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Chloride and Proton Binding in the E. coli 2Cl⁻:1H+ CLC Exchanger

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Chloride and proton binding in the 
E. coli 2Cl⁻:1H⁺ CLC exchanger

by

CATHERINE CHENAL

Dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of 
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CATHERINE CHENAL

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry to satisfy the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Chloride and proton binding in the E. coli CLC exchanger

by

CATHERINE CHENAL

Adviser: Professor Marilyn Gunner

The CLC family of membrane proteins is a ubiquitously expressed class of proton and usually voltage-activated chloride transporters involved in a myriad of physiological functions. Crystallographic structures identify up to three chloride binding sites: external, central and intracellular located in the inner half of the trans-membrane domain. The CLC proteins, except for the kidney isoforms, are gated by the extracellular side-facing gating Glutamate (E₁₄₈ in CLC-ec1, the E. coli exchanger), which is thought to undergo a conformational change upon protonation.

To sort out how the thermodynamic paths to H⁺ coupled Cl⁻ binding and conformational change in CLC-ec1 at the extracellular site are related, we constructed possible transport cycle intermediates with varied Eₓ conformations and used Multi-Conformational Continuum Electrostatics (MCCE) to simulate ligand titrations.

We find that Cl⁻ binding is strongly coupled to H⁺ binding at the external site; the Cl⁻ affinity is linearly correlated with the proton affinity, whose range is about 11 kcal/mol while the
range for the Cl\textsuperscript{−} affinity is about 20 kcal/mol, ensuring Cl\textsuperscript{−} binding and release with minimal protein rearrangement. The wide-ranging apo proton-affinity of E\textsubscript{x} is almost entirely controlled by its interaction energy with the protein backbone and is conformation-dependent: the outward open conformation is where the backbone interaction energy with E\textsubscript{x} is minimal and proton affinity is maximal. The 'helix cage' topology of the two sites in the selectivity filter, characterized by the electropositive hotspot resulting from the abutting of the N-termini of helix F and N, enables the binding of two negative charges and explains how two Cl ions can be bound within ~4 Å of each other, in spite of strong ion-ion repulsion.

Chloride binding data for the central site show that the central site affinity is not correlated to the E\textsubscript{x} apo pK\textsubscript{a}. We find that this site has a lower affinity than the exterior site. While Cl\textsuperscript{−} binding at the central site is also stabilized by backbone elements, it is hindered by rearrangements of S107 and Y445 that are often necessary upon Cl binding.

The main significance of this study of H\textsuperscript{+} and Cl\textsuperscript{−} binding in occluded structures resides in showing that binding at the external site initiates the H\textsuperscript{+}/Cl\textsuperscript{−} coupling and that the higher proton affinity of the gating residue once it leaves the helix cage enables two Cl ions to bind in close proximity. Thus this study provides atomistic details of the experimentally measured 2Cl\textsuperscript{−}:1H\textsuperscript{+} stoichiometry of binding in CLC-ec1.
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I would like to thank my committee members, Drs. Alessio Accardi, Ranajeet Ghose, Themis Lazaridis and Shaneen Singh for agreeing to be on my PhD dissertation committee and for their valuable feedback.

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INTRODUCTION

Biological cells and the compartments within are primarily identified by their membranes. Because the permeability of charged species across phospholipid membranes is very low, specific ion-selective transport proteins have evolved in order to control ion fluxes as needed.

1. Transport proteins: overview and nomenclature

One direct end-result of cellular respiration is the buildup of a proton concentration gradient of across the nearly charge-impermeable phospholipid bilayer membrane. This primary chemical potential is converted into the high energy bonds of a soluble species, typically ATP, which is referred to as the energy currency of the cell. Primary-active transporters, called pumps, are ATP-dependent transporters. In contrast, secondary-active transporters utilize the potential energy stored in transmembrane gradients to drive solute transport. A distinction is made between the channels, which only transport ions down their concentration gradient, and the co-transporters, which utilize the downhill gradient of one species to transport another. Co-transporters are called exchangers if the transported species are carried to opposite sides of
the membrane, else they are called symporters. Transporters are electrogenic if they transport a net charge across the membrane.

2. **Family portrait**

The **Chloride Channel (CLC)** family encodes ubiquitously expressed, homodimeric membrane proteins that are proton- and/or voltage-activated (Miller and White, 1984; Middleton et al., 1994; Ludewig et al., 1996; Maduke et al., 1999; Weinreich and Jentsch, 2001; Dutzler et al., 2002) (**Figure I**). In contrast with other channels such as Kcsa (Doyle et al., 1998), each CLC transporter subunit contains an independent conduction pore (Hanke and Miller, 1983; Ludewig et al., 1997; Dutzler et al., 2002; Zdebik et al., 2008; Robertson et al., 2010).

The CLC family of transport proteins includes both channels and exchangers\(^1\) (Accardi and Miller, 2004). The two subgroups have a low sequence identity (Accardi, 2015), but share a transmembrane fold (Estevez et al., 2003; Lin and Chen, 2003) that selects for mono-valent anions (Rychkov et al., 1998; Hebeisen et al., 2003; Accardi and Picollo, 2010), hence for Cl\(^-\), the physiologically prominent halide ion (Fahlke, 2001). Cl\(^-\) is transported down its electrochemical gradient by the channels sub-class. In the exchangers, protons (or Cl\(^-\) ions) can be pumped up their concentration gradient using the chemical potential of Cl\(^-\) ions (or or protons) (Stauber et al., 2012).

\(^1\) This is not a CLC family oddity: the SLC26 family of anion transporters with totally different functions does so as
Subsequent to the initial identification of the CLC family by cloning of CLC-0 from the electric ray (Jentsch et al., 1990), nine mammalian CLC genes were discovered. The channels subclass include CLC-1, CLC-2, and two kidney isoforms, CLC-Ka and CLC-Kb, while the five others, CLC-3 through CLC-7 are electrogenic exchangers all of which have a $2\text{Cl}^\,-:1\text{H}^+$ stoichiometry (Scheel et al., 2005; Picollo and Pusch, 2005; Neagoe et al., 2010; Graves et al., 2008; Leisle et al., 2011). CLC channelopathies result in kidney stones, deafness, blindness, epilepsy, osteopetrosis, lysosomal storage disease and defective endocytosis (Jentsch, 2008), and the dysfunction of CLC-3 is responsible for impaired cell migration (Schwab et al., 2012).

The major sources of the structure-function insights about CLC exchangers have come from the bacterial homolog structures of *S. enterica serovar typhimurium* and *E. coli* (Dutzler et al., 2002; Dutzler et al., 2003). These structures revealed that the conserved CLC protein fold (Estevez et al., 2003; Lin and Chen, 2003) results in a complex topology of 17 transmembrane helices per subunit (Dutzler, 2007), which are bent by at least 45° with respect to the membrane normal (Figure I1). Thus, the fold is conserved across phyla despite a very low overall sequence identity of about 20% (Mindell and Maduke, 2001) (Figure A9 in Appendix). There is to date no structural information on any mammalian exchanger. However, the truncated structure of the thermophilic red algae *C. merolae* comes closest since it is a Eukaryote (3ORG, Feng et al., 2010). It retains the topology of the other exchangers.
3. *CLC proteins* transport modes differ with location

The channel and exchanger subclasses of CLC segregate into different membrane systems. The channels are found in the plasma membrane where they maintain ion homeostasis, provide trans-epithelial transport, and control the excitability of muscles or neurons (Stauber et al., 2012). The Cl\(^{-}\)/H\(^{+}\) exchangers are found in intracellular/vesicular membrane systems where they provide a key role in the acidification of the endosomal and lysosomal lumen (Kornak et al., 2001; Jentsch, 2008). As the Cl\(^{-}\)/H\(^{+}\) exchangers transport two Cl ions in the opposite direction from one proton they transfer three charges across the membrane in each cycle. Cl\(^{-}\)/H\(^{+}\) exchangers in neutrophilic bacteria enable robust proton extrusion during an extreme acid challenge (Iyer et al., 2002). The related plant exchangers reside in the leaf vacuoles and exchange H\(^{+}\) with nitrate (De Angeli et al., 2006) via a single amino acid difference in the selectivity filter of the Cl\(^{-}\)/H\(^{+}\) exchanger (S107 to P107).

As the CLC exchangers are found wherever (de)acidification must occur, it was initially thought that they helped increase the ATPase mediated H\(^{+}\) accumulation in vesicles by the pumping of Cl ions (Estevez et al., 2003; Picollo and Pusch, 2005; Scheel et al., 2005), however, two recent *in vivo* studies have shown that the electrogenic nature of CLC-5 and CLC-7 is a functional feature for proper renal endocytosis (Novarino et al., 2010) and for normal lysosomal storage and bone resorption (Weinert et al., 2010), respectively. Thus, understanding how the coupling between Cl\(^{-}\) and H\(^{+}\) takes place is a key step in the development of drugs aimed to restoring it.
4. *Coupling and modulation*

The CLC exchangers maintain coupled, electrogenic ionic fluxes with a $2\text{Cl}^{-}:1\text{H}^+$ stoichiometry\(^2\)\(^\text{(Accardi et al., 2004; Accardi and Miller, 2004)}\), resulting in the net transport of three charges across the membrane. Most CLC exchangers are voltage-activated\(^\text{(Accardi and Miller, 2004; Smith and Lippiat, 2010; Stauber et al., 2012)}\), as seen by their allosteric response to voltage changes (characterized by a sigmoid i-V curve). In contrast, the E. coli exchanger (CLC-ec1) has an ohmic current-voltage curve (i.e. displays a linear response to voltage changes), so is not voltage-activated\(^\text{(Accardi et al., 2004; Yin et al., 2004)}\).

The exchangers $2\text{Cl}^{-}:1\text{H}^+$ stoichiometry of transport is independent of pH and of the varied transport rates among the CLC proteins\(^\text{(Accardi and Miller, 2004; Zdebik et al., 2008; Jayaram et al., 2011)}\). Five of the nine human CLC proteins (CLC-3 through CLC-7) are electrogenic exchangers\(^\text{(Scheel et al., 2005; Picollo and Pusch, 2005; Neagoe et al., 2010; Graves et al., 2008; Leisle et al., 2011)}\), while the other four are channels.

Early on, the CLC *channels* were found to be modulated by protons\(^\text{(Hanke and Miller, 1983)}\), but two more recent studies have found that they also export $\text{H}^+$ (without a fixed stoichiometry), and that this $\text{H}^+$ transport is the source of energy for a gating cycle asymmetry\(^\text{(Lísal and Maduke, 2008; Lísal and Maduke, 2009)}\). Hence, channels may be derived from a putative ancestral exchanger protein, retaining some antiport function which is now uncoupled from $\text{Cl}^{-}$ transport\(^\text{(Chen and Hwang, 2008; Miller, 2006; Niemeyer et al., 2009)}\).

\(^2\) The one exception is the 1:1 fluoride exchanger\(^\text{(Stockbridge et al., 2012)}\)
5. **Gating and modulation**

'Gating' refers to the pore opening events leading to conduction and the pore closing events leading to non-conduction {Hille, 1992}. Gating, as revealed by conductance patterns in most CLC proteins, is modulated by pH, the permeant anion and transmembrane voltage with activation by high [H+]_{out}, high [Cl]_{out} and depolarizing voltage; and with a closing rate that is [H+]_{in} dependent {Miller, 2006; Zifarelli and Pusch, 2010}. There are two exceptions to this generic modus operandi: CLC-5 is inhibited at low pH_{ext} {Picollo et al., 2010}, and CLC-2 opens upon hyperpolarization {Jentsch, 2015}. Considering the multiple ways to combine the three binary variables modulating the CLC channels (voltage (+/-), [Cl]_{out/in}, [H+]_{out/in}), the assignment of a dependence to a specific change in the protein is a complex task {Hanke and Miller, 1983; Zifarelli et al., 2008; Niemeyer et al., 2009; Zifarelli et al., 2010}.

