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A provisional transport mechanism for a chloride channel-type Cl^-/H^+ exchanger

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Chloride channel (CLC)-type Cl^-/H^+ exchangers are widespread throughout the biological world, and one of these, CLC-ec1 from *Escherichia coli*, has been extensively studied. The structure of this protein is known, and several of its mechanistic hot spots have been identified, but a mechanism for Cl^-/H^+ exchange has not previously been offered. We herein confirm by direct measurements of Cl^- and H^+ fluxes a Cl^--to-H^+ exchange stoichiometry of 2, and summarize experimental facts pertinent to the exchange mechanism. While the mechanism must involve a conformational cycle of alternating exposure of substrate-binding sites to the two sides of the membrane, CLC transporters do not adhere to a familiar ping-pong scheme in which the two ions bind in a mutually exclusive fashion. Instead, Cl^- and H^+ occupy the ion-binding region simultaneously. A conformational cycle is proposed that accounts for the exchange stoichiometry, several key mutants and the tendency of the protein to become uncoupled and allow 'slippage' of Cl^-.

Keywords: antiporter; chloride channel; transporter; conformational cycle

1. INTRODUCTION

The chloride channel (CLC) family of Cl^- transport proteins includes both Cl^- channels and proton-coupled Cl^- exchange transporters (Zifarelli & Pusch 2007). This clean mechanistic split within a single protein family—a circumstance unprecedented in membrane biology—presents to the biochemist a fascinating question: how can two very different ion-transport mechanisms, electrodiffusion through a watery pore versus coordinated movement of substrates via conformational cycling, be supported by a common molecular architecture? Over the past few years, we have been attempting to attack this question on several fronts, one of which is to understand in detail how the transporters work. In this contribution to a meeting on the 'ambiguous interface' between channels and pumps, we describe our current understanding of CLC-ec1, the founding and most experimentally tractable member of the CLC Cl^-/H^+ exchanger subfamily. This CLC homologue from *Escherichia coli*, one of two in this organism (Iyer *et al.* 2002), is readily purified in quantity and reconstituted in various membrane systems for functional analysis. Moreover, its crystal structure is known (Dutzler *et al.* 2002, 2003), along with structures of various functionally informative mutants. Using this combination of capabilities, we are beginning to develop a picture of how this protein exchanges Cl^- and H^+, the provisional mechanism described below.

(a) *Stoichiometry of coupled transport*

The discovery that CLC-ec1 is a Cl^-/H^+ exchanger transporter rather than a Cl^- channel emerged from a close examination of this protein's electrical behaviour in a chemically defined reconstituted membrane system (Accardi *et al.* 2004). In particular, the 'reversal potential'—the voltage at which transmembrane current is zero—was found to vary with Cl^- and H^+ transmembrane gradients according to the *a priori* prediction of an obligatorily coupled antiport mechanism:

$$V_r = \frac{1}{1+r}(rE_{Cl} + E_H), \tag{1}$$

where E_i is the Nernst potential of the indicated ion and r the Cl^-/H^+ exchange stoichiometry. The experimental results were explained well with $r=2$. Thus, a 10-fold gradient of Cl^- with symmetrical pH produces a reversal potential of approximately 40 mV, 2/3 of the value expected for an ideally selective Cl^- channel, while a three-unit pH gradient with symmetrical Cl^- yields a reversal potential of approximately 58 mV, one-third the Nernst potential for H^+. Br^-, the only known faithful Cl^- substitute, behaves similarly to Cl^- (Nguitragool & Miller 2006). These results are quantitatively satisfactory, but this measurement of Cl^--to-H^+ stoichiometry rests on an assumption: that the reversal potential represents thermodynamic equilibrium rather than a steady state. This assumption was validated in experiments following H^+ fluxes in reconstituted liposomes (Nguitragool & Miller 2006), but it would nevertheless be desirable to measure the exchange stoichiometry directly by rates of Cl^- and H^+ transport. Accordingly, we performed the experiment shown in figure 1. Reconstituted liposomes were loaded with high Cl^- (300 mM KCl) and high buffering power (40 mM citric acid acid/KOH) at pH 4.8, where this transporter is maximally active (Accardi *et al.* 2004). The liposomes were suspended in 1 mM KCl, 300 mM K^+ isethionate, with low (2 mM) buffer

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One contribution of 16 to a Discussion Meeting Issue 'Membrane transport in flux: the ambiguous interface between channels and pumps'.

