COMMENTARY

Q-cubed mutant cues clues to CLC antiport mechanism

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A recent *eLife* report from the Maduke laboratory at Stanford University (Chavan et al., 2020) describes a new CLC Cl^-/H^+ antiporter mutant crystal structure. Any publication dwelling on a single mutant of a long-studied protein provokes the question: is it meritorious—a deep dive into novel details that illuminate a molecular mechanism—or is it meretricious—a shiny object that merely delights the eye? I vote here for the former, being as enthused by this publication as by any on anion transport that I have read during my four decades in the field.

The CLC superfamily of anion transport proteins includes both ion channels and H^+-coupled anion antiporters built on identical structural plans (Jentsch and Pusch, 2018). These ubiquitous membrane proteins carry out their transport tasks in a multitude of biological contexts, regulating blood pressure and skeletal muscle excitability, facilitating acid resistance in enteric bacteria, modulating lysosomal pH, and countering environmental F^- toxicity in microbes, to cite just a few. Atomic-resolution structures of CLC-ec1, a Cl^-/H^+ antiporter from *Escherichia coli*, were solved nearly 20 yr ago (Dutzler et al., 2002; Dutzler et al., 2003), and despite a parade of many CLC structures over the years, this homologue remains the most deeply analyzed and serves as the go-to model for unraveling the mechanistic minutiae of transport.

A bit of background down in the CLC weeds places this new story in context. Secondary active transporters—antiporters and symporters—drive substrates thermodynamically uphill by using the free energy of dilution of secondary substrates, most often H^+ or Na^+, falling down preexisting gradients. These coupled transporters are often said to act by "alternating-access" mechanisms. Though widely used in the field, this term is a misnomer because it in no way denotes a specific mechanism. Rather, alternating access is the essential defining feature of coupled transport itself. In all transporters, regardless of any particular mechanism, aqueous substrates first bind exclusively from one side of the membrane and subsequently dissociate exclusively to the other. Transport can occur by vastly different conformational cycles involving strict rules for cosubstrate occupancy: from large, phosphorylation-linked nodding-donkey or rotary movements, to subtle configurational changes accompanying electron tunneling, but all demand sided alternation of substrate access to sites within the protein. The only alternative to transport by alternating access is transport by simultaneous access, and we have a time-honored name for proteins that do that—channels.

Most known antiporters switch sides in clothespin-like or elevator-like backbone movements, typically up to 20 Å, and they work by simple, easily visualized "ping-pong" mechanisms that strictly forbid simultaneous occupancy of the coupled substrates and permit the sided conformation switch only when substrate is bound. CLC antiporter mechanisms are fundamentally different, however, since Cl^- and H^+ ions occupy their distinct sites together at various stages of the exchange process (Accardi et al., 2005; Picollo et al., 2012). Moreover, the many CLC structures in the database all show essentially identical backbone conformations, suggesting a mechanism involving only rotameric movements of a single glutamate side chain (Dutzler et al., 2003; Feng et al., 2010). Although subsequent functional and spectroscopic evidence suggested that backbone rearrangements do occur during transport (Basilio et al., 2014; Khantwal et al., 2016), their details are unknown, their functional significance is unproven, and their displacements are thought to be much smaller than the movements known in other transporters.

Much work over the years has produced a basic picture of how CLC antiporters coordinate the stoichiometric, oppositely directed movement of two Cl^- ions and one H^+ ion through CLC-ec1 (e.g., Feng et al., 2010). Two anion-hungry sites lie in near the protein's center: the external site (S_{ex}), which is located toward the protein's extracellular surface, and the central site (S_{cen}) ~5 Å below it, which is closer to the intracellular side (Fig. 1 A). In the WT protein, a central Cl^- ion occupies S_{cen}, and the deprotonated carboxylate of the key external glutamate (E_{ex}) sits in S_{ex}. The central Cl^- is buried, occluded from extracellular solvent by the E_{ex} carboxylate above it and from intracellular solution by a conserved gate below. The cycle moving H^+ outward and Cl^- inward commences when intracellular H^+ moves

