilon = 4$ for ClC-ec1 protein and membrane, and $\varepsilon = 78$ for the surrounding water-like solution system. The focusing procedure is repeated for eight different locations of the system relative to the center of the 3D-grids (shifted half the fine grid spacing in each direction), and the resulting values are averaged. The total electrostatic energy of a system is then defined as

$$\Delta G_{\text{elec}} = \frac{1}{2} \sum_i^N q_i \Phi(r). \quad (2)$$

The electrostatic binding energy $\Delta G_{\mathrm{b,elec}}$ of the ith ion is defined as

$$\Delta G_{\mathrm{b,elec}} = \Delta G^{\mathrm{is}}_{\mathrm{elec}} - (\Delta G^{\mathrm{s}}_{\mathrm{elec}} + \Delta G^{\mathrm{i}}_{\mathrm{elec}}), \qquad (3)$$

where $\Delta G^{\mathrm{is}}_{\mathrm{elec}}$, $\Delta G^{\mathrm{s}}_{\mathrm{elec}}$, and $\Delta G^{\mathrm{i}}_{\mathrm{elec}}$ represent the electrostatic energies of the ion-occupied system, isolated ion-unoccupied system, and isolated ion in the solvent environment, respectively.

We would like to thank Yingying Zhuang at University of Washington for giving us many good suggestions in manuscript writing.

References

[1] Accardi A and picollo A 2010 *Biochim. Biophys. Acta* **1798** 1457
[2] Jentsch T J 2008 *Crit. Rev. Biochem. Mol. Biol.* **43** 3
[3] Miller C 2006 *Nature* **440** 484
[4] Jentsch T J, Neagoe I and Scheel O 2005 *Curr. Opin. Neurobiol.* **15** 319
[5] Achcroft F, Gadsby D and Miller C 2009 *Philos. Trans. R. Soc.* B **364** 145
[6] Lourdel S, Grand T, Burgos J, González W, Sepúlveda F V and Teulon J 2012 *Eur. J. Physiol.* **463** 247
[7] Faraldo-Gómez J D and Roux B 2004 *J. Mol. Biol.* **339** 981
[8] Maduke M, Pheasant D J and Miller C 1999 *J. Gen. Physiol.* **114** 713
[9] Accardi A, Kolmakova-Partensky L, Williams C and Miller C 2004 *J. Gen. Physiol.* **123** 109
[10] Dutzler R, Campbell E B, Cadene M, Chait B T and MacKinnon R 2002 *Nature* **415** 287
[11] Dutzler R, Campbell E B and MacKinnon R 2003 *Science* **300** 108
[12] Accardi A and Miller C 2004 *Nature* **427** 803
[13] Ko Y J and Jo W H 2010 *Biophys. J.* **98** 2163
[14] Accardi A, Lobet S, Williams C, Miller C and Dutzler R 2006 *J. Mol. Biol.* **362** 691
[15] Walden M, Accardi A, Wu F, Xu C, Williams C and Miller C 2007 *J. Gen. Physiol.* **129** 317
[16] Nguitragool W and Miller C 2007 *Proc. Natl. Acad. Sci. USA* **104** 20659
[17] Kuang Z, Liu A and Beck T L 2008 *Proteins* **22** 75
[18] Kuang Z, Mahankali U and Beck T L 2007 *Proteins* **68** 26
[19] Jayaram H, Accardi A, Wu F, Williams C and Miller C 2008 *Proc. Natl. Acad. Sci. USA* **105** 11194
[20] Miller C and Nguitragool W 2009 *Philos. Trans. R. Soc.* B **364** 175
[21] Picollo A, Malvezzi M, Houtman J C D and Accardi A 2009 *Nat. Struct. Mol. Biol.* **16** 1294
[22] Picollo A, Xu Y, Johner N, Bernèche S and Accardi A 2012 *Nat. Struct. Mol. Biol.* **19** 525
[23] Humphrey W, Dalke A and Schulten K 1996 *J. Mol. Graphics* **14** 33
[24] Mackerell Jr A D, Bashford D, Bellett M, Dunbrack R L, Evanseck Jr J D, Field M J and Fischer S 1998 *J. Phys. Chem.* B **102** 3586
[25] Phillips J C, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, Chipot C, Skeel R D, Kale L and Schulten K 2005 *J. Comput. Chem.* **26** 1781
[26] Davis M E and McCammon J A 1990 *Chem. Rev.* **90** 509
[27] Honig B and Nicholls A 1995 *Science* **268** 1144
[28] Holst M and Saied F 1993 *J. Comput. Chem.* **14** 105