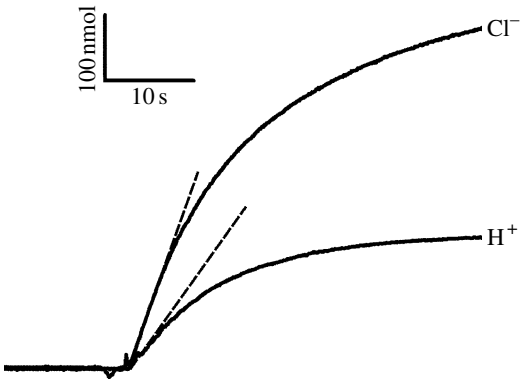

Figure 1. Direct measurement of Cl^-/H^+ stoichiometry. Liposomes were prepared for Cl^- and H^+ flux measurements (Nguitragool & Miller 2006; Walden *et al.* 2007) with CLC-ec1 at 1 μg protein/mg lipid, in the presence of high KCl and high buffering power, as described in text. They were suspended in a stirred cell in low-Cl^- solution with low buffering power, and the suspension was monitored electrochemically for efflux of Cl^- or influx of H^+, as indicated. The flux reaction was initiated by addition of valinomycin. A representative experiment is shown, with dashed lines representing initial rates for each ion.

at the same pH. Under these conditions, Cl^- will want to move 'downhill' out of the liposomes and thus drive H^+ influx against a pH gradient, but these fluxes can occur only if membrane polarization arising from electrogenic Cl^-/H^+ exchange is relieved by permeabilizing the membrane to K^+ with valinomycin (Accardi *et al.* 2004). The experiment was carried out in a stirred cell, where electrodes continuously monitored Cl^- concentration or pH of the suspension. In this way, the initial rate of Cl^- appearance and H^+ loss can be accurately determined under precisely the same conditions. Figure 1 illustrates such an experiment showing the initial rate of Cl^- efflux from the liposomes to be close to twice the rate of H^+ influx. In a series of such experiments, we obtained a Cl^-/H^+ flux ratio of 2.2 ± 0.3 ($n=6$), fully consistent with our earlier measurements by reversal potential.

(b) *Experimental facts bearing upon mechanism*

The classical approach to deriving a mechanism for a membrane transporter or channel is to subject the system to a detailed kinetic analysis. The pattern of kinetic changes as substrates, inhibitors and voltage are varied, and key regions of the protein are mutated, often provide testable clues about key molecular actors and conformational intermediates in the transport scheme. But we have been unable to apply this tried-and-true approach to CLC-ec1, for a prosaic but determinative reason: we have found no way to orient the protein in the membranes used for transport measurements. CLC-ec1 is studied by reconstituting purified protein into phospholipid membranes—liposomes for flux measurements and planar bilayers for electrical recording—in which the protein ends up in scrambled orientation, with roughly half facing each direction. Any kinetic measurement on such a system is undermined by this heterogeneity, and no satisfactory sided inhibitors of this protein have been found which can be applied to fully 'silence' one of the two orientations present (Matulef & Maduke 2005), as is routinely done in reconstituted ion channel studies (Park & Miller 1992; Heginbotham *et al.* 1999). This problem does not exist for transporters studied in biological membranes, as the proteins are reliably oriented by cellular protein-synthesis machinery; but measurements of CLC-ec1 have not yet been possible in native *E. coli* membranes. This is why the reversal potential—a 'null measurement' insensitive to orientation—has played such an important role in work on this transporter.