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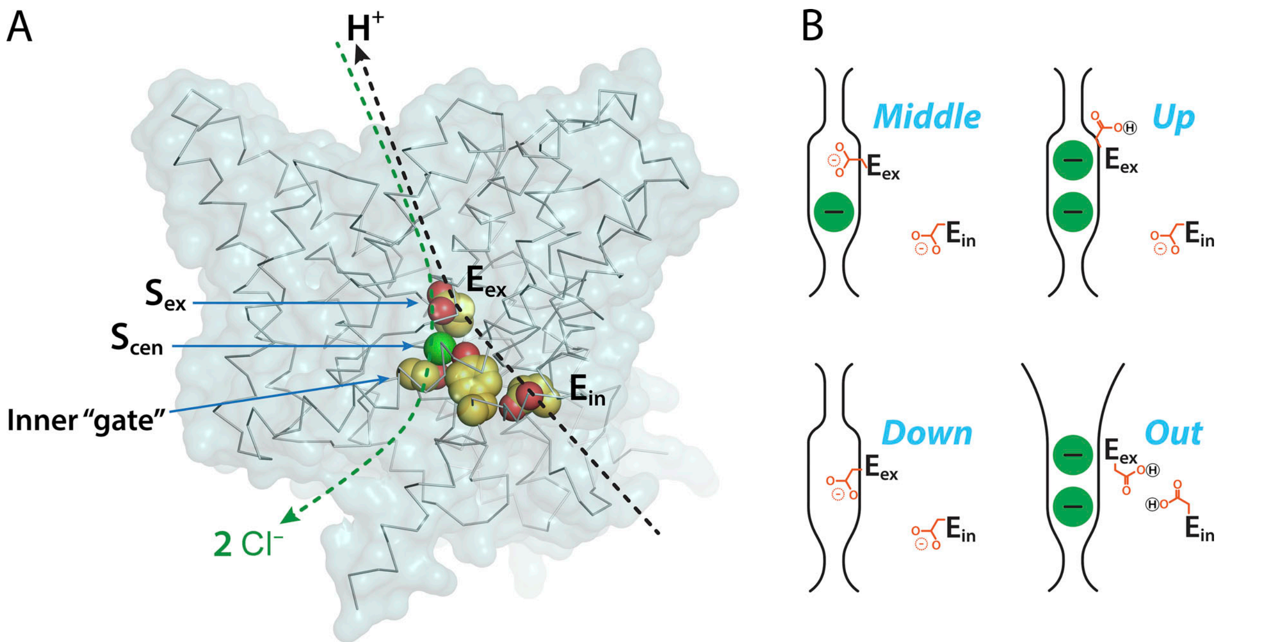

Figure 1. **Ion pathways in CLC transporters. (A)** CLC-ec1 (Protein Data Bank accession no. 1OTS), showing one of the two identical subunits. Key features, including anion-binding sites (S_{ex} and S_{cen}) and mechanistically critical glutamate residues (E_{ex} and E_{in}), provide the framework for ion transport in CLCs in which pathways for Cl^- and H^+ are shared through a portion of the protein and diverge at the center. **(B)** Four distinct rotameric configurations for E_{ex}. Cartoon depictions of the ion–pathway regions show E_{ex} in the previously known middle, down, and up configurations as well as in the new out position, which is accompanied by a configurational change of E_{in}.

through the protein to protonate E_{ex}. The now-neutral side chain, no longer stable in the anion-binding region, flips upward, delivering its proton to the outside solution, thereby unblocking a pathway for a second extracellular Cl^- to enter and occupy S_{ex}. This key antiport event couples Cl^- entry, H^+ exit, and the rotameric switch of E_{ex}, and the two bound Cl^- ions delineate an anion pathway running through the protein. How, then, does the intracellular proton reach the far-off carboxylate of E_{ex}? Early work identified a second key glutamate residue, internal glutamate (E_{in}), that appears to act as a waystation for H^+ transfer from intracellular solution to E_{ex} (Accardi et al., 2005; Lim and Miller, 2009). Proton coupling was abolished with this side chain replaced by nondissociable substitutes. The location of E_{in} near the intracellular side of the dimer interface, distant from the anion pathway, argued for a bifurcated pathway, with Cl^- and H^+ sharing a conduit from extracellular solution to the Cl^--binding region, then splitting off into separate pathways leading to the intracellular side (Fig. 1 A). The cycle continues when the E_{ex} deprotonated carboxylate re-enters the anion-binding region, pushing both Cl^- ions through the inner gate and delivering them to the intracellular side.