6. **Binding sites and key residues**

Each subunit of the CLC protein transports Cl⁻ through a conduction pathway indicated in x-ray structures by three Cl⁻ binding sites: Sₓ, Sᶜ and Sᵢ, for external, central and intracellular site, respectively {Dutzler et al., 2003} (Figure 1). The binding affinity of the exchangers has been measured *in crystallo* {Lobet and Dutzler, 2006} and *in vitro* {Picollo et al., 2009; Picollo et al., 2012} yielding affinities in the millimolar range for Sₓ and Sᶜ and at least ten-fold lower affin-
ity for $S_i$, the intracellular site. The higher $K_d (> 20 \text{ mM})$ of $S_i$ provides a basis for its exclusion from a mechanistic role in the exchange {Picollo et al., 2009; Picollo et al., 2012}.

The selectivity filter is the short rectilinear portion of the path that includes $S_x$ and $S_c$. It is gated by specific residues on each end. This is expected: channels only need one gate to control downhill flux {Hille, 1992}, but an exchanger needs at least two gates to effect the transport conformational cycle {Jardetzky, 1966; Forrest et al., 2011; Drew and Boudker, 2016}. For an exchanger, the affinity for Cl¯ must vary through the reaction cycle {Jardetzky, 1966; Forrest et al., 2011}. For a proton coupled exchanger, this anion affinity must somehow be coupled to the changing proton affinity of at least one group.

*The extracellular side gating residue: $E_x$*

$E_x$, the gating Glutamate (E148), is common to channels and exchangers alike (except for the kidney isoform) in all the sequences inspected (Appendix Figure A9 {Altschul et al., 1997; Altschul et al., 2005}) and is a key residue of the external gating mechanism {Traverso et al., 2003}. It blocks $S_x$ by moving in and out of the binding site. $E_x$ is the proton acceptor that controls gate opening and transport on the extracellular side {Dutzler et al., 2003; Accardi et al., 2005; Scheel et al., 2005; Jayaram et al., 2008; Zdebik et al., 2008; Zifarelli et al., 2012}. It also confer the protein its main pH dependence of Cl¯ transport {Niemeyer et al., 2003; Accardi and Miller, 2004; Bell et al., 2006; Zdebik et al., 2008; Garcia-Celma et al., 2013}. Thus, in the Alanine and Glutamine mutants (E148A, E148Q {Dutzler et al., 2003}) H⁺ transport is abolished but Cl¯ conduction is maintained {Accardi and Miller, 2004}. $E_x$ is common to channels and ex-
changers alike (except for the kidney isoform) with ~80% identity {Mindell and Maduke, 2001} (Figure A9 in Appendix).

Parenthetically, the existence of a single titratable group involved in the transport and binding processes had been long ago suggested by electrophysiological characterizations of CLC channels {Hanke and Miller, 1983; Rychkov et al. 1996; Ludewig et al. 1997; Fahlke, 2001; Chen and Chen 2003}; the bacterial structures provided a hard proof.

**The intracellular side gating residues: Ser_c and Tyr_c**

Ser_c and Tyr_c, the central Ser and Tyr residues (S107 and Y445), which coordinate the central site, were identified as the inner gate in CLC-ec1 and all other exchangers {Accardi and Miller, 2004; Accardi et al., 2006; Jayaram et al., 2008; Basilio et al., 2014}. Both residues can occlude S_c {Lobet and Dutzler, 2006; Jayaram et al., 2008; Accardi et al., 2006; Basilio et al., 2014}.

Tyr_c is responsive to conformational changes of remote helical domains {Basilio et al., 2014}, implying that it may be the key residue for coordinating domain movements to pore opening on the intracellular side. Residues with small side-chains mutated at this position uncouple or degrade the 2:1 stoichiometry are small residues {Walden et al., 2007}. Although conserved in channels, the central Tyrosine was found ineffective at blocking methane thiosulfonate reagents in CLC-0 {Lin and Chen, 2003}, strongly implying that only the external gate was at play in the channel sub-class.
Ser c can switch the selectivity from Cl\(^-\) to NO\(_3^-\) when mutated to Proline {Zifarelli and Pusch, 2009} as found in the A. thaliana exchanger {De Angeli et al., 2006}. No other residue has been identified with roles in selectivity.

_The intracellular side ‘proton glutamate’ E\(_i\)_

Most exchangers also rely on a ‘proton glutamate’ E\(_i\) (E203), on the intracellular side {Accardi et al., 2005; Zdebik et al., 2008; Grieschat and Alekov, 2012} for coupled exchange. The mutant E203Q no longer transports H\(^+\) (and in the case of CLC-5, this same mutation also abolishes the Cl\(^-\) flux, attesting to subtle variation of unknown origins {Picollo et al., 2010}). The two key glutamates, E\(_x\) and E\(_i\) are over 13 Å apart: a giant leap for a proton. Several molecular dynamic simulations have investigated how protons might transfer between the two Glutamic acids. One way is to enable proton hopping by the the Grotthius mechanism via water wires. Simulations show this is feasible over the full distance between the key Glutamates {Han et al., 2013}, or over a reduced distance when E\(_i\) side-chain rotates towards E\(_x\) {Wang and Voth, 2009}. However, in spite of the importance of this ionizable residue in many exchangers, coupled exchange can be found with Threonine or Valine instead of E203, as in the C. merolae exchanger {Feng et al., 2010}, or Isoleucine as in the C. koseri bacterium {Phillips et al., 2012}. This indicates that the local environment, which includes strictly conserved E202, may enable proton capture and release.
7. **Site topology**

$S_x$ and $S_c$ are $\sim 4$ Å apart and located in an electropositive 'helix cage' \{Aqvist et al., 1991\} formed by the N-termini of helix F and N. $E_x$ also competes for this site (Figure I2) \{Dutzler et al., 2002\}. The stabilization motifs for $S_c$ comprise the backbone dipoles of GSGIPEI in helix D and LY in helix R, while those for $S_x$ comprise the dipoles of GR/KEGP in helix F and GxFxP in helix N (Figure I2). These sequences are at least 80% conserved \{Mindell and Maduke, 2001\}.

An early continuum electrostatics (CE) study noted, in passing, the significant contribution to ion stabilization from the backbone \{Faraldo-Gomez and Roux, 2004\}. An early MD simulation of Cl$^-$ conduction in CLC-ec1 \{Cohen and Schulten, 2004\} also noted the possible importance of the backbone elements. In contrast, another MD simulation \{Ko and Jo, 2009\} investigated the stabilization energy contributed by atoms in the coordination shell of Cl$^-$ at either $S_x$ or $S_c$ as the ions were found completely dehydrated as they passed through the filter. However, these authors analyzed the nearby atoms based their polarity in a way that downplayed the importance of the backbone. Nevertheless, none of these CE or MD investigations estimated the dependence of the sites Cl$^-$ affinity on their interaction with the backbone, nor the relationship between the helix cage and the stoichiometry of Cl and H transport.
The two closely spaced anionic binding sites are formed by the complex CLC protein fold, which is made up of an inverted topological repeat (ITR) whereby two structurally similar halves are rotated about 180° with respect to the other half (Figure I1). Thus, the structure of helices B through I is the rotated structure of helices J to Q in each monomer {Dutzler et al., 2002; Dutzler 2007}.

ITRs occur in several classes of secondary-active transporters and in other anion exchangers, such as AE1 of erythrocytes {Yamaguchi et al., 2010; Bonar et al., 2013} and the SLC26
family of electrogenic Cl⁻/bicarbonate exchangers {Ohana et al., 2011; Jentsch, 2015}. It enables the alternate-access mechanism {Forrest and Rudnick, 2009; Radestock and Forrest, 2011}.

8. Transport paradigm

The first proposed transport scheme, called the alternate-access mechanism {Jardetsky, 1966}, described the coupling of the gating events in the transport cycle of secondary-active transporters. Here, the substrate binding site(s) accessibility to either one side of the membrane or the other is achieved via coupled conformational changes of the extracellular and intracellular gates. Thus, initially, this mechanism implied anti-cooperative binding of the two substrates. Modern refinements of the Jaredtsky mechanism no longer support this de facto anti-cooperativity as many exchangers are found to bind both their substrate simultaneously {Drew and Boudker, 2016}. Indeed, this appears to be the case with the CLC exchangers, which bind Cl⁻ and H⁺ simultaneously and cooperatively {Accardi and Miller, 2004; Accardi et al., 2005; Accardi et al., 2006; Nguitragool and Miller, 2006}, with the same binding stoichiometry as that of transport {Picollo et al., 2012}.

One definitive feature shared by the several types of transport falling under the alternate-access paradigm {Drew and Boudker, 2016} is the necessity of conformational changes. Conformational changes are an integral part of gating and often can be divided into domain-level ('heavy gates', g_H) and residue-level ('light gates', g_L) conformational changes {Forrest et
Thus, in CLC exchangers, \( E_x \) and \( \text{Tyr}_c \) would be the light gates, while helical domains would be heavy gates.

**Figure I2. Conserved sequence motifs of the binding sites forming the selectivity filter**

The coordination cage of the external site is built by the \( E_x \) and F357 motifs located at the N-terminal end of helices F and N, respectively. The structure shown, \( E^{UP} \), uses the back-mutated Q148E in 1OTU as its starting structure, so that \( E_x \) is in the 'UP' orientation, that leaves the external site free to bind \( \text{Cl}^- \).

**9. Structural conformations derived from crystal structures of CLC**

The transmembrane portion of all crystallized CLC proteins is superimposable with a very small RMSD (i.e. about 0.6 Å\(^2\)). Thus, either the crystallization conditions so far used do not stabilize different conformations involving changes in entire protein domains that are habitually

---

\(^3\) The authors speak of 'thin' and 'thick' gates.
depicted in the alternate access mechanism, or that these changes are in fact more limited in the CLC exchangers. The least speculative inference we make about the common transmembrane domain is that it is a low energy conformation. Nevertheless, the known conformational states of E\textsubscript{x} stated above show the intrinsic lability of the gating residue side chain and suggest a link with its proton affinity.

**Figure I3. Gating residue conformations from x-ray crystals**

In the scheme of the alternate access mechanism, the E. coli WT structure represents a closed state, in which the sites S\textsubscript{c} and S\textsubscript{i} are filled with Cl\textsuperscript{−} {1OTS, Dutzler et al., 2003}. The known conformational changes in CLC proteins are shown in Figure I3: the conformations of E\textsubscript{x} side-chain is the major discriminant. A crystal structure of CLC-ec1 in the apo state {2EXZ, Lobet and Dutzler, 2006} shows E\textsubscript{x} charged side chain deeper into S\textsubscript{x} than in WT (Figure I4). The E\textsubscript{x}...
Glutamine mutant (1OTU) shows a conformational change ('UP') via the neutralized Gln side-chain swinging out of the $S_x$ site.

It is presumed that $E_x$ will undergo a similar conformation change upon protonation. This transition has been seen in computer simulations (Bostick and Berkowitz, 2004; Cohen and Schulten, 2004; Gervasio et al., 2006; Miloshevsky and Jordan, 2010), and via a combination of MD simulation and F-NMR (Khantwal et al., 2016). The later study also established remote domain motions linked to the protonation of $E_x$, a possibility that was discovered in a meta-dynamical simulation (Krivobokova et al., 2012).

The UP conformation results in an apparent ‘outward-open’ state that allows for an ion to bind $S_x$ in the crystal structure (1OTU, Dutzler et al., 2003). An additional conformation of the external gate is suggested by the sole Eukaryote structure to date, a thermophilic red alga C. merolae (3ORG, Feng et al., 2010) where $S_x$ and $S_i$ are bound and $E_x$ side chain extends into the apo central site in a ‘DOWN’ conformation.
Figure I4. Conformations in topological context

The coordination cage of the external and central sites is built by the E$_x$ (E148) and F$_x$ (F357) sequence motifs located at the N-terminal end of helix F (teal color; stick residues: L$_{145}$G$_{146}$R$_{147}$E$_{148}$G$_{149}$P$_{150}$xxV$_{152}$), and helix N (orange; stick residues: G$_{354}$G$_{355}$I$_{356}$F$_{357}$A$_{358}$P$_{359}$M$_{360}$). S$_c$ is additionally coordinated by the central Ser and Tyr residues, S107 and Y445 (not shown here, but in Figure SI.9). The meeting of these two N-termini at a mid-membrane location is the result of the inverted topology repeat of the CLC fold {Dutzler et al., 2002; Forrest and Rudnick, 2009}. APO: 2EXW, x-ray apo {Lobet and Dutzler, 2006}; WT: 1OTS, S$_x$ blocked by E$_x$ side-chain {Dutzler et al., 2003}; MID: modified 1OTS to move E$_x$ by ≈1.4Å to allow space for bound S$_x$; UP: back-mutated E148Q mutant 1OTU {Dutzler et al., 2003}. Green spheres depict the bound S$_x$ and S$_c$ sites, (S$_x$ in the foreground).

