Prerequisites to proton transport in the bacterial ClC-ec1 Cl^-/H^+ exchanger

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Chloride channel (CLC) genes are expressed in species ranging from bacteria to humans, in which nine of them are in mammals. CLC proteins were originally thought to function only as Cl^- selective ion channels, but it later became clear that many operate as Cl^-/H^+ exchangers in which the transport of Cl^- in one direction is stoichiometrically coupled to the transport of H^+ in the opposite direction (1–3). The CLC ion channels are expressed in plasma membranes, where they stabilize, for instance, the resting membrane potential in skeletal muscle fibers and balance salt transport in the kidney, inner ear, and other epithelial tissues (4). The Cl^-/H^+ exchangers, also known as antiporters, are rather confined to the membranes of endosomes, lysosomes, and synaptic vesicles, where they have been implicated in the acidification of these intracellular compartments. The first high-resolution crystal structures of Cl^-/H^+ exchangers from bacteria were resolved more than a decade ago (5, 6). Along with exquisite biophysical characterization, they provided crucial details about the molecular aspects of the CLC antiporter function. However, although the chloride permeation pathways in Cl^-/H^+ exchangers are rather well characterized today, the mechanisms of the H^+ translocation process remain unclear. In PNAS, Han et al. (7) used molecular dynamics (MD) simulations of the ClC-ec1, a CLC exchanger from *Escherichia coli* (6) to specifically address this issue. The predictions of their calculations were tested by additional experiments, providing a robust description of the molecular prerequisites to proton transport in ClC-ec1 and a framework for refining models of the Cl^-/H^+-coupled transport in CLCs.

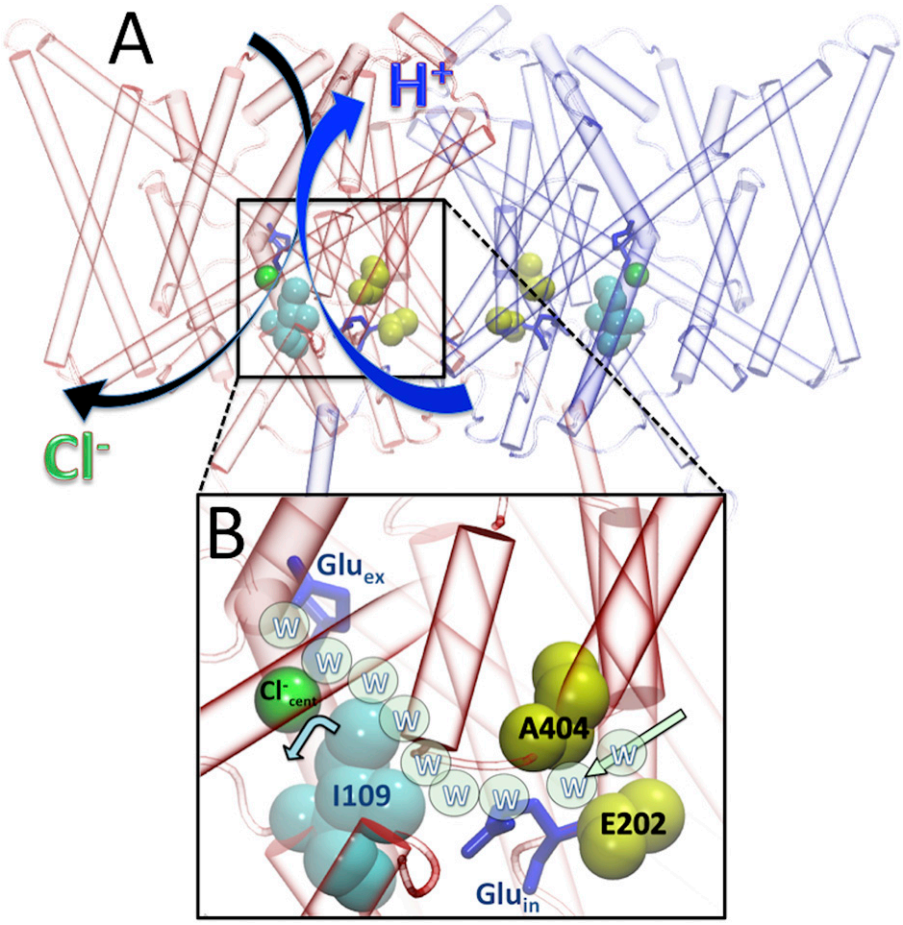

Fig. 1. Proton permeation pathway in CLC-ec1. (*A*) Topology of the ClC-ec1 dimer viewed from the membrane in a cartoon representation. The black and blue arrows represent the putative Cl^- and H^+ pathways in one domain. (*B*) Zoom into the region encompassing the residues critical to proton transfer. A404 and E202 forming the entry portal (light green arrow) of the water molecules are shown as yellow spheres. The critical I109 is highlighted as cyan in a space filling model. The broken cyan arrow indicates the motion it should undergo to allow for the formation of a continuous water (small light spheres) wire able to conduct protons through a Grotthuss shuttling mechanism between Glu_{in} and Glu_{ext} (shown as blue sticks). The central Cl^- ion crucial for the water wire stabilization is shown as a green sphere.