Workers in the field, although differing on details, agree on the cycle's basic outline, and all acknowledge that it raises fundamental, unresolved difficulties. How can protons traverse the ∼12-Å hydrophobic stretch between E_{in} and E_{ex}, since protonation of E_{in} (mimicked by Gln substitution) seems not to cause the movement of this key side chain (Accardi et al., 2005)? Why are H^+ coupling and pH dependence of transport retained in E_{in} substitutions by histidine or lysine, residues with protonation chemistry that is very different from glutamate (Lim and Miller, 2009), as well as in certain CLC homologues lacking a dissociable side chain at the E_{in} position? Why would electrically neutral E_{ex}, protonated extracellularly in its up rotamer, plunge down into the anion-binding region to displace a Cl^- ion at S_{ex}? Moreover, a dirty little secret typically left unexpressed in cartoons (but see Khantwal et al., 2016) haunts the picture: with E_{ex} in its proton-accessible up rotamer snorkeling to the outside, the extracellular pathway is still too narrow for Cl^- to pass through. And what's going on with the Cl^- pathway's inner gate, which appears closed in all antiporter structures? Explaining away these mechanistic soft spots has required much hand-waving, molecular dynamics simulation, and ad hoc proposals: a full-down E_{ex} rotamer with its carboxylate in S_{cen}, crystallographically observed in several homologues but only indirectly inferred in CLC-ec1 (Vien et al., 2017; Park et al., 2019), to reduce the separation of the two H^+-transfer glutamates to "only" 8 Å; proton-conducting water wires transiently connecting E_{ex} to E_{in} or directly to intracellular solvent to obviate the need for E_{in}; or protein breathing dynamics to transiently allow extracellular Cl^- access to S_{ex} or to open the inner gate to intracellular Cl^-. While none of these klooges outrages biophysical propriety, taken together they leave a sour taste in the mouth when trying to come up with a satisfactory Cl^-/H^+ antiport scheme.

The new CLC-ec1 structure provides plausible answers to most of these questions. The protein is mutated to mimic a form of the antiport cycle, which Maduke's group (Khantwal et al., 2016) had previously shown with fluorinated NMR probes to undergo some sort of backbone rearrangement at low pH, a physiologically relevant condition for this homologue, which helps *E. coli* survive passage through the stomach (Iyer et al.,