10. Open questions addressed in this study

In spite of extensive physiological experiments and computer simulations on the CLC exchangers, there persists a few nagging questions: How does the structure enable the two sites in the dehydrated selectivity filter to bind two anions within 4 Å of each other, meaning, how are the desolvation penalty and the ion-ion repulsion compensated? All prior studies have ei-
ther recognized the importance of the backbone interactions as the key element of Cl\textsuperscript{-} stabilization at the external and central sites without establishing any linkage with E\textsubscript{x} proton affinity. Thus we may ask: how do the interactions with the backbone affect the proton affinity of E\textsubscript{x} when no Cl\textsuperscript{-} is bound, and how is this linked to the gating function of E\textsubscript{x}.

Conformational changes in CLC proteins were detected throughout the transport cycle (Accardi and Pusch, 2003; Bell et al., 2006; Elvington et al., 2009; Miloshevsky et al., 2010; Krivobokova et al., 2012; Basilio et al., 2014; Abraham et al., 2015). Domain movements on the extracellular and intracellular sides have been detected either in vitro (Bell et al., 2006; Elvington et al., 2009; Basilio et al., 2014) or via simulation (Miloshevsky et al., 2010), but their role in determining the gating Glutamate conformational change is still unknown. This is so because the conformations of E\textsubscript{x} are the main feature differentiating the structures. Recall, E\textsubscript{x} is a multi-tasker: to enable Cl\textsuperscript{-} binding at the external site, E\textsubscript{x} has to move away, grab or lose a proton and, somehow, coordinate its movement away from the central site with the intracellular gate. To date, only the protonation of E\textsubscript{x} has been shown as the linkage between its conformational change and the occupancy of the external site (Dutzler et al., 2003; Bostick and Berkowski, 2004; Cohen and Schulten, 2004, Ko and Jo, 2009). Thus, we may ask several questions pertaining to the intrinsic energetics of the gating residue: what is its intrinsic pK\textsubscript{a} (in the apo protein), how its proton affinity changes with conformation and number of bound Cl ions, and what is the basis for a possible linkage between Cl\textsuperscript{-} and H\textsuperscript{+} binding.
While the Multi-Conformational Continuum electrostatic framework (MCCE) keeps the backbone protein fixed, it simulates proton and electron chemistry {Alexov and Gunner, 1997; Georgescu et al., 2002}, which to date cannot be done by standard molecular dynamics programs. Since ligand binding energies can also be estimated, it can provide insights into proton-coupled binding reactions.

The focus of this study is the characterization of Cl⁻ binding at the Sₓ and Sₐ sites along with that of the proton affinity (pKₐ) of Eₓ, the gating glutamate. We find that even the limited structural starting points we used yields an astonishing range of pKₐ in the apo state (ΔpKₐ~9). Our search for the energetic source for this range pointed to the backbone's electrostatic interaction energy as the dominant source. As Cl⁻ binding is coupled to Eₓ protonation, the range of Eₓ proton affinity contributes to the ability of the protein to control Sₓ affinity for Cl⁻. Our simulations lead to a step-wise mechanism of exchange with a 2Cl⁻:1H⁺ stoichiometry?
MATERIAL AND METHODS

1. Simulation with Multi-Conformation Continuum Electrostatic (MCCE)

MCCE is a structure-based, continuum electrostatic and molecular mechanics program {Alexov and Gunner, 1997; Georgescu et al., 2002; Song et al., 2009} that enables in silico titrations of protons {Alexov and Gunner, 1997; Georgescu et al., 2002}, electrons {Zheng and Gunner, 2008} and ions {Song and Gunner, 2009}. MCCE samples multiple side-chain conformations given a fixed-backbone structure {Song et al., 2009}. Monte Carlo (MC) sampling finds the Boltzmann distribution of residue conformation together with ligand binding, redox and protonation states to equilibrium at a given pH, ionic strength and ligand concentration. The proton affinity (pK_a) of each ionizable residue is determined from the resultant pH dependence of the residue's protonation as a function of pH, while the K_d of a ligand is determined by changing the chemical potential of the ligand in solution.

The electrostatics interactions in MCCE are calculated with DelPhi {Nicholls and Honig, 1991} which solves the linearized Poisson-Boltzmann equation. This equation encapsulate the requirements for modeling the equilibrium partitioning of charges in regions of different dielectric potential and the influence of mobile charges on the local potential at an implicit salt concentration of 150 mM. Membrane proteins are surrounded by an array of neutral atoms forming a low dielectric (ε=4) slab that mimics the hydrophobic core of a membrane bilayer. For CLC-ec1, the slab thickness is 26Å. The dielectric constant of protein, \( \varepsilon_p \), is 4, while it is 80 in the wa-
ter. PARSE charges and radii \{Sitkoff et al., 1994\} are used for amino acids. Amber94 \{Cornell et al., 1995\} provides the Lennard-Jones and torsion parameters. The Cl Lennard-Jones parameters are taken from \{Jensen and Jorgensen, 1994\}. The Cl ion Born solvation energy in solution is -21.925 kcal/mol. The Cl Lennard-Jones parameters are taken from \{Jensen and Jorgensen, 1994\}.

MCCE generates a structure with all possible conformations of all the non-hydrogen atoms of each residue side-chain, called rotamers \{Song et al., 2009\}. Then, each rotamer is assigned proton positions and protonation state (for ionizable residues), yielding multiple conformers for each rotamer. The choice of residue conformers defines all the microstates subject-ed to Monte Carlo sampling. For the CLC exchanger, MCCE calculates a microstate energy as the sum of electrostatic and van der Waals interactions between non-bonded atoms and the energy for proton, and ligand transfer from solution or reaction partners at ambient pH and ligand chemical potential.

2. **Calculation of the proton affinity of the gating residue**

The proton affinity is given as pK'_{4.5}, which describes the pH where the protonation free energy would be zero with the rest of the protein retaining the same ionization and conformational states as at pH 4.5 where the CLC-ec1 exchanger protein is maximally active \{Iyer et al., 2002\}. This measure is also called intrinsic pK_a \{Kieseritzky and Knapp, 2011; Mahankali, 2006; Beck et al., 2006\} or pK_{a,MAX} \{Faraldo-Gomez and Roux, 2004\} (with the latter measure calculat-
ed at the single conformer level). The pK'\textsubscript{4.5} rather than the pK\textsubscript{a} is used here because the residue of interest is changing its protonation at pH 4.5 on chloride binding. In contrast, the pK\textsubscript{a} measures the ionization cost at a different pH, and thus includes the effects of pH changes on ionization and conformation in the rest of the protein. The $\Delta G$ for moving in situ from the neutral to the ionized form is $2.303RTm(pH - pK')$ kcal/mol, where $m$ is 1 for acids and -1 for bases. Thus, at 25°C and pH4.5 the energy to ionize a residue in situ is $1.364m(4.5 - pK'_{4.5})$ kcal/mol, and $pK'_{4.5}$ is defined as:

$$pK'_{4.5} = pH_{4.5} - pK_{a,sol} + \frac{\Delta G_{prot}}{1.364m} \tag{1.a}$$

where

$$\Delta G_{prot} = (\Delta G_{soln} - \Delta G_{prot}) = \Delta \Delta G_{dsol} + \Delta G_{bkb} + \Delta G_{tors} + \Delta G_{vdw} + \Delta G_{res,pH4.5} + T\Delta S \tag{1.b}$$

Eq. 1 defines the classical partitioning of the energy terms that are accounted for by the simulation, where $pK_{a,sol}$ is the solution pK of the residue; $\Delta \Delta G_{dsol}$ is the loss of solvation energy (also known as Born energy or reaction field energy) that results from the transfer of a charge in solution into the protein; $\Delta G_{bkb}$ is the interaction between the residue or ligand with the backbone atoms; $\Delta G_{tors}$ is the torsion energy; $\Delta G_{vdw}$ is the van der Waals energy; $\Delta G_{res,pH4.5}$ is the residue pairwise interaction energy at pH4.5, and $T\Delta S$ is an entropy correction term (Song et al., 2009).
3. **Calculation of the Cl\textsuperscript{−} binding affinity**

MCCE provides the relative not absolute binding affinity. There are two conformers for each Cl\textsuperscript{−}: one in a given position in the protein and one in solution. The bound Cl ion may have multiple non-solution conformers if translation has been enabled. As detailed in a previous Cl\textsuperscript{−} binding study (Song and Gunner, 2009), the free energy of binding can be simplified as:

\[
\Delta G_b = \langle \Psi_{\text{holo}} \rangle + \mu_i - \langle \Psi_{\text{apo}} \rangle - \langle U \rangle - \mu_{\text{grid},0}
\]  

(2)

where \(<\Psi_{\text{holo}}>\) is the free energy of the protein and bound ion for a given value of the chemical potential, \(\mu_i\), and \(<\Psi_{\text{apo}}>\) is the free energy of the apoprotein, \(<U>\) is the free energy of adding an ion to solution, \(\mu_{\text{grid},0}\) is the reference solution chemical potential with concentration defined in ions/Å\textsuperscript{3}. In MCCE, \(<U>\) includes the reaction field energy and implicit van der Waals interaction of the ion with the solvent. The last term in Eq. 2 converts the molar units of the standard reference concentration (\(C_0\)) into protein or grid space units, i.e. moles are converted to number of ions and liters to Å\textsuperscript{3}, so that: \(C_0' = 6.02\times10^{-4} \text{ ions/Å}^3\), and

\[
\mu_{\text{grid},0} = RT \log \left( \frac{C_0'}{1\text{ion/Å}^3} \right) \text{ is -4.4 kcal/mol.}
\]

MCCE samples both apo and holo states together in the Monte Carlo simulation. The occupancy of bound ion [Cl\textsuperscript{−}] and bulk ion [Cl\textsuperscript{0}] concentration at a given site is:

\[
\frac{[\text{Cl}^\text{−}]}{[\text{Cl}^0]} = e^{-\frac{\langle \Psi_{\text{holo}} \rangle}{kT} + \frac{\Delta \mu}{NkT}}
\]

\[
= \frac{e^{-\left(\frac{\langle \Psi_{\text{holo}} \rangle}{kT} + \Delta \mu / kT \right)}}{e^{-\left(\frac{\langle \Psi_{\text{apo}} \rangle}{kT} + \langle U \rangle / kT \right)}}
\]  

(3)
The chemical potential titration simulates the binding of the ligands by changing the chemical activity, $a = \Delta \mu / NkT = (\mu_i - \mu_{\text{grid},0})/NkT$, followed by Monte Carlo sampling of the conformer ensemble yielding the fractional occupancy of each conformer at pH4.5. The simulations were run without a voltage-difference or ionic gradients across the membrane. The $K_{d,\text{rel}}$ is obtained from the relative chemical potential ($\mu_{\text{rel}}$) by interpolation of the conformer occupancy curve at half saturation.

