Molecular basis of CLC antiporter inhibition by fluoride

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Supporting Information Placeholder

ABSTRACT: CLC channels and transporters conduct or transport various kinds of anions, with the exception of fluoride that acts as an effective inhibitor. Here, we performed sub-ns DFT-based QM/MM simulations of the *E. coli* anion/proton exchanger ClC-ec1 and observed that fluoride binds incoming protons within the selectivity filter, with excess protons shared with the gating glutamate E148. Depending on E148 conformation, the competition for the proton can involve either a direct F^-/E148 interaction or the modulation of water molecules bridging the two anions. The direct interaction locks E148 in a conformation that does not allow for proton transport, and thus inhibits protein function.

The CLC family encompasses anion channels and anion/proton exchangers across the three kingdoms of life [1-6] and fulfills various cell functions. Human CLC channels and transporters contribute to the regulation of cellular excitability, epithelial ion transport or Cl^- and pH homeostasis in intracellular organelles.[7-9] Mutations in genes encoding these proteins cause a variety of diseases, including muscle overexcitability, deafness, epilepsy, intellectual disability, nephrolithiasis and osteopetrosis. [10-11] The significant physiological importance as well as the intriguing co-existence of voltage-gated anion channels and anion/proton transporters in one gene family makes the CLC family a highly interesting topic for studying the chemical basis of transmembrane ion transport.

The Cl^-/H^+ antiporter ClC-ec1 was the first member of the CLC family that was studied by X-ray crystallography.[12-13] The protein mediates the transmembrane exchange of Cl^- for H^+ with a 2:1 stoichiometry.[14] Anion/proton exchange occurs in a permeation pathway limited by two glutamates, one pointing towards the intracellular side (E203, the so-called proton glutamate)[12, 15] and the other toward the extracellular side (E148, the so-called gating glutamate).[12, 14, 16-17] Protons from the cytosol bind the carboxyl group of E203 and subsequently reach E148 via a water wire[18-21] between these two residues. After protonation, E148 rotates outward and thus exposes itself to the external side of the channel (from a *down* to an *up* conformation), to release the proton to the extracellular side and to open a permeation pathway that allows for chloride transit.[12, 22-23] CLC anion channels and transporters allow for the transport of various anions, with significant permeability not only for Cl^-, but also for larger and polyatomic anions, such as Br^-, I^-, NO_3^- and SCN^-.[24-28]

Transport of F^- anion, which is smaller than Cl^-,[29] has not been extensively studied across the CLC proteins. Intriguingly, it is negligibly permeant through CLC-1 and CLC-2[25, 30] and it inhibits anion/proton exchange in ClC-ec1.[25, 31-33]

Indeed, flux assays and current measurements show that reconstituted transporters do not transport F^-. Fluoride efflux from CLC-ec1-containing liposomes is indistinguishable from protein-free liposomes,[33] while other anions can pass the protein-containing liposomes even without exchange with H^+.[27] Neutralization of the gating glutamate by mutation to Ala permits high F^- conductance and effective F^- equilibrium binding. [33] In contrast, mutants that only disrupt the anion pathway's inner gate (Y445A) or impair H^+ binding from the cytoplasmic side (E203Q) are still highly selective for Cl^- over F^-.[33-34] Hence, F^- inhibition is likely to be caused by specific F^--H^+ interactions at the central binding site, rather than by a strong Cl^-/ F^- selectivity of the anion conduction pathway.

X-ray studies on E148Q ClC-ec1 show that Q148 (in *down* conformation) directly interacts with the F^- within a hydrogen bond distance.[33] A neutral E148 in *down* conformation could keep F^- blocked in the protein binding site through a strong hydrogen bond interaction, whereas protonation of E148 triggers the *down/up* transition and hence proton release to the extracellular side with chloride as main anion and in absence of fluoride.[18, 22]