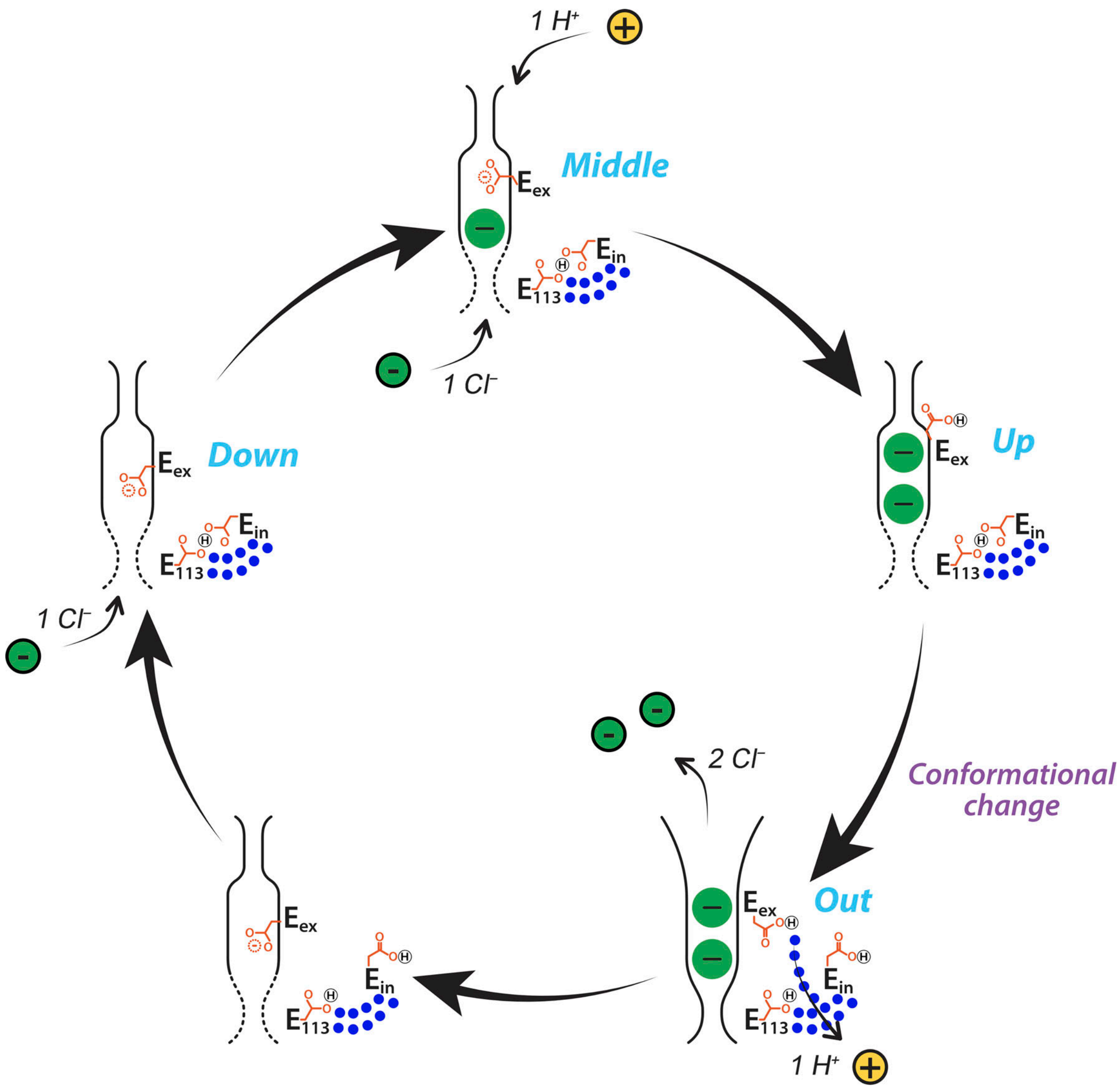

Figure 2. **Nearly magic-free mechanism for CLC Cl^-/H^+ transport.** Cartoon depictions of the ion-binding regions show how the rotary movement of E_{ex} through its four configurations (middle, up, out, and down), coordinated with Cl^-/H^+ binding/unbinding/translocation, can achieve 2:1 Cl^-/H^+ antiport. For clarity, one direction of the transport cycle is depicted; however, the mechanism works in both directions. This mechanism avoids previous magic, in that (1) it does not require deprotonated E_{ex} to compete with Cl^- for the anion pathway, (2) the opening of the extracellular pathway in the out conformation provides a clear pathway for Cl^- ions, and (3) the rotation of E_{in} away from E113 allows H^+ transport along water pathways and is thus consistent with the observation of coupled transport in CLC homologues that lack a titratable residue at the E_{in} position. The only remaining magical step concerns the inner gate, which is depicted here with dashed lines to indicate the uncertainty as to how and/or when the inner gate opens.

2002). The crystallized protein, designated QQQ, substitutes glutamine as a protonated surrogate for E_{ex}, E_{in}, and a third glutamate that H-bonds with E_{in}. The structure reveals a novel backbone conformation in four of the protein's 18 membrane-embedded helices. With maximum C_α movement of only 3 Å, the shift from WT and virtually all other CLC structures is subtle, but it profoundly alters both the Cl^- and H^+ pathways in three suggestive ways.