4. Structures preparation

4.1 Parent structures:

The set of structures prepared for our simulations were initiated from three x-ray structures with conformationally distinct $E_x$ (E148) positions. The missing residues were not modeled. In silico mutations were made with the side-chain completion algorithm in MCCE. All structures were subjected to GROMACS energy minimization {Hess et al., 2008; Hornak et al., 2006} with Cl$^-$ ions in each of the three sites, except for the APO (2WX) which was kept free of Cl$^-$. The coordinates of the Cl$^-$ bound at the external site came from 1OTU when it was not present in the initial structure. Table M1 summarizes the preparation of the structures, $E_x$ protonation state for energy minimization, and type of affinity calculation.
The string identifying the simulated structure bears the orientation of E<sub>x</sub> as a superscript. E<sup>Sx</sup> is the WT structure (1OTS) {Dutlzer et al., 2003} that was simulated with the E<sub>x</sub> side-chain in S<sub>x</sub> and 2 Cl<sup>-</sup> bound in S<sub>c</sub> and S<sub>i</sub>. The original E<sup>Sx</sup> carboxyl group is 0.9 Å away from CL<sub>x</sub> coordinates, thus into S<sub>x</sub> (Figure M1). E<sup>MID</sup> denotes a structure with E<sub>x</sub> side-chain displaced outward by 1.2 Å from the E<sup>Sx</sup> side-chain position to accommodate the Cl<sup>-</sup> at the external site (Figure M1). 1OTS-derived E<sup>DN</sup> has E<sub>x</sub> side-chain moved to match the downward orientation in the C. merolae structure (3ORG) {Feng et al., 2010}. In E<sup>DN</sup>, E<sub>x</sub> fits between Cl<sup>-</sup>-bound S<sub>x</sub> and S<sub>c</sub> sites. E<sup>UP</sup> has E<sub>x</sub> rotated outward as in the E148Q mutant (1OTU) {Dutlzer et al., 2003}, from which it is derived. E<sup>UP</sup> and E<sup>Sx</sup> were used to obtain the Asp mutants Q148D and E148D, respectively. Lastly, two other structures were simulated in their original state: E<sup>APO</sup>, the structural apo structure (2EXW, E<sub>x</sub> into S<sub>x</sub>), which has E<sub>x</sub> in the S<sub>x</sub> and the side-chains of Ser<sub>c</sub> and Tyr<sub>c</sub> in S<sub>c</sub>; and E148A, the Alanine mutant (1OTT, {Dutzler et al., 2003}). Figure M2 summarizes the conformations of the gating residue in the dataset.
### Table M1. Experimental dataset

<table>
<thead>
<tr>
<th>Name</th>
<th>$N$, chains</th>
<th>$E_x$ state for energy minimization</th>
<th>$#Cl^-/N$</th>
<th>Affinity calculation</th>
<th>Treatment besides EM</th>
<th>$E_x$ side-chain orientation post EM</th>
<th>Parent PDB (res.)</th>
<th>Bound sites in crystal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E^{Sx}$:WT</td>
<td>2</td>
<td>$E_x$ ionized</td>
<td>2</td>
<td>$H^+</td>
<td>S_c, S_i$</td>
<td>None</td>
<td>Same, into $S_x$</td>
<td></td>
</tr>
<tr>
<td>$E^{DN}$</td>
<td>2</td>
<td>$E_x$ ionized</td>
<td>3</td>
<td>$H^+$ and $Cl^-$</td>
<td>WT backbone; $E_x$ down similar to 3ORG conformation</td>
<td>Points downwards, but allows for bound $S_s$ and $S_c^{(b)}$</td>
<td>1OTS:WT (2.5 Å)</td>
<td>$S_s, S_c (E_x$ blocks $S_i)$</td>
</tr>
<tr>
<td>E148D</td>
<td>2</td>
<td>$D_x$ ionized</td>
<td>3</td>
<td>$H^+$ and $Cl^-$</td>
<td>E148D mutation</td>
<td>Different in each unit; can be same as parent or sideways &amp; down</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E^{Mid}$</td>
<td>12</td>
<td>10 with $E_x$ ionized; 2 with $E_x$ neutral</td>
<td>3</td>
<td>$H^+$ and $Cl^-$</td>
<td>$E_x$ side chain pulled away and up from $S_x$</td>
<td>Same, allows for bound $S_s$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E148Q</td>
<td>2</td>
<td>n.a.</td>
<td>3</td>
<td>$Cl^-$</td>
<td>None</td>
<td>Same, UP</td>
<td>1OTU:E148Q (3.3 Å)</td>
<td>$S_{s}, S_c, S_i$</td>
</tr>
<tr>
<td>Q148D</td>
<td>2</td>
<td>$D_x$ ionized</td>
<td>3</td>
<td>$H^+$ and $Cl^-$</td>
<td>Q148D mutation</td>
<td>Different in each unit; still UP but closer to $S_s$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E^{up}$</td>
<td>6</td>
<td>4 with $E_x$ ionized; 2 with $E_x$ neutral</td>
<td>3</td>
<td>$H^+$ and $Cl^-$</td>
<td>Q148E reversed mutation</td>
<td>Same, UP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E148A</td>
<td>2</td>
<td>n.a.</td>
<td>3</td>
<td>$Cl^-$</td>
<td>None</td>
<td>NA</td>
<td>1OTT:E148A (3.0 Å)</td>
<td>$S_s, S_c, S_i$</td>
</tr>
<tr>
<td>$E^{apo}$</td>
<td>4</td>
<td>$E_x$ ionized in 2 chains</td>
<td>0</td>
<td>$H^+$</td>
<td>None</td>
<td>Same, into $S_x$</td>
<td>2EXW:APO (3.2 Å)</td>
<td>None, x-ray apo</td>
</tr>
</tbody>
</table>

**Table M1. Summary of experimental dataset preparation and structural parents.** **Name:** The string identifying the simulated dimeric structure bears the orientation of $E_x$ as a superscript. $N$ is the total count of protein chains, taken as independent subunits, for each type of structure. $S_s$, $S_c$ and $S_i$ denote the $Cl^-$ binding sites (external, central and intracellular, respectively) that can be bound in x-ray structures. (a): Whenever added, the extracellular $Cl^-$ ion coordinates are those from 1OTU. (b): In 3ORG, $E_x$ side-chain is oriented downwards into the empty central site, where it sits in between $S_s$ and $S_c$ in $E^{DN}$ and during energy minimization, the $Cl^-$ ions found a new position laterally offset by 1 Å so the 1OTS central ion was kept. All mutations of the gating residue started with the parent's conformation.
Figure M1. $E^{\text{MID}}$: Modified conformation of the gating Glutamate, E148 to allow Cl$^-$ binding at $S_x$.

The $E^{\text{DN}}$ structure was obtained by matching the downward orientation of $E_x$ side chain from the $C.\ merolae$ (3ORG) structure in the backbone of 1OTS. This position allows Cl$^-$ to be bound at both $S_x$ and $S_c$ sites. However the parent backbone conformations differ. $E_x$ conformation in the DOWN structure is close to that of E209 (the equivalent residue in 3ORG), but in the context of the $E.\ coli$ backbone $E_x$ does not extend as far towards the central site. As a consequence, there is still room for Cl$^-$ at $S_c$. Figure A1.a shows the outcome of the 148 position hybridization using the $E.\ coli$ 1OTS backbone and matching the side-chain conformation to that in $C.\ merolae$ 3ORG.

In $C.\ merolae$, 3ORG, where Trp replaces CLC-ec1 Arg147, the displaced backbone segment apparently allows $E_x$ to reach deeper into $S_c$ than in the 1OTS-derived $E^{\text{DN}}$ structure, which prevents binding at the central site. A homology model (Zhang, 2008; Roy et al., 2010; Yang et al., 2015) of $E_x$ sequence motif of the second isoform of $C.\ merolae$ has the familiar $E_x$ conformation in $S_x$ as seen in the $E.\ coli$ WT (1OTS) structure (Figures A1.b), although it has Pro substituted for Trp. It is not known whether this downward conformation occurs in $E.\ coli$, but it ap-
pears that a close match to the conformation in 3ORG cannot be achieved without movement of the 1OTS backbone. (Further details can be found in Appendix.)

**Mutants:** The conservative E148D mutant has been found to drastically reduce the WT transport rate in CLC-ec1 and CLC-cm {Feng et al., 2012}, and reduce the open probability of the CLC-0 channel {Traverso et al. 2006; Zifarelli et al. 2008, Zifarelli and Pusch, 2010}. We performed this mutation in order to assess whether these perturbations were a consequence of altered energetic factors such as H\(^+\) and Cl\(^-\) affinity, or degrees of conformal freedom.

Cl\(^-\) binding was calculated for E148A and E148Q mutants. These are channel-like mutants that transport Cl\(^-\) and not H\(^+\) {Dutlzer et al., 2003}. These mutated structures provide additional points of reference for the Cl\(^-\) affinity in absence of unfavorable carboxyl group/ion interactions. The conformations of the gating residue in the dataset are summarized in Figure M2.

**Missing residues:** The missing/unresolved residues in E\(^{APO}\), E148Q, WT (M1 to L16 and E461 to T473 in chain A; M1 to R17 and K459 to T473 in chain B) were not modeled. The absence of these solvated residues results in up to 2 missing charges per unit at pH 4.5.
Figure M2. Gating residue conformations in the dataset

S_x

S_c

Figure M2. Gating residue conformations in the dataset in the apo (Cl^-unbound) state. S_x and S_c denote the location of the external and central sites. Green circles indicate the position of Cl^- bound in either site. Superimposed E_x from the A and B chains are shown when they differ (in E148D and Q148D). E^{DN}: adds 3ORG E_x conformation to WT (1OTS) backbone; E^{APO}: 2EXW, structural apo; WT: 1OTS, apo external site; E^{MID}: WT with E_x side-chain position to accommodate an ion in S_x; E^{UP}: 1OTU with reversed mutation of E148Q; Asp mutants: E^{148D} derived from WT, and Q^{148D} from E^{UP}.

4.2 Energy minimization (EM)

Waters and any ligands other than substrate were deleted from the structures. All structures were energy minimized using the steepest descent algorithm using 2000\(^4\) steps with 0.01 nm step size and 100 kJ/mol/nm tolerance (Hess et al., 2008), given the Amber force field along with GBSA implicit solvent model (Hornak et al., 2006). Ionizable residue states were identical in all minimizations except when E_x (or D_x) was fixed to be either ionized or neutral (Table M1). E113 and E417 were kept neutral. All His were neutral with proton on NE except for H284 were the ND tautomer was used.

\(^4\) This high number of steps is a source of stabilization of the starting structures (Dr. Lazaridis, personal communication).
5. Simulations nomenclature

Each Cl\(^-\) binding simulation is identified by its Cl occupancy state at \(S_x\) and \(S_c\) and the state of the gating residue, which appears as a superscript 0, for neutral (protonated), or -1 for ionized (deprotonated). The site letter X or C denotes a freely titrating \(S_x\) or \(S_c\) binding site, respectively. The site letter together with a 0 or 1 means that the site is titrating alone or with the other site bound and fixed (non-titrating), respectively. For example, \(X1^{-1}\) represent the Cl\(^-\) binding simulation (or state) of the external site (X) with the central site bound and the gating residue ionized. The names 'CL\(_x\)' and 'CL\(_c\)' are used whenever there is a need to refer to the bound Cl ion in \(S_x\) or \(S_c\).

6. Reporting of error

The MCCE method has an error bound of about 3 to 4 kcal/mol. MCCE overstabilizes charges, especially with low protein dielectric constant, \(\varepsilon_{prot}\), leading to depressed pK\(_a\) for stable acids (Gunner et al, 2011). This method error is stated here and meant to be kept in mind, but is not reported with the simulation data. Instead, averages are reported with their standard deviation to show the dispersion over the group they represent (e.g. average over the two chains, or over a structure type).
7. *Estimation of the energy of moving from $E^{\text{MID}}$ to $E^{\text{UP}}$ positions:*

The energy of conformational change of $E_x$ was estimated from the difference in $E_x$ proton affinity between $E^{\text{MID}}$ and $E^{\text{UP}}$. As these structures show little to no change in conformation, apart from $E_x$ positions, other possible contributions to the energy differences of $E_x$ were ignored.

We calculated the energy differences for the three state transitions between the thermodynamic cubes of $E^{\text{MID}}$ and $E^{\text{UP}}$ ($00^{-1}$, $0C^0$ and $XC^0$, Figure A5), which are part of a step-wise pathway leading to 2:1 exchange. To this aim, we used a reconstructed Boltzmann distribution for a population comprised of the 5 most favorable $E_x$ conformers in each state and structure (there are rarely more than five occupied conformers in any given run of the dataset). The average energy of the 5-conformer population in each state was used to calculate the $E^{\text{UP}} - E^{\text{MID}}$ difference, which represents the transition estimate (see Table A4) for the two representative structures that were also used in the thermodynamic cubes (Figure A5).