For these reasons, our investigations of CLC exchangers have proceeded haltingly through a series of functional and structural glimpses at the protein that have provided only sporadic hints at how movements of Cl^- are linked to those of H^+. This section summarizes the salient results from which a proposal for a transport mechanism emerges.

2. EACH SUBUNIT ACTS AS A TRANSPORTER

Many multi-subunit proteins work through cooperative movements of the subunits. Oxygen binding by haemoglobin, gating of many types of ion channels, and allosteric control of cooperative enzymes are familiar examples in which concerted quaternary rearrangements achieve the protein's functional purpose. So we might expect that the conformational cycle of CLC-mediated Cl^-/H^+ exchange requires relative movement of the two subunits of the homodimer. But this appears not to be the case. Fully coupled, kinetically competent Cl^-/H^+ exchange is carried out by a 'straitjacketed' construct of CLC-ec1, highly constrained by four cross-subunit covalent cross links (Nguitragool & Miller 2007). This result implies that the transport mechanism is contained within each individual subunit, a situation recalling double-barrelled fast gating in the CLC channel subclass (Middleton *et al.* 1994, 1996; Ludewig *et al.* 1996). In developing a mechanism, then, we view the homodimer simply as two transporters glued together and working in parallel, and can thus focus on ionic movements through the single subunit, unworried by complexities of cross-subunit interactions. CLC-5, a mammalian CLC transporter, also behaves in this fashion (Zdebik *et al.* 2008).

3. Cl^- MOVES ALONG A PATHWAY OF THREE ANION-BINDING SITES

The crystal structure of CLC-ec1 (figure 2), in which Cl^- (or Br^-) ions are readily visible (Dutzler *et al.* 2003; Accardi *et al.* 2006), immediately suggests that these ions move through the protein along a defined pathway. The 'outward-open' conformation of the protein reveals three Cl^- ions, denoted internal, central and external, along a gently curving trajectory connecting the internal and external aqueous phases. The internal and external Cl^- ions are partly exposed to their respective solutions, but the central ion is dehydrated in both 'open' and 'closed' states, with the coordination shell composed of polar groups from the protein: three backbone amides (G149, I356, F357), two side-chain hydroxyls (S107, Y445), and amazingly, the carboxylate oxygen of a deprotonated glutamate residue (E148).

We note right away that this is an unusual arrangement of substrates within an exchange transporter. Familiar cartoons of coupled-transport mechanisms—as well as crystal structures of certain coupled transporters (Abramson *et al.* 2003; Huang *et al.* 2003)—show a substrate-binding site around which the protein pivots to alternately face internal and external solutions via wide aqueous vestibules. But no such vestibules are seen in CLC-ec1, and the Cl^- ions are arranged in a channel-like disposition spanning the membrane width. The structure suggests a transport mechanism in which a Cl^- ion entering from the extracellular side at the 'top' of the pathway must move into the central site and eventually exit to the cytoplasm from the internal site, forcing single-file movement of the Cl^- ions in front of it. But it is not known as to how these ion movements occur or how they are coordinated with oppositely directed H^+ movement.

4. PROTONS ALSO MOVE ALONG A PATHWAY WITH GLUTAMATE GATES

Mutagenesis studies (Accardi & Miller 2004; Accardi *et al.* 2005) have identified two surface-exposed glutamate residues as key players in proton transport—E148 near the extracellular side, denoted Glu_{ex}, and E203, denoted Glu_{in}, on the intracellular side (figure 2). When deprotonated, the Glu_{ex} carboxylate group occupies the external anion-binding site and thereby blocks off the central Cl^- ion's extracellular access (Dutzler *et al.* 2003). This side chain acts as a proton-activated extracellular 'gate' to the Cl^- pathway; in a mutant with Glu_{ex} replaced by Gln to mimic an always-protonated carboxylate, the side chain swings out to open a pathway for Cl^- to the outside solution. If Glu_{ex} is replaced with non-protonatable side chains, H^+ transport is completely abolished, but Cl^- transport persists, albeit at approximately five-fold lower rate (Accardi & Miller 2004; Jayaram *et al.* 2008). Thus, Glu_{ex} participates in transport of *both* substrates, transferring H^+ between extracellular solution and the protein machinery, while opening or closing the extracellular side of the Cl^- pathway according to its pronation state. This crucial residue thus participates in three reactions essential for an H^+-coupled Cl^- transport cycle: protonation, conformational change and subsequent Cl^- binding to its transport pathway.