CLC proteins assemble as dimers. The Cl^-/H^+ exchangers structures (5, 6) revealed that each subunit contains an ion translocation pathway in which three binding sites for the anions line up: the internal and external sites (S_{int} and S_{ex}, respectively) are in direct contact with the intra- and extracellular solutions, respectively. The central site, S_{cen}, located in the middle of the bilayer, is isolated from both aqueous phases. It is partially coordinated by the hydroxyl oxygen atoms of S107 and Y445 (Tyr_{cen}) and by the side chain of E148. In the WT ClC-ec1 structure, the negatively charged (E148 or Glu_{ex}) side chain occupies S_{ex} competing with Cl^-; the chloride anion occupies S_{ex} only when E148 is removed or protonated. Adopting this conformation, Glu_{ex} therefore blocks access of extracellular anions to S_{cen} and was shown to be important for the voltage-dependent gating of CLCs (8).

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Glu_{ex} is also crucial for the exchange/coupling of H^+ to Cl^- (1). Another glutamate (E203 Glu_{in}) partially buried in the intracellular vestibule (Fig. 1), located on the opposite side of the membrane, and found specifically in Cl^-/H^+ exchangers modulates the transport of protons. Early mutagenesis experiments suggested that Glu_{ex} and Glu_{in} are the extra- and intracellular H^+ acceptors in ClC-ec1 (2), and their role was confirmed in other CLC transporters (1): when mutated to nonprotonatable residues, the CLC exchangers lose the ability to transport H^+, whereas Cl^- transport is retained. Although it was shown that for some CLC channels Glu_{in} does not play the same uniformly necessary role as Glu_{ex} (9), the two residues are believed to be the H^+ entry and exit points in ClC-ec1. These exquisite details still leave us with a long-standing question: how does a proton cross the ∼14-Å long gap lined mostly by hydrophobic residues that separates the two Glu residues? Discovering the rules underlying this crossing is a major aim in deriving the CLC exchange mechanism.

One of the suggestions put forward (10–12) is that excess protons (H^+) translocate within the gap via a Grotthuss shuttling mechanism (13) that can be made possible by the rearrangement of protonatable protein residues and water molecules. It was further noted (11) that the experimentally observed proton flux in CLCs, ranging from few per millisecond (14) to a few tens per second (15), is 8–11 orders of magnitude slower that the hopping time across water molecules. This supports the assertion that the H^+ transfer process in Cl^-/H^+ exchangers is rather a rare event and that if it is mediated by solvent proton-transport competent configurations, these are probably not stable enough to be captured in crystal structures.

This has triggered a number of molecular modeling studies (11, 12, 16, 17), all aimed at determining how a water wire can fit into the hydrophobic gap between Glu_{ex} and Glu_{in} in a conformation that allows for a Grotthuss shuttling mechanism. The present study (7) stands out as the most insightful and robust one. Indeed, Han et al. show that under normal pressure and temperature conditions, water molecules can spontaneously hydrate the hydrophobic region by penetrating the internal structure from the cytoplasmic bulk phase. Quite interestingly, the central region between Glu_{ex} and Glu_{in} can accommodate enough molecules to form transiently continuous water wires. It experienced within the ∼500-ns MD sampled run frequent emptying and refilling events. The lifetimes of such transient structures were found in the nanosecond timescale range, i.e., long enough to allow for H^+ shuttling between the two extreme Glu residues.

Another significant insight of this work (7) is to provide a mechanistic view of the prerequisites for such water structures to occur. Scrutiny of the simulation runs revealed that (*i*) water molecules mostly enter each monomer through a portal lined by residues E202, Glu_{in}, and A404 located near the dimer interface; (*ii*) this requires a minute conformational change of I109 belonging to the GSGIPE CLC amino acids signature sequence; and (*iii*) the presence of Cl^- in S_{cen} plays a critical electrostatic role in the stabilization of the water wires (Fig. 1).

Impairing any of the three prerequisites to the formation of water wires extending from Glu_{in} to Glu_{ex} has a severe consequence on the H^+ transport in ClC-ec1.

Han et al. (7) test these predictions through a series of supplementary simulations and exquisite experiments: mutagenesis of the portal polar lining residue E202 was already known to yield a significant effect on H^+ transport (18). Here the authors show that mutation of the most important residue of the portal, A404, to a bulkier one impairs the proton transfer as well, with a relatively mild effect on the Cl^- pathway. Mutagenesis experiments were also performed to test whether and how H^+ transport is sensitive to I109 substitution. Mutations of the latter to five residues all resulted in a reduction of the proton turnover rate and had no apparent effect on the Cl^- pathway. Finally, comparative simulations with and without the Cl^- ion in S_{cen} confirmed that the presence of the anion promotes and stabilizes the formation of water wires and therefore promotes H^+ transfer, which is consistent with data showing a correlation between proton coupling and the central site occupancy (19).

Han et al. (7) therefore demonstrate that impairing any of the three prerequisites to the formation of water wires extending from Glu_{in} to Glu_{ex} has a severe consequence on the H^+ transport in ClC-ec1, which strongly suggests that the latter indeed involves a shuttling pathway. Their study describes a remarkable minute setting involving elements of the structure not thought of before to be functionally coupled. The molecular ingredients of the cross-talks between these elements and the Cl^- pathway are yet to be fully characterized. The findings allow us, however, to start discriminating between the various kinetic models proposed to describe Cl^-/H^+ exchanger transport cycles.

Many other intriguing questions remain. How do these H^+ pathways evolve during the working cycle of the CLCs? How do they compare between CLC homologs? The progress achieved by Han et al. (7) in combining simulations and experiments shows that answers to these questions are also within reach.

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