The difference between $E_x$ all-conformer average MFE and the 5-conformer average MFE was used as the error bound for the procedure just described. The maximal value (nearing 6 kcal/mol) is found in XC of $E^{\text{MID}}$ and is due to the ionized conformers of the smaller population as they are normally not found in this run. The estimate for the apo state transition is the least uncertain as the difference is less than 1 kcal/mol.
RESULTS

The Cl\(^-\) affinities at the external (S\(_x\)) and central (S\(_c\)) sites and their dependence on the proton affinity of the gating residue at position 148 (E\(_x\), Q\(_x\) or D\(_x\)) were determined. As the gating residue is not solvent accessible in all structures studied, which are close to their x-ray parents, this study pertains to binding in the context of occluded structures. The average gating residue H\(^+\) affinities and Cl\(^-\) affinities of S\(_x\) and S\(_c\) along with their standard deviations can be found in Table A1 in Appendix. The analysis will primarily compare the behavior of the structures with E\(_x\) in the UP and MID positions (E\(^{UP}\) and E\(^{MID}\)).

1. **E\(_x\) proton affinity and relative Cl\(^-\) affinity of the external site, S\(_x\)**

The proton affinity of E\(_x\) in the absence of Cl\(^-\) (e.g. in the apo state), is strongly correlated with its position, as shown by the comparison between the affinity in E\(^{MID}\) and E\(^{UP}\) (Figure R1, a): simply moving E\(_x\) relative to S\(_x\) in a generally static protein structure leads to a remarkable change proton affinity of \(\approx 8 (\pm 2)\) kcal/mol. In both E\(^{MID}\) and E\(^{UP}\), the E\(_x\) side-chain has no torsional energy and its van der Waals energy is 0 (\(\pm 1\)) kcal/mol. Thus the *ad hoc* E\(_x\) side-chain conformation of the MID construct is not the source of this difference.

In the E\(^{APO}\) or E\(^{Sx}\) structures, where E\(_x\) is in S\(_x\), the average \(\Delta G_{protn.}\) is about 14 (\(\pm 2\)) kcal/mol (corresponding to a pK'\(_{4.5}\) of -6); it is 12 (\(\pm 2\)) kcal/mol in the E\(^{MID}\) structures where E\(_x\) is
near $S_x$ and reaches 2 ($\pm1$) kcal/mol in $E^{UP}$, which has the backbone from the E148Q mutant (Table A1). Thus, $E_x$ will remain ionized in the protein in the absence of Cl$^-$. (It is found partially protonated in the apo state of the two $E^{UP}$ subunits energy-minimized with $E_x$ neutral.) The energy to protonate the $E_x$ at pH 4.5 presents a substantial penalty for protonation especially when $E_x$ is near the Cl$^-$ binding site.

Cl$^-$ titrations as a function of the Cl$^-$ chemical potential were performed at pH4.5, with all residue side-chains free to titrate and explore different rotamers on the fixed backbone (Figure R1, c), and when $E_x$ is fixed neutral or ionized (Figure R1, d, e).

With CL$_x$ (Cl$^-$ bound at $S_x$), $E_x \Delta G_{protn}$ (Figure R1, b) is almost identical in all structures averaging -4 ($\pm2$) kcal/mol. Hence, CL$_x$ is sufficient to protonate $E_x$, thus the reaction taking place is a Cl$^-$-binding induced protonation. Thus, $E_x \Delta G_{protn}$ is always unfavorable in the apo state and favored with Cl$^-$ bound to $S_x$. In terms of pK$_x$, $E_x$ pK$_{a}$ is always below 4.5 in the apo state and above 4.5 with Cl$^-$ bound to $S_x$ (Figure R1, a, b). There are small changes in protonation throughout the protein but $E_x$ is the sole residue that shows a significant change in protonation when Cl$^-$ binds to $S_x$. 
The Cl\(^-\) affinity varies by 20.0 kcal/mol despite there being little conformational variation, except for the position of E\(_x\). As S\(_x\) is filled, E\(_x\) becomes protonated. The energy of the coupled reaction can be dissected using the thermodynamic cycle shown in Figure R2. Paths A and a represent the proton binding to E\(_x\) in the apo or Cl\(^-\) bound state, respectively. Paths B and b represent the Cl\(^-\) binding to S\(_x\) when E\(_x\) is fixed neutral (\(\Delta G_b^0\); X0_E01 in Fig. R1 panel d) or ionized (\(\Delta G_b^i\); X0_E-1 in Fig. R1 panel e). Path C is the coupled H\(^+\)/Cl\(^-\) binding reaction when both ligands are free to titrate (\(\Delta G_b^{\text{free}}\); X0 in Fig. R1 panel c).
Figure R2. Thermodynamic cycle for proton-coupled Cl⁻ binding at $S_x$. Arrow directions indicate binding events. Protonation of the gating residue $E_x$ occurs with the binding of a Cl⁻ ion. **Paths A and a:** H⁺ binding affinity of the residue in the apo state: $\bigcirc$, or Cl⁻ bound $S_x$ state: Cl$\scriptstyle S_x$, respectively. **Paths B and b:** Cl⁻ binding affinity when $E_x$ state is fixed neutral ($\Delta G_b^\text{neu}$) or ionized ($\Delta G_b^\text{i}$), respectively. **Path C:** coupled H⁺, Cl⁻ binding affinity with $E_x$ free to titrate ($\Delta G_b^\text{free}$). The energy of binding Cl⁻ with ionized $E_x$ ($\Delta G_b^\text{i}$) leads to paths A and B being favored in either the $E_MID$ or $E_{UP}$ subset. Unlike $E_{MID}$, $E_{UP}$ also yields favorable $\Delta G_b$.

Relative Cl⁻ binding energies for paths $b$, $B$ and $C$ are obtained from the Cl⁻ occupancy computed by the Monte Carlo (MC) simulation of the Cl⁻ chemical potential titration, while the proton affinity of $E_x$ in paths $a$ and $A$, pK$'$, 4.5 (Eq. 1), is computed with the Mean Field Energy (MFE) approach where all other ionizable residues in the multi-conformational space are set to their equilibrated state at pH4.5 as determined by MC sampling (Methods Eqn. 1). The comparison between MFE- and MC-derived energies for Cl⁻ binding is shown in Figure A5. The MFE values are about 1 pK unit lower than the more accurate values derived by MC. This is an expected outcome of the MFE estimates partly due to the averaging of the other conformers into a low dielectric environment for a given titratable residue, which favors the ionized state {Honig and Nicholls, 1995; Alexov and Gunner, 1997}. These energies are used over the entire thermody-
namic cycle for one example of a protein structure each in the E\textsuperscript{UP} and E\textsuperscript{MID} subsets (Figure A6): small adjustments of < 2 kcal/mol were needed to obtain closed cycles (Table A5).

Relating the chloride and proton affinities to the reaction cycle in Figure R2, we observe the following. Binding a Cl\textsuperscript{−} with ionized E\textsubscript{x} (X\textsubscript{0-E-1}=ΔG\textsubscript{b}\textsuperscript{i}, Path b) incurs a small, 3 kcal/mol penalty versus X\textsubscript{0} (ΔG\textsubscript{b}\textsuperscript{free}, Path C) for E\textsuperscript{MID} and E\textsuperscript{UP} alike, yet it is easier to do in E\textsuperscript{UP} since E\textsubscript{x} pK\textsuperscript{′}\textsubscript{4.5} is very close to its solution value. The Cl\textsuperscript{−} binding affinity with protonated E\textsubscript{x} (X\textsubscript{0-E01}=ΔG\textsubscript{b}\textsuperscript{n}, Path A) is remarkably increased in all the MID-like structures (Table A1).

Even though the coupled Cl\textsuperscript{−} and H\textsuperscript{+} binding reaction (X\textsubscript{0}=ΔG\textsubscript{b}\textsuperscript{free}, Path C) is favored in either structure (see the E\textsuperscript{MID} and E\textsuperscript{UP} thermodynamic cubes in Figure A7), the strength of the coupling resides in the E\textsuperscript{MID}, the more occluded structure, where E\textsubscript{x} side-chain resides close to the helix cage. The proton-coupled Cl\textsuperscript{−} affinity, (ΔG\textsubscript{b}\textsuperscript{free}, X\textsubscript{0}), in the different structures is correlated with the energy required to bind a proton to E\textsubscript{x} in the apo state. Thus, the energy of protonating E\textsubscript{x} contributes to the cost of Cl\textsuperscript{−} binding (Table A1; Figure R3). Yet, even with a neutral E\textsubscript{x}, S\textsubscript{x} binds Cl\textsuperscript{−} about 5 kcal/mol more tightly in E\textsuperscript{UP} structures indicating that other structural factors trapped in the WT 1OTS and E148Q 1OTU structures influence the Cl\textsuperscript{−} affinity to S\textsubscript{x}.

The Cl\textsuperscript{−} affinity in the coupled reaction (ΔG\textsubscript{b}\textsuperscript{free}, X\textsubscript{0}) varies with the conformation of E\textsubscript{x}. Structures can be divided into the UP- or the WT-like (E\textsuperscript{MID} or E\textsuperscript{Sx}). The results with E\textsuperscript{DN} and both Asp mutants are more closely related to the E\textsuperscript{MID} structures. With E\textsuperscript{DN}, the side chain is still relatively close to S\textsubscript{x} (3.2 Å away from S\textsubscript{x} compared to 6.0 Å in UP).
We think that the extremely tight affinities seen when $E_x$ is fixed protonated or UP (Table A1) reflect an unaccounted cost that include the conformational change of $E_x$. Thus, in the apo state $E_x$ will be ionized and will block the external site. With $C_{L_x}$, $E_x$ must become protonated and also move into the UP positions. Hence, we estimated (Methods) the free energy of the $E_{\text{MID}} \rightarrow E_{\text{UP}}$ transition as if it were commensurate with $E_x$ protonation energy in order to obtain a reaction cycle between these two structures yielding the experimental $2\text{Cl}^-:1\text{H}^+$ stoichiometry.
Figure R3. Dependence of the coupled reaction path on the Cl\(^-\) and H\(^+\) affinities at S\(_x\):

(a) E\(_x\) or D\(_x\) proton affinity, in the apo state, \(\Delta G_{\text{protn.}}\) (Methods Eqn. 1);
(b): the relative Cl\(^-\) binding affinity of the external site when E\(_x\) or D\(_x\) is fixed neutral, \(\Delta G_b^\text{n}\); (c): Sum = \(\Delta G_{\text{protn.}}\) + \(\Delta G_b^\text{n}\) (corresponding to path C in the thermodynamic cycle). Empty blue circles: E\(_{\text{UP}}\) with E\(_x\) neutral during energy minimization, which are excluded from best fit line since E\(_x\) is partially protonated in the apo state; filled blue circles: E\(_{\text{UP}}\) with E\(_x\) ionized during EM; Filled red circles: E\(_{\text{MID}}\); Triangles: Asp mutants; Diamonds: E\(_{\text{DN}}\).

There is a strong correlation (R\(^2\) 0.9) of the gating residue apo \(\Delta G_{\text{ioniz.}}\) to the distance between its carboxylic O-atoms and S\(_x\) (Figure R4.b). However, calculated energetic terms (described in Methods Eqn. 1) are better comparable to a free energy than distance. The energy terms that contribute to the variation in \(\Delta G_b^\text{n}\) and E\(_x\) apo \(\Delta G_{\text{ioniz.}}\) were investigated (Figures A8).
The major component for $E_x$ or $\text{Cl}_x$ is the positive electrostatic potential from the backbone amides of the N-termini of the two helices that come together to make the binding site, characterized as an alpha helix cage (Aqvist et al., 1991) (Figure R4.4, Figure A3). The range of backbone interaction energy with the gating residue accounts for the range of apo $\Delta G_{\text{ioniz}}$ of about 10 kcal/mol. The chloride-backbone interaction energy is also large and favorable with an average value of 26 ($\pm$1) kcal/mol for $\text{Cl}_x$ and 24 ($\pm$1) kcal/mol for $\text{Cl}_c$ (Table A4, Figure A3). None of the other energy terms (vdW interaction, residue pair-wise interaction, desolvation) that are expected to contribute to the proton affinity of a buried residue explain any of the variation in $\Delta G_0^n$ and $E_x$ apo $\Delta G_{\text{ioniz}}$ (Figure A8).