Here, we investigate the molecular basis of fluoride inhibition on the ClC-ec1 transport cycle by multiscale molecular simulations. Our model system consists of the CLC-ec1 X-ray structure,[12] embedded in a POPC[35-36] bilayer in the presence of counterions (Fig.1), in which F^- replaces the Cl^- in the central binding site in both the subunits. Our project takes advantage of a recently developed, massively parallel DFT-based QM/MM interface between the CPMD and GROMACS codes[37-38] that was run on the computational facilities at the Juelich Supercomputing Center. Overall, this has allowed us to model 16 ps of DFT-based QM/MM molecular dynamics (MD) and 455 ps of well-tempered metadynamics (MTD[39]) free energy calculations[37-38] (at the B3LYP[40-41] and BLYP levels of theory, respectively). Since the crystallographic structure lacks water molecules inside the channel, we obtained the average solvation around the anion within the transporter core through 300 ns of classical MD simulations using the CHARMM force field. [42]

The central region is hydrated with up to 12 water molecules, forming a continuous water chain connecting E203 and E148 (Fig. 1A and S1). The E148 side chain (in its deprotonated state) does not change conformation during the simulation time (Fig. S2).

On average, two water molecules lie in between E148 and F^- and four solvent molecules coordinate the fluoride in its binding site, where Y445 and S107 side chains complete the coordination around the anion with either direct or water-mediated H-bonds. (Figure S1 and S2).

As mentioned above, the proton transfer (PT) from E203 to E148 is a crucial step in ClC-ec1 proton transport.[19, 22]

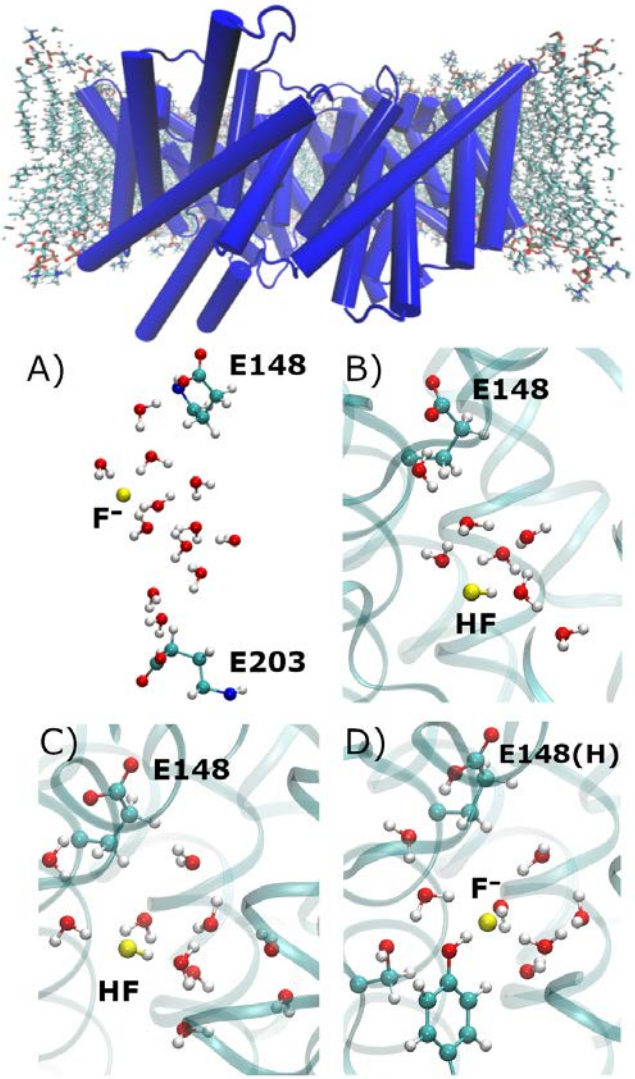

FIGURE 1. QM/MM MD simulations at B3LYP/CHARMM level of theory for different initial proton locations. **Top panel** *E. Coli* CLC antiporter (CLC-ec1) in a lipid bilayer. Water and counterions are not shown for clarity. **Bottom panel A)** Snapshot from our classical MD simulation. Here twelve water molecules form a hydrogen bond network connecting E203, E148 and F^- (yellow sphere). The excess proton is added to different water molecules in five simulations. Only three are shown here. **B)-D)** QM/MM configurations after 0.5 ps. The proton, after hopping through a chain of water molecules, has already formed either H-F (B-C) or protonated E148 species (D). The starting configurations, as well as the other two trajectories, are depicted in Fig. S3A-E. The QM region includes F^-, E148, R147, hydronium, the water molecules connecting them, and in **D)**, also Y445 and S107.