On the cytoplasmic side, Glu_{in} is located near the subunit interface, approximately 20 Å away from the Cl^- pathway's opening to this side. As with Glu_{ex}, substitution of Glu_{in} by non-protonatable residues severely impairs H^+ coupling, while retaining Cl^- transport at a somewhat lower rate than wild type (Accardi *et al.* 2005), as also documented in two mammalian CLC transporters (Zdebik *et al.* 2008). We have proposed that Glu_{in} exchanges protons between the internal aqueous phase and the protein interior in a way functionally analogous to Glu_{ex} on the outside. Crystal structures, however, do not show any side chain rearrangement of the E203Q mutant, so we do not have a clear a picture, as for Glu_{ex}, of how the proton is delivered into the protein by Glu_{in}.

Since these two 'proton-transfer glutamates' are separated from each other by 15–20 Å, the proton taken up by one must cross the membrane to be delivered to the opposite solution by the other. In the absence of large conformational changes that bring Glu_{ex} and Glu_{in} into close proximity—an unlikely circumstance—the proton must traverse a pathway between these two residues. Such a pathway overlaps the Cl^- pathway on the extracellular side, and splits off from this to reach Glu_{in}, as cartooned in figure 2. Protonation of these glutamates must be somehow coordinated with Cl^- ion occupancy of the three anion-binding sites. Discovering the rules underlying this coordination is a major aim in deriving an exchange mechanism.

5. A TYROSINE GATE FOR Cl^- MAINTAINS H^+ COUPLING

We have so far arrived at a rough picture of two ionic pathways running across the protein. The Cl^- pathway is gated on the external side by the Glu_{ex} side chain, which adopts closed-deprotonated and open-protonated conformations. An internal gate appears to be formed by two hydroxyl-bearing residues that coordinate the central Cl^- ion, Y445 (denoted the 'central' tyrosine, Tyr_c) and S107. Likewise, the H^+ pathway is capped on each end by the two proton-transfer glutamates, but the intermediate proton-binding sites between these are unknown. These considerations focus attention on Tyr_c, which lies near the intersection of the two pathways, coordinates the central Cl^-, and blocks access to the intracellular solution. It also lies about halfway between the two H^+-transfer glutamates. It is not surprising, then, that substitutions here profoundly affect the coupling of H^+ to Cl^- transport (Accardi *et al.* 2006; Walden *et al.* 2007).

Mutations of Tyr_c uncouple H^+ from Cl^- movement to varying extents according to the volume of the substituted side chain. For example, with large residues (Phe, Trp) at this position, Cl^-/H^- exchange proceeds with only a slight weakening of wild-type stoichiometry, but with Ala or Gly substitutions, Cl^- movement is unaccompanied by H^+; intermediate-volume side chains (Leu, Met) produce partially coupled Cl^- transport. The protein behaves as though it suffers an inherent tendency to allow Cl^- ions to 'slip' through the inner gate, which is kept tight uniquely by Tyr_c, but which is degraded by any other residue at this position, smaller residues producing more slippage.