**Figure R4.** Energetic determinants of the gating residue apo $\Delta G_{\text{ioniz}}$

**Figure R.4.** Energetic determinants of the gating residue apo $\Delta G_{\text{ioniz}}$. Relationship between the gating residue ($g_{\text{Res}}$) apo $\Delta G_{\text{ioniz}}$, and its free energy of interaction with the backbone (a), and the average distance between its carboxylic O atoms to $S_x$ position (b). Blue dots: $E^{\text{UP}}$; Red dots: $E^{\text{MID}}$; Circles $E^{\text{APO}}$; Diamonds: $E^{\text{DN}}$; Triangles: Asp mutants. The shaded area indicates the van der Waal radius of the Cl$^-$ ion.
The backbone interaction energy plotted as a running sum (Figure A3) identifies two structural motifs of nine residues each, that stabilize Ex and the external and central sites. The first one (residues 145 to 153) includes the sequence motif of the gating Glu, G\textsubscript{146}xE\textsubscript{148}G\textsubscript{149}P\textsubscript{150}, in the N-terminal end of helix F; the other one (residues 354 to 362) includes the sequence motif of conserved F357, G\textsubscript{355}xF\textsubscript{357}xP\textsubscript{359}, in the N-terminal end of helix N (with x denoting a non-conserved position).

The abutting of the N-terminal ends of helix F and helix N results from the inverted topological repeat (ITR) of the CLC fold {Dutzler, 2002} (Figure I2), which create an electropositive hotspot {Yin et al., 2004}, roughly in the middle of each subunit. The backbone elements stabilizing the binding sites originate from the first two turns of each helix end, and their energy can represent from ~70% to 90% of the total interaction energy between the backbone and the anions (Ex\textsuperscript{−} or the Cl ions). Thus, these very short-range interactions form a 'helix cage' {Aqvist et al., 1991}, which, as we will see, can accommodate two negative charges (Figure A6).

Since the total Ex|backbone interaction energy in the E\textsuperscript{APO} structures is -23 (±1) kcal/mol versus -19 (±1) in the E\textsuperscript{MID} structures, we can estimate that the displaced Ex side chain to the MID position has lost ~4 kcal/mol of favorable interaction energy with the backbone. This amount was also the estimate of a 'barrier' to pulling an ion from S\textsubscript{C} to S\textsubscript{x} in an MD simulation {Cohen and Schulten, 2004}. Further details of the interaction with the backbone can be found in Appendix.
2. *E*$_x$ proton affinity and relative Cl$^-$ affinity of the central site, $S_c$

The central site was likewise singly titrated keeping $S_x$ empty. When $E_x$ is free to titrate (0C), on average, Cl$^-$ binds $S_c$ with similar affinity in the UP and MID structures (Figure R5, panel c). Most of the variation in affinity emanates from the interaction energy with residues, principally $S_{rc}$ and $T_{rc}$ whose OH groups can rotate into the central site, weakening the Cl$^-$ affinity by almost 10 kcal/mol if neither side-chains is positioned optimally for Cl$^-$ binding. The unfavorable alternative orientation of these polar residues shows how they can compete with Cl$^-$ for binding the site so they can act as a gate.

Binding to the central site raises the $E_x$ proton affinity by 4 pH units in $E^{\text{Mid}}$ to a pK'$_{4.5}$ of 1, and by 2 pH units in $E^{\text{Up}}$ to a pK'$_{4.5}$ of 5. Thus, in the $E^{\text{Up}}$ structures, $E_x$ can be almost 70% protonated in the presence of CL$_c$ even when CL$_x$ is not bound. In the $E^{\text{Sx}}_x$ or $E^{\text{Mid}}_x$ positions, the pK shift is not sufficient to lead to proton binding.
Figure R5. Free energy of protonation of E_x and relative free energy of Cl^- binding at S_c

We have seen that the relative free energy of binding at S_x is related to the E_x proton affinity in the apo state since a proton needs to be bound as S_x is filled (Fig. R3-a). There is no such relationship for S_c (Fig. R6-a), so Cl^- binding at S_c is not coupled to proton uptake. Yet, the sites affinities are similarly correlated and dependent when ΔG_{b,rel}^{neu} is plotted versus ΔG_{b,rel}^{free} (Figs. R3-b and R6-b).
Figure R6. Dependence of the coupled reaction path on the Cl⁻ and H⁺ affinities for the central site

3. **Simultaneous titrations at $S_x$ and $S_c$**
CLx and CLc are only 4 Å apart. The average ion-ion repulsion amounts to 15 (±0) kcal/mol in all structures but EDN where it is 10 (±0) kcal/mol (due a lateral position shift there). This strong repulsion creates a challenge for simultaneous Cl⁻ binding at the two sites. When both sites titrate freely (Table A1, XC), for a given structure, the site with the strongest Cl⁻ affinity in the single site titration is filled first with a ΔGb,rel similar to that found in the single titration, requiring much higher [Cl⁻] to fill the second site.

Binding at the central site is weaker in the EUP or E148Q structures because Serc and TyrC have moved into Sc, a 'trait' inherited from the 1OTU parent structure (where helix F N-ter shows some distortion, see Gating residue conformations in Appendix).

Binding at the external site is weaker in all the structures with WT 1OTS as a parent, because this structure is that of the CLC-ec1 protein crystallized with a charged carboxylic group into Sx and an anion in Sc. Also, due to Ex close proximity to the helix cage there, it can still engage in an intramolecular bond with its amide H-atom, which stabilizes a more occluded conformation. Lastly, the depressed apo pK' in these structures translate to more work needed by the incoming Cl⁻ in order to bind Sx concurrently with Ex protonation, since they are coupled. Thus, Sx is filled first in EUP and Sc is filled first in EMID.

These structural differences persist in titrations at either site with the other one Cl⁻ bound and fixed (Table A1, states X1 and 1C, Figure R7 panel c), however there, the ion-ion repulsion is now in effect. The titrations where one site is bound and fixed are mandated by the thermodynamic cycle of the reaction (Figure A6), but can be interpreted to represent binding of a second ion with the first one locked in by a gate.
Figure R7. Free energy of protonation of $E_x$ and relative free energy of $Cl^-$ binding to $S_x$ and $S_c$ when the other site is bound.

Distribution of the free energy of protonation of $E_x$ with no Cl$^-$ bound (Panels 1.a and 2.a: same data); in the Cl$^-$ titration of $S_x$ (Panel 1.b) and $S_c$ (Panel 2.b). Relative free energy of Cl$^-$ binding at $S_x$ (Panel 1.c) and $S_c$ (Panel 2.c) with $E_x$ free to titrate; $E_x$ fixed neutral (d); $E_x$ fixed ionized (e). The symbols and identifiers are as described in Fig. R1.

4. Discussion

Our analysis of the thermodynamics of Cl$^-$ and H$^+$ binding at pH4.5 and of their structural basis in CLC-ec1 uncovered key features and provided several conclusions that helped derived a mechanism (Figure R8).
The stabilization of anions in $S_x$ and $S_c$, the two binding sites found within 4 Å of each other in the selectivity filter, originates from the favorable interaction energy with the backbone elements of the two abutting N-terminal ends of helices F and N (Figure A3). This electro-positive hotspot, or helix cage, results from backbone dipoles localized in the first two turns of each N-terminal ends and corresponds to sequence motifs with 80% identity: the $E_x$ and the $F357$ motifs. The helix cage can accommodate two anions, be they $E_x^-$ with $CL_c$ or $CL_x$ with $CL_c$. The $Cl^-$ ions are almost equally stabilized by the helix cage, although $S_c$, which is slightly offset from it (Figure I1), also relies on the backbone elements of the inner gate residue motifs, Ser$_c$ and Tyr$_c$. $E_x$ in the apo state is slightly less favorably stabilized by the backbone elements of the N-termini motifs, yet these determine its depressed apo $pK'$ (Table A2, Figure R4a). Additionally, is a competitor to $Cl^-$ binding so the favorable backbone interactions are the first source of cooperativity for binding two $Cl$ ions in spite of the strong $Cl-Cl$ repulsion (Table A4).

The $Cl^-$ and $H^+$ binding results provide an explanation for the source of the $1H^+:2Cl^-$ coupling stoichiometry. We find that $E_x$ protonation is strongly coupled to $Cl^-$ binding at $S_x$. In fact, we can strongly concur with experimental data that $E_x$ protonation is $Cl$-induced {Picollo et al., 2012}. The strength of the coupling is anti-correlated to the degree of openness of the structure: the more open structure, $E_{UP}$, has the greatest $Cl^-$ affinity and the weakest $H^+$ affinity, ensuring that $H^+$ release occurs after two $Cl$ ions are bound. $Cl^-$ binding at $S_x$ is coupled to $E_x$ protonation with a strength that varies with the structure and the position of $E_x$ side-chain: protonation and $Cl^-$ binding are disfavored when its carboxyl group is within hydrogen-bonding distance (~3 Å) of the helix cage in the more occluded structures. In contrast, a bound $Cl^-$ at $S_c$ will raise its $H^+$ affinity, but without reaching full protonation; this is the state found in WT 1OTS.
We also found that binding at the central site is sensitive to the positions of Ser_c and Tyr_c. These results lead to the overall exchange mechanism (Figure R8).

Mechanistically then, and assuming that the non-conducting state is an apo state, an incoming Cl\(^-\) from the extracellular solution will destabilize ionized E_x side-chain in S_x and will induce a conformational change that will bring it outside of the helix cage. The need for this conformation is deduced from the extremely tight Cl\(^-\) affinities seen when E_x is fixed protonated (Table A1).

The strong coupling at S_x forces an assumption for changes in the conformation of the heavy gate components on the external side, \(\Delta g_H^{X}\): given that the light gate, \(g_L^{X}\) (E_x side-chain) must move upon protonation to enable Cl\(^-\) binding at S_x, then some components of \(g_H^{X}\) must open to allow entry of Cl ions in the extracellular vestibule. This we believe, is equivalent to the transition between the 'outward-facing, occluded' (OF\_occluded) and the 'outward-facing, open' (OF\_open) conformations recently proposed in a multi-pronged, NMR/MD simulation study of conformations in CLC-ec1 (Khantwal et al., 2016). Then, \(g_H^{X}\) must close again once E_x is protonated so that H\(^+\) is not released before the two sites are filled. On the intracellular side, movement of the Tyr_c and Ser_c, the intracellular light gate, \(g_L^{I}\), into S_c as E_x moves in to S_x to produce a stable apo state, which otherwise not favored by electrostatics. Our proposed mechanism (Figure R7) suggests that full occupancy of the filter (Step 6) triggers the inner gate opening (in the direction depicted), which agrees with the strength of the ion-ion repulsion in a more occluded structure (e.g. E^{MID}). This is consistent with \(\Delta g_H^{X}\) above mentioned: once the Cl ions are in the protein will contract again from the external side, in preparation for the next conformation, e.g. OF\_occluded→Inward Facing. This last conformation would be responsible for the
forceful return of newly ionized $E_x$ (in Step 9), towards its APO conformation, expelling $CL_c$ and the return to the apo state with $\Delta g^f$.

The apo state seems to be needed {Matulef and Maduke, 2007; Miller and Nguitragool, 2009; Basilio at al. 2014; Khantwal et al., 2016} to obtain the 2:1 stoichiometry without resorting to transient gating by extracellular Cl ions at the external site ($Cl^-$ backflow) {Feng et al., 2010}. The reorganization of $Ser_c|Tyr_c$, which move into $S_c$ in the $E^{APO}$ is a likely contributor in stabilizing the apo state.

In contrast to other models which detail the binding of two Cl ions at once {Matulef and Maduke, 2007; Nguitragool and Miller, 2009; Feng et al., 2012; Basilio at al. 2014; Khantwal et al., 2016}, we chose to depict a step-wise translocation of $Cl^-$ from $S_x$ to $S_c$ because our data indicate that the $E_x^-|CL_c$ state is the most stable (Figure A6), agrees with the WT 1OTS structure and with experimental data yielding a model with two independent gating steps {Grieschat and Alekov, 2014}. Additionally, the importance of the central site for the coupled exchange {Nguitragool and Miller, 2006} is represented in states 4 and 9, which are the only states where the central site is bound and where $E_x$ can be protonated or ionized, respectively. Thus in effect, the central site is implicated with the proton transfer.