First, the external anion pathway now widens to a diameter of ∼3 Å. With the neutral Q_{ex} expelled from S_{ex} in this vestibule, the two Cl^- ions may freely move in single file between their binding sites and extracellular solution.

Second, the Q_{ex} side chain adopts a rotamer never before observed in any CLC antiporter. The electrically neutral headgroup abandons the anion pathway entirely, embedding itself in a cluster of nearby aromatic residues, a configuration recently suggested from simulations to form an external proton conduit (Leisle et al., 2020). The E_{ex} of CLC-ec1 is thus seen to adopt four distinct rotameric configurations (Fig. 1 B). Two of these, down and middle, are deprotonated and they occupy S_{ex} and S_{cen}, respectively; one of these, up at the protein–water surface, enjoys a pH-dependent equilibrium with extracellular water; and the new, protonated out rotamer avoids the anion pathway altogether. If relevant to the transport cycle, this configuration would eliminate the problem of a protonated carboxyl group competing with Cl^- in an anion-binding site.

Third, the Q_{in} side chain, released from its H-bonding partner, also adopts a new rotamer, flipping upward within air-kissing distance (5–6 Å) of Q_{ex} (Fig. 1 B). Remarkably, this movement, accompanied by local rearrangements, opens a pathway wide enough to potentially allow water to fill the space between Q_{ex} and intracellular solution. This observation raises the possibility that E_{in} does not directly transfer intracellular protons but instead is gated by pronotation to connect the protein's center to intracellular solution via a water-mediated H^+ pathway.

To test the pertinence of these unexpected structural results to the antiport mechanism, Maduke, in a COVID-appropriate, physically distanced collaboration, enlisted the spectroscopic muscle of Mchaourab at Vanderbilt and the computational power of Tajkhorshid at the University of Illinois (Chavan et al., 2020). The former group used double electron–electron resonance to observe pH-dependent, Å-scale distance changes between judiciously chosen residues. They confirmed the movements crystallographically predicted upon acidifying the WT protein and showed that QQQ distances were pH insensitive and matched the low-pH values of WT. Tajkhorshid's computational contribution provided a surprising insight into the nature of a putative H^+ pathway: molecular dynamics simulations of QQQ showed robust formation of water wires connecting intracellular solvent directly to Q_{ex} in the widened region observed in the QQQ structure. This result supports the authors' proposal that intracellular H^+ protonates E_{ex} via a water wire rather than via E_{in}. The role of E_{in} would instead be to promote the filling of this conduit with water upon a protonation-driven rotameric flip, thus rationalizing the conundrum regarding the absence of protonatable residues in some CLC homologues. To functionally test this, H^+ coupling was measured for an array of E_{in} substitutions and was found to be present in all, albeit at H^+/Cl^- stoichiometry that was substantially lower than the WT value of 0.5 (e.g., 0.1 for Ala and 0.013 for Gln).

These new observations and inferences lead to a Cl^-/H^+ antiport scheme that eliminates much of the magic from standard proposals (Fig. 2). The protein's extracellular side now offers two pathways, physically close but not congruent—a Cl^- pathway opened when the out form of E_{ex} is protonated, and rotameric acrobatics between out and up conveying the transported proton to and from solution. On the intracellular side, the proton, handed off to a water wire, exchanges with solution, leaving E_{ex} poised to enter the anion pathway in a down rotamer that displaces the central Cl^- ion. The mechanism posits an elegant rotary movement of E_{in}—up → out → down → middle → up—that keeps the carboxyl headgroup out of the anion pathway when protonated and occupying it only when deprotonated. The rotation's net chirality depends on the ion gradients determining the direction of net Cl^-/H^+ antiport. Remaining for future work is investigating the opening of the inner Cl^- gate below S_{cen}, which must somehow coordinate with the configurations of the extracellular pathways—currently a magic step in all proposed schemes. It will not escape the reader's eye that the mechanism is far more elaborate than ping-pong schemes of conventional antiporters. This complexity, the authors conjecture, may reflect constraints arising from a feature of CLC-mediated antiport that is so far unique in membrane biology: the opposite charges of the coupled ions.

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