6. THE CENTRAL Cl^- ION IS LINKED TO H^+ COUPLING

In parallel to this functional pattern, the degree of H^+ coupling, as measured from the Cl^-/H^+ transport stoichiometry, strikingly correlates with a structural feature: the *anion occupancy of the central Cl^- binding site* determined using Br^- as a crystallographically useful Cl^- substitute in structures of the Tyr_c mutants (Accardi *et al.* 2006). In tightly coupled mutants, Br^- binds to this site as in wild type, but in the uncoupled, small-residue substitutions, Br^- is absent. Since these uncoupled mutants transport Cl^- at roughly wild-type rates, we do not imagine that a large barrier to Cl^-

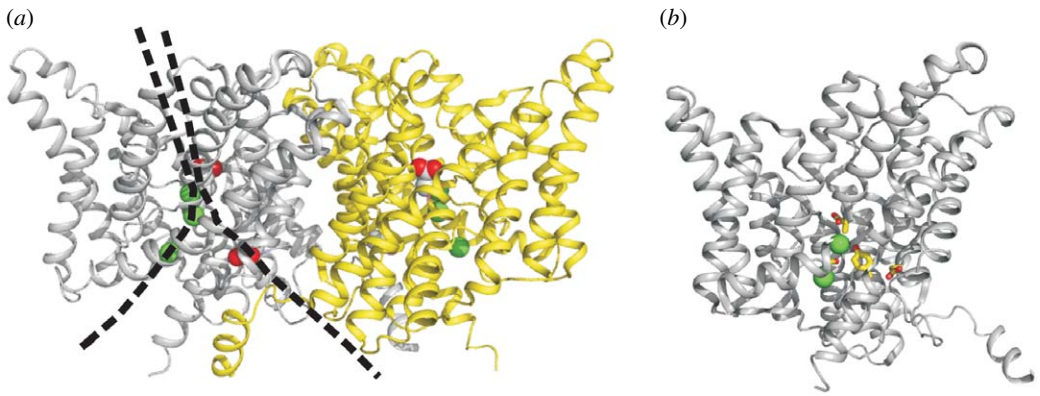

Figure 2. Crystal structure of CLC-ec1. CLC-ec1 is shown oriented with extracellular side up. (*a*) Outward-open structure of the homodimer, using the mutant E148Q (accession 1OTU), is depicted to show Cl^- ions (green spheres) in all three anion-binding sites. Space-filled residues show locations of the extracellular and intracellular proton-transfer residues Glu_{ex} and Glu_{in}, respectively. Dashed lines shown in one subunit represent Cl^- and H^+ pathways. (*b*) Magnified view of the ion-binding region in an individual subunit of the outward-closed wild-type structure (accession 1OTS), with stick-rendered side chains indicating the proton-transfer glutamates (Glu148, Glu203) and inner Cl^- gate residues (Ser107, Tyr445).