5. **Step-wise transport model for coupled $2Cl^-:1H^+$ exchange**

The $Cl^-$ affinities of the two sites along with the $H^+$ affinity of $E_x$ enabled the building of a thermodynamic cube for each $E^{UP}$ and $E^{MID}$ subsets (Figure A7), which we used to obtain a
pathway leading to the $2\text{Cl}^-:1\text{H}^+$ stoichiometry within each cube. This pathway was then augmented with the estimates for the state-to-state MID⇌UP transitions (Table A3). The proposed exchange mechanism (Figure R8) closely follows the combined paths of Figure A6 with possible intermediate states.

Figure R8. Stepwise model for $2\text{Cl}^-:1\text{H}^+$ exchange ($\text{Cl}^-\text{-inflow}/\text{H}^+\text{-outflow direction}$)

**Walk-through:** The mechanism starts in the apo state (STEP 1) with $E_x$ ionized and in its closed conformation. There the inner gate residues also adopt the closed conformation seen in the APO structure where an overlap of $S_c$ by the side-chains of Ser$_c$ and Tyr$_c$ creates a clash blocking $\text{Cl}^-$ binding. A conformational change in the apo state initiates the opening of the outer gate (STEP 2), followed by the coupled protonation of $E_x$ with $\text{Cl}^-$ binding at the external site (STEP 3). After translocation of the ion from the external to central site, another ion binds the newly vacated external site (STEP 4, 5). Another conformation at the outer gate occurs with the fully loaded state, the most favorable configuration with 2 Cl ions and protonated $E_x$ (STEP 5), so that only the inner gate opens (STEP 6) and releases the first bound $\text{Cl}^-$. We postulate that the fully loaded state constitutes a gate-coordination signal for the intra gates to open. Another ion translocation takes place (STEP 7) and the inner gates open again to release the second $\text{Cl}^-$ (STEP 8) while the unknown gates coordinating mechanism ensures the return of $E_x$ side-chain to its closed position with concurrent release of $\text{H}^+$ to the outside solution (STEP 9). The high cost of unbinding the central site is reduced by the inner gate residues re-entry into the central site.
Transition estimates:

The model reflects the estimated MID ↔ UP conformational energy in three transitions. The first is Δapo, the apo\textsuperscript{MID} → apo\textsuperscript{UP} transition in the apo state with E\textsubscript{x} ionized (∼12 kcal/mol) taking place from step 1 to step 2. This cost would represent the work needed to introduce 2 Cl\textsuperscript{−} ions to approach the entrance of the pore via conformational changes domain-level motions would belong to the heavy gate (g\textsubscript{H}) component of the gating mechanism, while side-chain movements would constitute the light gate one (g\textsubscript{L}) {Forrest et al., 2011} and could be H\textsuperscript{+}-dependent {Elvington et al., 2009}. A candidate for Δapo would be a conformational change of helix O relayed to helix N (whose N-terminal flanks one side of the external site, and where conserved F357 resides). Helix O (inner g\textsubscript{H}) has been found to directly interact with residue Y445 (inner g\textsubscript{L}) {Basilio et al., 2014}, thus the closing of the inner gate may initiate the opening of the external one. The second one, Δ0C, occurs in the singly bound central site (0C\textsuperscript{MID} → 0C\textsuperscript{UP}, ∼3 kcal/mol, step 4). The third one, ΔXC, takes place in the fully loaded state (XC\textsuperscript{0,MID} → XC\textsuperscript{0,UP}, step 5): if the inner gate were to open with E\textsubscript{x} UP, channel mode would ensue, thus we surmised that the fully loaded state triggers the coordinated closing of the external gate with the opening of the inner one in order to release Cl\textsuperscript{−} bound at the central site. As E\textsubscript{x} proton affinity in E\textsuperscript{UP} is close to its pK\textsuperscript{′}_{4,5}, this implies that the deprotonation of E\textsubscript{x} side-chain in E\textsuperscript{UP} must be accompanied with other conformational changes that provide the energy to bring it back into S\textsubscript{x}. 
6. **Conclusions and perspective**

We find that both the $S_x$ and $S_c$ Cl$^-$ affinity is coupled to $E_x$ proton affinity ([Fig. R3 and R6](#)), but with surprising molecular details. $E_x$ protonation (H$^+$ capture) is induced by Cl$^-$ binding to $S_x$ only, while Cl$^-$ binding at $S_c$ reduces the protonation cost from 10 to 6 kcal/mol. As $E_x$ protonation is linked to its springing out of the helix cage, the filled central site provides a source of positive cooperativity by making $E_x$ protonation less costly for the second Cl$^-$ binding.

The CLC fold itself, i.e. the protein backbone is another source of cooperativity. We found that $E_x$ proton affinity in the apo state is determined by its interaction with the backbone: $E_x$ proton affinity increases with decreasing backbone interaction energy ([Fig. R4.a](#)).

The extreme positive potential formed by the helix cage rather than residues ensures there is weak coupling to protons except for the special gating residue, $E_x$, which is competing for the same site. Additionally, the helix cage stabilizes the Cl$^-$ ions better than the $E_x$ ionized carboxyl group, so the backbone contribution to the anion binding affinity of the sites increases by at least 5 kcal/mol by substituting Cl$^-$ for carboxyl$^-$ ([Fig. A3, Table A4](#)). Moreover, the helix cage is a cooperative feature as it stabilizes two anions ([Fig. A3](#)), a feature recovered in the combined energy step diagram for the reaction cycle ([Figure A6](#)): the lowest states are those with two charges (XC or 0C|$E_x^{-1}$). The apo structure with only one charge in the binding region ($E_x^{-1}$) is at higher electrostatic energy than the states with two negative charges, but the motions of $E_x$ and Ser$_c$|Tyr$_c$ into their binding sites stabilize the apo structure.

The sites stabilization by the helix cage, a signature of the CLC fold, together with their interactions with the gating residues side-chain ($E_x$ and Ser$_c$|Tyr$_c$) offer an appreciation of the
structural basis of their affinities: Both Cl$^-$ binding sites are subjected to nearly the same conditions bearing on the strength of their Cl$^-$ affinities: these will not vary as long as $\alpha F$ and $\alpha N$ stay aligned and as long as the respective gating residue, $E_x$ for $S_x$ and $Ser_c$ and $Tyr_c$ for $S_c$, has a side-chain conformation that is optimal for binding.

Experimental data have so far not revealed a disruption of the selectivity filter even under various pH conditions, or when performing mutations or cross-linking reactions {Accardi et al., 2006; Lobet and Dutzler, 2006; Robertson et al., 2010; Picollo et al., 2012; Basilio et al., 2014}. However, a recent study investigating the change of the distance, $\Delta r$, between three pairs of residues C$\alpha$ from the N-termini of helix F and helix N as result of collective motions. They found that up to 8 collective motions in these helical domains could produce $\Delta r > 1.5\AA$, a change greater than was made with our E$^{\text{Mid}}$ position. Perhaps a FRET study could find out the nature of this motion: Could it produce an enlargement of the helix cage, and thus lead to a reduction, albeit transiently, of the selectivity filter Cl$^-$ binding affinity for at least one site, e.g. $S_x$?
Table A1. Averaged Cl\(^-\) affinities of S\(_x\) and S\(_c\) and H\(^+\) affinities of the gating residue at pH4.5

<table>
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<tr>
<th></th>
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<td>(S_x^0)</td>
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Table A1. Averaged Cl\(^-\) affinities of S\(_x\) and S\(_c\) and H\(^+\) affinities of the gating residue at pH4.5. The doublets of values represent the affinity and its standard deviation (s) in the given structure type (\(E_{\text{UP}}, E_{\text{MID}}, E_{\text{DN}}, E148D, Q148D, E148A, E148Q\)). The two-character run identifier specifies the titration condition of each site: \(X0\) and \(X1\): \(S_x\) titrates freely while \(S_c\) is unbound, or bound and fixed, respectively; \(0C\) and \(1C\): \(S_c\) titrates freely while \(S_x\) is unbound, or bound and fixed, respectively. The run identifier superscript denotes the fixed state of \(E_x\) in that run: 0: neutral, -1: ionized, while absence of superscript means \(E_x\) was free to titrate. The error associated with Continuum Electrostatic methods typically is about \(\pm 3.0\) kcal/mol, which happens to be the maximal s.e. of the dataset if ED is excluded (the variation there is due to the different conformation of \(E_x\) in the two subunits). NA values indicate that the equilibrium constant lies outside the titration range and in the unfavorable regime.
Table A2. Average pK' and pK' shifts of the gating residue

<table>
<thead>
<tr>
<th>Run</th>
<th>E_x, pK'_{4.5} (s.d.)</th>
<th>pK' shifts vs. apo state of same run</th>
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<td>E^{UP}</td>
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<td>Q148D</td>
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<td>E^{MID}</td>
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<tr>
<td>E^{DN}</td>
<td>-4 (1)</td>
<td>9</td>
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Table A2. Average pK' and pK' shifts of the gating residue. The pK shifts are computed using the apo pK' of the same structure. The structural apo (E^{APO}) or WT (E^{x}) apo pK' is about -6.

Table A3. Estimates of the MID to UP transition (2 chains)

<table>
<thead>
<tr>
<th>Average MFE (kcal/mol)</th>
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<td>Difference</td>
<td>UP - MID</td>
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<td>-2</td>
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Table A3. Estimates of the MID to UP transition. The transitions when E_x is neutral or ionized were obtained from the difference in the mean field energy (MFE) between the 5 most favorable neutral and the 5 most favorable ionized conformers of E_x, respectively. These values are not dataset averages: the conformer population comes from one representative chain in the UP subset and one representative chain in the MID subset; these same two chains affinities were also used to build the thermodynamic cube.

Table A4. ΔG_{inter.} between the backbone and E_x or bound Cl⁻ ions

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<tr>
<th>Backbone ΔG_{inter.} with:</th>
<th>Difference viz. E_x</th>
<th>Cl⁻</th>
<th>Cl⁻ repulsion (kcal/mol)</th>
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<td>(kcal/mol)</td>
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<tr>
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Table A4. ΔG_{inter.} between the backbone and E_x or bound Cl⁻ ions. The bound Cl⁻ ions in S_{x} (CL_{x}) and in S_{c} (CL_{c}) are better stabilized by the backbone than the E_x side-chain in the apo state by at least 5 kcal/mol, except in DOWN. The value for E_x in E^{MID} is -23 kcal/mol, while that of the Cl ions averaged over EA and EQ is -28 kcal/mol. The favorable backbone ΔG_{inter.} completely overcomes the ion repulsion when both sites are bound in E^{UP}.
Table A5. Adjustments in the Cl$^-$ and H$^+$ affinities for closed thermodynamic cycles (2 chains)

<table>
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<tr>
<th>Sites</th>
<th>ΔG$_{b,rel}$</th>
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<th>New</th>
<th>MID adj.</th>
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<td>X1$^C$</td>
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<tr>
<td></td>
<td></td>
<td>X0</td>
<td>1.8 0.0</td>
<td>1.8</td>
<td>-4.8 -0.5</td>
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<tr>
<td></td>
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<td>X0$^C$</td>
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<td>6.6</td>
<td>10.2 0.2</td>
<td>10.4</td>
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Table A5. Adjustments in the Cl$^-$ and H$^+$ affinities for closed thermodynamic cycles (2 chains). The Cl$^-$ affinity is obtained via Monte Carlo simulations of chemical potential titrations, while the H$^+$ affinity of E$_x$ is computed via the Mean Field Energy (MFE) framework, thus it is likely that the inclusion of these quantities in a thermodynamic cycle does not yield a cycle sum of 0. The states (runs) listed combine into a thermodynamic cube representing the equilibria of a three object-system (2 Cl$^-$ binding sites, 1 ionizable group). The adjustments reported here result in a cycle sum of -0.1 kcal/mol in UP and of 0.1 kcal/mol in MID, and they are within the method error of about ±3 kcal/mol. The data is that of a representative chain in each of the UP and MID subsets.
Gating residue conformations

\( E^{\text{MID}} \)

The MID conformation was obtained after manipulation of \( E_x \) side-chain to allow an ion to bind \( S_x \) and estimate the site \( \text{Cl}^- \) affinity with the side-chain in close proximity. This ad hoc position is devoid of van der Waals clashes or torsion energy: it is as well stabilized as the UP position. It fits between the WT 1OTS and UP 1OTU conformations seen in an MD simulation when \( E_x \) is protonated (Ko and Jo, 2009).

