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:

\[ V_r = \frac{1}{1 + r} (rE_{Cl} + E_{H^+}). \]

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⁻ (Nguiragool & 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 (Nguiragool & 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/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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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 central ion coordinated by four cross-subunit hydrogen bonds. 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. CI⁻ 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 Gluex, and E203, denoted Gluin, on the intracellular side (figure 2). When deprotonated, the Gluex 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 Gluex replaced by Gluin to mimic an always-protonated carboxylate, the side chain swings out to open a pathway for Cl⁻ to the outside solution. If Gluex 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, Gluex 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 protonation 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, Gluin is located near the subunit interface, approximately 20 Å away from the Cl⁻ pathway’s opening to this side. As with Gluex substitution of Gluin 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 Gluin exchanges protons between the internal aqueous phase and the protein interior in a way functionally analogous to Gluex 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 Gluex, of how the proton is delivered into the protein by Gluin.

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 Gluex and Gluin 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 Gluin 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 Gluex 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, Tyrc) 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 Tyrc, 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 Tyrc 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 Tyrc, 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 Tyrc 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⁻...
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−/HCO3− 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
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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: Glu₁₉₉ which acts as an outer gate for both Cl⁻ and H⁺ pathways; Glu₁₀₁, the intracellular jumping-off or -on point for protons; the inner gate for Cl⁻, formed in part by Tyr₁₃ 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 Glu₁₉₉ 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 Glu₁₀₁ (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 Glu₁₀₁ 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 Glu₁₀₁ 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 Glu₁₀₁ approaches the outer site and expels the central Cl⁻ intowards 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 Glu₁₀₁ and the central Cl⁻ ion; this region is devoid of any dissociable side chains, except for Tyr₁₃ whose hydroxyl group is not required for coupled Cl⁻/H⁺ exchange (Accardi et al. 2006; Walden et al. 2007). Finally, the ‘destabilisation’ of the inner gate by over-packaging 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 Glu₁₀₁ 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 Ca²⁺ 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 Tyr₃ 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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REFERENCES