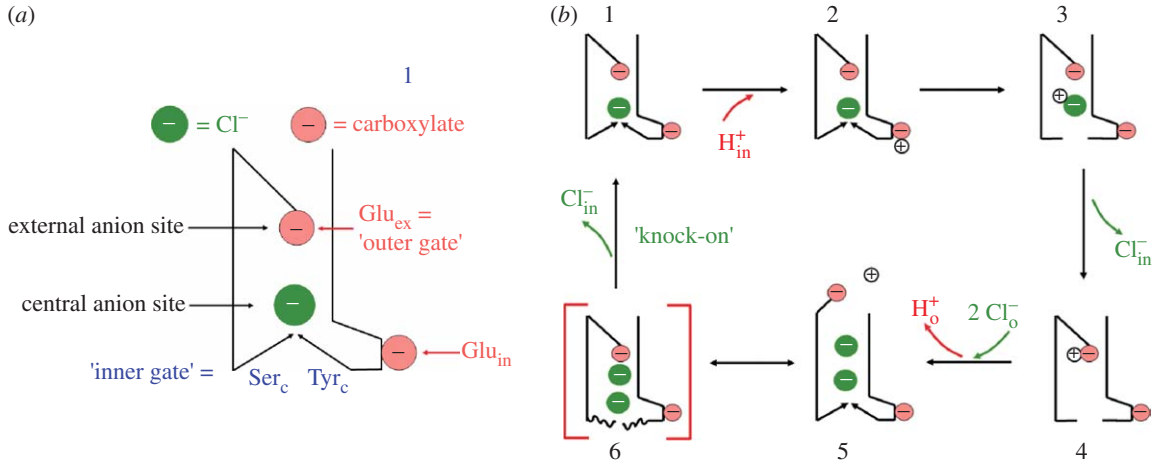

Figure 3. Mechanism of CLC-mediated Cl^-/H^+ exchange. (*a*) Icon depicting ions and residues involved in coupling mechanism. (*b*) Minimal 6-state mechanism described in text.

movement is created by these mutations, only that the equilibrium binding energy here is made less favourable. These results suggest that the central Cl^- ion is somehow involved in H^+ coupling, an idea buttressed by an additional observation: that the pseudohalides SCN^- and $SeCN^-$ are transported robustly by the wild-type protein, *without any accompanying* H^+ *movement* (Nguitragool & Miller 2006). Moreover, the central anion-binding site is empty in crystals of wild-type protein grown in $SeCN^-$. So once again, H^+ coupling is lost if an anion fails to occupy the central site. For these reasons, we proposed (Accardi *et al.* 2006) that a required step in proton coupling is protonation of the central Cl^- ion at some point in the transport cycle, i.e. the transient formation of HCl. Although this may seem a chemically deranged idea in light of the strong-acid nature of aqueous HCl, in the anhydrous environment of the central site, protonation of a Cl^- ion might not be so implausible. Nevertheless, this remains a working hypothesis that has not been experimentally tested.

(a) *A mechanism of* Cl^-/H^+ *exchange*

A familiar hallmark of exchange-transport mechanisms in tightly coupled systems such as the Cl^-/HCO_3 exchanger AE1 (Knauf *et al.* 2002) or H^+-coupled exchangers such as EmrE (Soskine *et al.* 2004) and NhaA (Arkin *et al.* 2007) is mutually exclusive binding of the two substrates, and the 'ping-pong' kinetics arising from it. If (i) both inward- and outward-facing conformations of the protein can bind substrates, and (ii) both substrates can never be bound simultaneously, and (iii) the protein can undergo the conformational change only when substrate is bound, then strict, stoichiometric exchange necessarily follows. But CLC-ec1 operates in a fundamentally different way, since the transported Cl^- and H^+ ions bind simultaneously, at least at some points in the transport cycle. This conclusion follows from two kinds of experimental results. First, crystal structures reveal Cl^- bound at the normal transport sites in Gln mutants that mimic protonated forms of both H^+-transfer gates (Dutzler *et al.* 2003; Lobet & Dutzler 2005). No such simultaneously occupied states could exist in a ping-pong exchanger. Second, as described above, even when both H^+-transfer gates are present, H^+ cannot move from one to the other when Cl^- fails to occupy the central site. This kind of synergistic binding of the two transported substrates is strictly forbidden in ping-pong mechanisms. Our conclusion—soft, to be

sure—that a key step in coordinating ion movements is the binding of a transported proton to a transported Cl^- ion is plainly inconsistent with ping-pong behaviour.

These considerations lead to a provisional proposal (figure 3) for Cl^-/H^+ exchange by CLC-ec1 (and by implication other members of the CLC exchanger-subfamily). The mechanism seeks to explain the coordinated movement of Cl^- and H^+ along each ion's pathway. For the Cl^- pathway, we consider only two of the anion-binding sites, the central and outer, to play essential roles in the mechanism, and will ignore the inner site, which is directly exposed to the intracellular solution in crystal structures. The key actors in this mechanism, indicated by the icon in figure 3, are: Glu_{ex}, which acts as an outer gate for both Cl^- and H^+ pathways; Glu_{in}, the intracellular jumping-off or -on point for protons; the inner gate for Cl^-, formed in part by Tyr_c; and the central Cl^- ion itself.