\( E^{\text{UP}} \)

In our conservative setup, the UP conformation of \( E_x \) is the same as that of the Gln mutant. In the only MD simulation showing the conformation of protonated \( E_x \) (Ko and Jo, 2009), the side-chain is closer to \( S_x \) than the Gln amido group is in 1OTU. There is a positional change of the gating residue due to a distortion in the tip of helix F (Figure A0). If this distance were due to a rotation of the entire helix F, the angle would be over 50°, a domain movement out of reach in ns-long MD simulations. It is unknown to us what the time scale of an MD simulation would need to be to obtain a distortion at the end of a 6-turn-long helical segment.

\[ \theta = \Delta / r_{\text{helix}} \]
Figure A0. Helix F distortion at N-terminal end in CLC-ec1 structures

Figure A0. Helix F distortion at N-terminal end in CLC-ec1 structures: Helices F (blue) and N (orange) in two E. coli structures. 2EX. (Lobet and Dutzler, 2006): Structural apo, 2EXW: E148, 2EXY: Q148 1OT. (ions removed for clarity) (Dutzler et al., 2003): 1OTS: E148, 1OTU: Q148. The position of the gating residue Cα-atom differ by Δ=1.4 to 1.2Å in the 1OT- and 2EX-pair, respectively. In contrast, in positions beyond T151 of helix F (about the second turn), as with any helix N positions, the Cα-Cα distance is identical (Δ~0.5Å).

E148D and Q148D

The Aspartate mutants were setup in the WT and UP backbone (E148D and Q148D, respectively). The residue shorter side-chain can rotate by 110° in Q148D, and by 135° in E148D. These mutants are the only ones found with different conformations both in the apo or bound states. In the apo state, E148D can adopt a MID-like position (same Cα-Cβ direction as that of E in E^MID), while in the bound state, it can be found in a 'down-and-out' conformation: outside of the helix cage and directed towards I186. The Q148D mutant can adopt an occluded form in the apo state that is not as deeply reaching into the helix cage as in E^APO because of the displacement of helix F noted above, else it is UP-like. Mechanistically, this wide ranging conformational freedom can be disruptive, yet how it can be related to the Asp mutant ability to block transport (Feng et al., 2012), or to drastically depress it (Traverso et al., 2005) is still unclear.
Without a change in backbone conformation, $E_x$ cannot adopt the down conformation seen in the *C. merolae* structure (3ORG) where helices F and N have slid downward over 1Å relative to WT ($F357-C_{\alpha}$ is closer to the external solution, while $E_x-C_{\alpha}$ is closer to the central site by this distance) (Figure A1).

**Figure A1:** Gating Glu downward conformations in E.coli and C. merolae

The downward conformation in 3ORG (Feng et al., 2010), may not have been found if the other CLC isoform had been used. The two *C. merolae* isoforms are rather anomalous in their substitution of the Arg or Lys residues that are usually found at the position preceding $E_x$ (i.e. $R147$ in E. coli).
Figure A2: Alternate sequence motif of C. merolae isoform with WT backbone conformation

Figure A2: E. coli, 1OTS (green: residues R147 and E148; 2.5 Å resolution); C. merolae, 3ORG (cyan; residues W209 and E210; 3.5 Å resolution) and the other isoform of C. merolae (pink, residues P190 and E191; accession # gi 544213481 (Nozaki et al., 2007)). The homology model for the short segment of the C. merolae isoform was obtained from the Zhang lab using the I-TASSER program (Zhang, 2008; Roy et al., 2010; Yang et al., 2015).

The isoform from which 3ORG is derived has the bulky aromatic Trp residue at this position, while the other has Pro. Figure A2 suggests that even a Pro residue at position 147, which usually harbors either Lys or Arg residue, would adopt the WT backbone conformation. Thus, the different backbone of 3ORG is likely due to the presence of the bulky Trp residue at position 147 and not to the x-ray resolution difference. The downward conformation of E_x with the WT backbone, E^{DN}, produced a lateral shift of ~1 Å in the position of the two Cl ions at S_x and S_c. There, E_x conformer in E^{DN} sits in between S_x and S_c and allows for binding of two Cl ions, unlike the situation in 3ORG where the backbone shift enables E_x side chain to occupy the central site. This shift of the ion positions in E^{DN} leads to a slightly different backbone stabilization. In the apo state, E_x in E^{DN} is stabilized by its own sequence motif backbone elements as equally as in E^{MID}, but it gains stability from the backbone interaction with the F357 sequence motif so that
overall, its backbone interaction energy is at a level nearly midway between $E^{\text{apo}}$ (structural apo) and $E^{\text{MID}}$ (Figure A3, sequence region from the F357 motif and beyond). However, this conformation in the bound state (Figure A3, middle and bottom panels), while still highly favorable, is 2 to 4 kcal/mol less beneficial to ion stabilization.
Stabilization of the $S_x$ and $S_c$ binding sites by the backbone inverted topological repeat

Figure A3. $E_x$ and $Cl^-$ binding sites backbone interaction energy running sum

Figure A3. $E_x$ and $Cl^-$ binding sites backbone interaction energy running sum. Running sum of the backbone interaction energy averaged over chains A and B in representative structures (kcal/mol). Top panel: with $E_x$ in the apo state. Middle and bottom panels: with $Cl$ bound at $S_x$ ($Cl_x$) and $Cl$ bound at $S_c$ ($Cl_c$). There are only two sequence motifs that contribute significantly to stabilizing $E_x$ in the apo state and $Cl_x$ via the backbone elements: the first corresponds to $E_x$ sequence motif ($L^{145}G^{146}R^{147}E^{148}G^{149}P^{150}xV^{152}$; red box), the other one corresponds to the motif of conserved F357 (GxFxPxL; green box), which includes nearly the entire loop between $\alpha J$ and $\alpha K$ (S350-F357) and the first four residues of $\alpha K$. $Cl_c$ is stabilized by the central Serine sequence motif (purple box), as is $E_x$ in the DOWN structure in the apo state, but to a lesser extent.
The running sum of the interaction energy between the backbone and the bound Cl ions at \( S_x \) or \( S_c \) reveals that the same structural motif stabilizes the two sites almost equally (Fig. A3). The two motifs, 'E\(_x\) motif' and 'F357 motif' are located at the N-terminal of helices F and N, respectively. The inverted topological repeat of the CLC fold, whereby two \textit{structurally similar} halves are rotated about 180°, brings these two ends almost face-to-face half-way in the transmembrane region forming a helix cage (Aqvist et al., 1991). The external site is better stabilized than the central site by about 2 kcal/mol (26 vs. 24 kcal/mol), despite the central site being helped by the backbone elements of its coordinating, and inner gate residues, Ser\(_c\) (S107) and Tyr\(_c\) (Y445).

In the apo state, \( E_x \) is ionized and similarly stabilized by the backbone elements of the same motifs, although to a lesser extend as the spherical ions fit the helix cage better. The strongest bond stabilizing \( E_x \) is an intramolecular bound with its own NH atom (~3 kcal/mol), which it loses upon rotating in the UP position (Fig. A4).

Thus the selectivity filter structure stabilizes two anions. The evaluation of this interaction provides, to our knowledge, the first explanation for the robustness of the 2Cl\(^-\):1H\(^+\) stoichiometry amid various ion concentration and membrane potential conditions (Accardi, 2015).
Figure A4. Strongest amide hydrogen-bonded residues in the structural motifs of $S_x$.

Only the strongest interactions are shown for clarity. This energy, in the form of a new hydrogen bond with $\text{CL}_x$ and amide hydrogen atoms, is available once $E_x$ rotates outward. The 5 bonds depicted, G146, R147, E148, F357 and A358 amount to about 10 kcal/mol, or 40% of the total backbone interaction energy with $\text{CL}_x$. 
Figure A5. Cl⁻ binding affinity of the external site: energy from MC titration versus MFE energy.

Figure A5. The Sₓ-bound ion total energy calculation in the mean field energy approximation (MFE) captures 90% of the binding affinity ($pK_{d,n} = -\log(K_d^n)$) obtained from the Monte Carlo titration (with $E_x$ fixed neutral). The exclusion of 2 outliers from the best fit line (black line) identifies a systematic under estimation on the part of the MFE method of about 1pH unit.
Figure A6. Energy step diagram for $2\text{Cl}^-:1\text{H}^+$ binding

Figure A6. Energy step diagram for $2\text{Cl}^-:1\text{H}^+$ binding. Reaction pathway for $2\text{Cl}^-$ inflow, $1\text{H}^+$ outflow in a representative run of the MID and UP subgroup. The favored path in MID was matched in the UP subset. The energy levels of the UP structure were positioned according to the $(\text{MID} \rightarrow \text{UP})_{\text{apo}}$ energy estimate. The combined cycle uses the most favorable states. Binding of $\text{Cl}^-$ at the external site promotes gate opening and protonation (transition to the UP conformation). Bound $\text{Cl}^-$ translocates to the central site and returns to a more constricted state until the second $\text{Cl}^-$ ion binds the newly vacated external site, followed by release of the central site. Another $\text{Cl}^-$ translocation step returns to the MID conformation before the release of the second $\text{Cl}^-$, and finally the $\text{H}^+$. +add: the up data is shifted by the energy estimate of the conformational change between the up and mid structures (+12 kcal/mol).

Figure A7. Thermodynamic cycles in UP and MID (2 chains) for 2 $\text{Cl}^-$ binding sites and $E_\text{x}$

Figure A7. Thermodynamic cycles in UP and MID (2 chains) for 2 $\text{Cl}^-$ binding sites and $E_\text{x}$. Vertices: $\text{Cl}^-/\text{H}^+$ occupancy states denoted by a 2-character, 1-subscript identifier: the first character pertains to $S_x$, the other to $S_c$, and the superscript denotes the charge state of $E_\text{x}$. Vertical arrows: $\Delta G_{\text{prot}}$ (H$^+$ affinity) of $E_\text{x}$ in kcal/mol and corresponding pK$'$ in parentheses. Horizontal arrows: $\Delta G_{\text{b/rel}}$ of $\text{Cl}^-$ binding at $S_x$ (kcal/mol). Slanted arrows: $\Delta G_{\text{b/rel}}$ of $\text{Cl}^-$ binding at...
$S_c$ (kcal/mol). Boxes values on the dashed arrows are estimates of the corresponding MID-$>$UP transitions under the hypothesis that these are commensurate with $E_x$ proton affinity (kcal/mol).

**Figure A8.** Other components of the energy of $E_x$ and their dependence with $E_x$ apo pK'.

**Figure A8.** Other terms contributing to $E_x$ total energy in the apo state: van der Waals interaction energy (top); Residues pairwise interaction energy (middle); Desolvation energy (bottom). Filled circles: chain B; Unfilled circles, chain B; Dotted circles: $E_{DN}^N$ structure.
Table A6. Species list in alignment with accession numbers

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<td>gi:19883990 E. coli CLCa</td>
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<td>AtCLC-a, Arabidopsis thaliana</td>
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<td>gi:1652876 Synechocystis sp. PCC6803, cyanobacterium Jayaram et al., 2011</td>
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<td>gi:167008040 Citrobacter koseri ClCB Phillips et al., 2012</td>
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Figure A9. Protein sequence alignment. BLASTP alignment using default parameters {Altschul et al., 1997; Altschul et al., 2005}. Shown are the alignment portions for the three key residues of the selectivity filter: Ser_{c}, E_{x} and Tyr_{c}. The alignment shows the degree of conservation in the transmembrane region (common fold) of these proteins. Red: 80-100% identity; orange: 60-80% identity.
<table>
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<tr>
<th>Short</th>
<th>Full citation</th>
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<tbody>
<tr>
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