We performed five independent QM/MM MD simulations, in which one proton is added in different positions of the water network connecting F^-, E203 and E148 (Fig. 1A). In all circumstances, PT processes via the water wire occur already within less than 1 ps, either to E148 or to F^-, leading to the formation of HF. The resulting acids

(protonated E148 and HF) do not dissociate afterwards (Fig. S3A-E). No significant conformational changes of E148 occurred over the remaining simulation time. The formation of a stable HF molecule is fully consistent with the well-known halogens' acid-base properties: HF dissociation in water is rather unfavorable (ΔG=4.3 kcal/mol), while this is not the case for other binary acids including HCl (ΔG= -9.5 kcal/mol[29]). However, in case of Cl^- ion, the PT process toward the E148 could include a transient state in which the proton binds the chloride for a short time,[43] while a direct interference of the F^- with the excess proton has only been hypothesized so far.[44]

Next, we investigate whether E203 may affect these PT pathways. By including E203 and its hydration sphere in the QM region, our QM/MM simulations mostly reproduced the same results as above. Yet, in one QM/MM simulation, the proton does migrate to E203 (Fig. S4E). We thus conclude that, in the presence of fluoride, E203 only serves as part of the proton pathways towards the two negatively charged groups, while playing no role for fluoride inhibition as proton acceptor, as established by the experiments.[33]

To estimate the relative stability of the two states characterized by the proton bound to either the F^- anion or E148, we investigated the free energy landscape associated with the PT between the two anions with two intervening water molecules (Fig. 2A) via QM/MM MTD simulations. The free energy, as a function of chosen collective variables (the fluoride- and E148-proton distances) shows two minima at d(H-F) = 1.0 Å, d(H-E148) = 1.7 Å, and d(H-F) = 1.6 Å, d(H-E148) = 1.0 Å, corresponding to the two protonation states (Fig. 2A). The minima are similar in free energy (~1 kcal/mol) and they are separated by a free energy barrier of a few kcal/mol[45] (Fig. S5). In this conformation, the proton goes back and forth, through two water molecules, from F^- to E148 in a kind of competition/sharing mechanism. The position of the E148 side chain may change depending on its protonation state.[18, 22] In particular, the calculations so far relied on the E148 crystallographic conformation, in which the carboxylic side chain is exposed to the external side of the permeation pathway (*up*). In contrast, E148 in its *down* conformation is exposed to the inner part of the channel, where it can interact with the anion. We therefore investigated the *down/up* conformational change by calculating the associated free energy as a function of E148's N-Cα-Cβ-Cγ (χ_1) torsional angle, which dictates the transition (Figure 2C and D). In both cases the anion is present in the binding site as F^- and HF when E148 is protonated and deprotonated, respectively.