All steps in the mechanism are, of course, reversible, but for clarity the cycle is described running clockwise, as with large ion gradients, Cl^- high on the outside, H^+ high on the inside. The cycle begins (state 1) with the Cl^- pathway closed on both ends and both H^+-gates deprotonated. The outer and central anion sites are occupied by the Glu_{ex} carboxylate and a Cl^-, respectively, and the carboxylate group is buried, with its protonatable oxygens facing inward, inaccessible from aqueous solution. An intracellular proton binds to Glu_{in} (state 2) and moves along its pathway to protonate the central Cl^- ion (state 3). At this point we introduce a key coupling rule: that whenever a proton occupies the anion-binding region, the inner gate opens. The HCl thus formed hands its proton off to the nearby Glu_{ex}, as the Cl^- ion escapes to the intracellular solution (state 4). The outer gate, now protonated, swings open as the inner gate closes, and two Cl^- ions enter from the outside solution (state 5). The Glu_{ex} side chain loses the proton to the outside and so wants to close, but Cl^- in the outer site discourages this. Instead, an unstable state 6 forms in which the carboxylate of Glu_{ex} approaches the outer site and expels the central Cl^- inwards as the inner gate becomes transiently destabilized by the high density of negative charge in this 'triple-ion' state. The protein, now deprotonated, closed and occupied by a single Cl^-, returns to state 1.

The mechanism exhibits a few glaring flaws. First, the opening of the inner gate upon HCl formation is an *ad hoc* feature unsupported by any experimental evidence; moreover, the physical nature of inner-gate opening is completely unknown, since all crystal structures of CLC-ec1 show this gate closed. Second, we have no idea as to how the proton navigates the 10 Å separating Glu_{in} and the central Cl^- ion; this region is devoid of any dissociable side chains, except for Tyr_c, whose hydroxyl group is not required for coupled Cl^-/H^+ exchange (Accardi *et al.* 2006; Walden *et al.* 2007). Finally, the 'destabilization' of the inner gate by over-packing the protein with three anions (state 6) is invoked for no reason other than to make the mechanism work.

Despite these ambiguities, the mechanism has its virtues. First, most of the states postulated have been observed crystallographically, using mutants representing protonated or open gates—state 1 (wild type), state 2 (E203Q), state 4 (Y445A) and state 5 (E148Q). Second, the mechanism effortlessly accounts for the 2-to-1 stoichiometry of Cl^-/H^+ exchange; this stoichiometry follows from the anion-binding region's two sites, one of which binds either Cl^- or the Glu_{ex} carboxylate, while the other binds only Cl^-. Third, the channel-transporter duality of the CLC family mitigates some of the awkwardness of the triply occupied state 6; such a transient, three-ion state is an essential step in 'knock-on' mechanisms of ion permeation through Ca^{2+} and K^+ channels, whereby concerted movement of two ions in single file is driven by the entry of an 'extra' ion into the pore (Armstrong & Neyton 1991; Zhou & MacKinnon 2003). Fourth, the uncoupling caused by small-residue substitutions at Tyr_c is naturally understood in terms of a 'leaky' inner Cl^- gate in this mechanism. Moreover, the abolition of H^+ coupling with non-halide anions such as SCN^- is explained by invoking an inability of these anions to be protonated during the transport cycle. Finally, this mechanism makes it easy to envision how the subclass of CLC channels might have evolved as 'broken transporters' (Miller 2006) in which the inner gate, or its coordination with the outer gate, was lost.

We emphasize that this mechanism is provisional and that future experiments will almost certainly require its modification. The crucial postulate of direct protonation of the central Cl^- ion cries out for experimental verification, which will be difficult but perhaps possible with modern spectroscopic techniques. At the very least, the mechanism provides an anchor to prevent us from drifting too far into the foggy seas of mutagenesis, crystallography and functional analysis of membrane transport proteins.

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