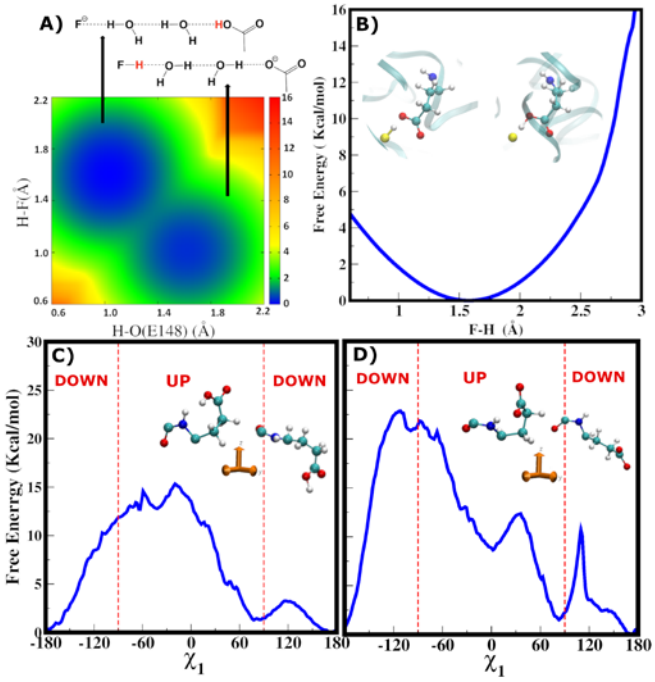

FIGURE 2. Free energy profiles (in kcal/mol) emerging from QM/MM MTD simulations at BLYP/CHARMM level of theory. **A)** Free energy associated with the PT process between fluoride and E148 modulated by two water molecules. The schematic representation of the two free energy minima is displayed on the top of the figure. Here, E148 is in the *up* conformation. **B)** Free energy profile associated with the direct PT process between F^- and E148 in *down* conformation. The minimum is reached at H-F and H-O(E148) (Fig. S8D) distances of 1.5 Å and 1.0 Å respectively. No barrier separates the state in which the proton is bound to the fluoride (H-F 1.0 Å). **C-D)** Free energy as a function of E148's χ_1 dihedral angle for E148 protonated **C)** and deprotonated **D)**. The relative positions of E148 carboxyl group above or below the backbone unit define the *up or down* conformations. The transition from one conformation to the other is indicated by dashed red lines.

In the presence of F^- anion, the *down* conformation is far more favored than the *up* one regardless of the protonation state, in agreement with previous calculations.[23, 46] In addition, *up* is a local minimum only if E148 is deprotonated (Fig. 2C and 2D). Upon protonation, the *up* → *down* transition is actually a barrier-less process. There is no longer a minimum near the *up* conformation region.

Once exposed to the internal side of the channel, E148 can form a direct hydrogen bond with F^-. To investigate the nature of this interaction, we performed a MTD-based free energy calculation using the H-F distance as a collective variable. It turns out that the proton is fully shared by fluoride and E148, as shown by the presence of a single minimum in the H-F free energy (Fig. 2B). This may be consistent with the similarities of the pKa value of Glu (4.2)[47] and HF (3.2)[29]. While this direct interaction

was invoked already as the key structural determinant for the inhibition mechanism,[33] our findings point to a much more complex scenario than a simple H-bond interaction: the H-F distance ranges between 1 Å (HF-E148) and 2 Å (F- HE148, Fig. 2B).

Bringing the proton from its complex with E148 and F^- to the bulk requires the *down* → *up* transition of the protonated E148. The estimated free energy barrier of this transition (~15 kcal/mol, Fig. 2C) is much higher than the corresponding one for chloride (5 kcal/mol).[22] The inverse (*up* → *down)* process is basically barrier-less (Fig. 2C), leading to the rather stable[48] F-H-E148 triad structure (Fig. 2B).

These considerations lead us to suggest the following mechanism of inhibitions: protons coming from the extracellular side will migrate spontaneously to the protein cavity and there will be trapped there by E148 (in *down* conformation) and F^- (Fig. 2B). This explanation is consistent with the available experimental data on CLC-ec1, from the formation of a fluoride-Gln H-bond in the E148Q mutant to the fact that the E148A variant allows for F^- transport.[33]

In conclusion, we here identify the high affinity of both F^- and E148 for protons as the basis of the transport inhibition of the CLC anion/proton exchangers from *E.coli*. Our hypothesis predicts impaired fluoride inhibition of CLC channels that lack a glutamate at this position, like the renal CLC-K,[49] and restored block in mutant channels with re-inserted glutamate.[50] The comparative analysis of fluoride inhibition in multiple CLC channels and transporters, some of which differ in binding affinity and selectivity of binding sites within the anion transport pathway,[51] may identify additional determinants of fluoride block across this important anion channel/transporter family.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the ACS Publications website.

Computational details for the system setup, classical MD, QM/MM and QM/MM MTD simulations. Hydration and structural analysis of the MD simulations. Snapshots of the QM/MM dynamics illustrating the proton pathways. Convergence of the FE profiles. Population analysis. (PDF)

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The authors declare no competing financial interest.

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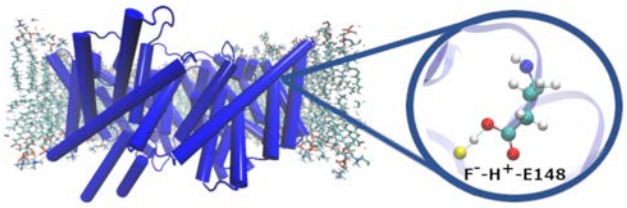
F⁻-H⁺-E148