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CLC channels and transporters: proteins with borderline personalities

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Abstract

Controlled chloride movement across membranes is essential for a variety of physiological processes ranging from salt homeostasis in the kidneys to acidification of cellular compartments. The CLC family is formed by two, not so distinct, sub-classes of membrane transport proteins: Cl^- channels and H^+/Cl^- exchangers. All CLC's are homodimers with each monomer forming an individual Cl− permeation pathway which appears to be largely unaltered in the two CLC sub-classes. Key residues for ion binding and selectivity are also highly conserved. Most CLC's have large cytosolic carboxy-terminal domains containing two cystathionine β-synthetase (CBS) domains. The C-termini are critical regulators of protein trafficking and directly modulate Cl− by binding intracellular ATP, H + or oxidizing compounds.

This review focuses on the recent mechanistic insights on the how the structural similarities between CLC channels and transporters translate in unexpected mechanistic analogies between these two sub-classes.

Introduction

Historically channels and transporters have been thought of as distinct and separate classes of membrane proteins with unrelated three-dimensional architectures and supporting opposite thermodynamic functions. Channels form water-filled holes through which substrates freely diffuse across a membrane. A "gate" opens and closes these pores in response to external stimuli such as a change in the membrane potential, binding of a ligand, a change in temperature or stress on the membrane itself. Thus, channels efficiently dissipate pre-existing electrochemical gradients but are incapable of mediating the energetically uphill substrate movement necessary to create these gradients. This task is carried out by transporters that couple energy consumption to the movement of substrate against an electrochemical gradient.

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Primary transporters derive the energy for uphill transport from chemical reactions, such as ATP hydrolysis, while secondary transporters dissipate the gradient of one (or more) substrate to catalyze uphill transport of the other(s). The substrate translocation pathway of the transporters is delimited at its ends by two gates that are never simultaneously open. This coordination ensures that, at any given moment, access to the substrate binding sites can happen only from one side of the membrane. This ensures the stoichiometric coupling between the transported substrates and the protein's conformational cycle and allows for uphill transport. Recent discoveries have further weakened the distinction between channels and transporters: CFTR is a Cl^- channel that bears structural and sequence homology to ABC transporters [1], many neurotransmitter transporters show channel-like "leaks" [2-4] and a single point mutation in the voltage sensing domains transforms a K^+ channel into a H^+ transporter [5]. Uncoupling of the transporters' gates can lead to an unbroken pathway directly connecting the opposite sides of a membrane, allow free diffusion and functionally transform the transporter into an ion channel [6-8].A most surprising discovery has been that the CLC family of chloride transporting proteins is split in half between Cl^- channels and H^+/Cl^- exchange transporters, rather than being solely populated by Cl^- channels as had been believed since its discovery [9-13]. Thus, the CLC's and CFTR, a Cl^- channel that belongs to the super-family of ABC transporters, belong to protein families whose members mediate transport through two completely different mechanisms. The CLC channels are localized to the plasma membrane of cells where they play key physiological role such as determining the resting membrane potential in skeletal muscle fibers, balance salt transport in kidney, inner ear and other epithelial tissues [14]. The exchangers on the other hand appear to be confined to the membranes of cellular compartments such as endosomes, lysosomes, osteoclastic lacunas, plant vacuoles and synaptic vesicles. There is ample and convincing evidence that the CLC transporters play a key role in acidification of the compartments where they reside: impaired transporter function through a knock-out in mice and bacteria or as a consequence of a naturally occurring mutation in humans invariably result in impaired pH regulation [13-20]. What is less clear, however, is how these CLC transporters facilitate acidification of these compartment. A H^+/Cl^- exchanger is not an efficient electrical shunt for the H^+-pumps that are known to be primarily responsible for building the pH gradient in these cellular sections; for every 2 Cl^- entering the compartment 1 H^+ is extruded, effectively increasing by ~30% the energy cost of the formation of the pH gradients. It will be interesting to see how essential the coupling of H^+ and Cl^- fluxes by these proteins is to ensure correct acidification.

While the two CLC sub-classes have opposite functional properties, mediating active and passive transport, there is a large body of evidence indicating that the CLC channels and transporters share the same basic architecture. This suggests that that only modest changes are needed to adapt a single structural scaffold to support these two opposite transport mechanisms. Thus, the CLC's provide us with a unique opportunity: we can investigate the molecular determinants that underlie channel and transporter within a structurally defined and unified context.

In this review we will summarize the main recent advances in our understanding of how CLC channels and transporters function and of how the two subclasses are related.

Structure of the transmembrane region and molecular basis of Cl^- binding

The high resolution crystal structure of the bacterial CLC exchanger CLC-ec1 [21-22] provides a molecular picture of the arrangement of the transmembrane domains of the CLC proteins. CLC-ec1 is a homodimer with two identical subunits (Fig. 1A). Each sub-unit has 18 α-helices, tilted with respect to the plane of the membrane, with different lengths and contains an internal antiparallel repeat [21]. Each sub-unit has three binding sites for anions: the internal and external sites, S_{int} and S_{ex}, are in direct contact respectively with the intra- and extra-cellular

solutions. The central site, S_{cen}, is in the middle of the bilayer and isolated from both sides of the membrane. A Cl^- ion in S_{cen} is partly or completely dehydrated and coordinated by the hydroxyl oxygen atoms of S107 and Y445, also known as Ser_{cen} and Tyr_{cen}, by the main-chain amide nitrogen atoms of G149, I356, F357 and by the side chain of E148, Glu_{ex} [21-22]. In the WT structure the negatively charged Glu_{ex} side chain occupies S_{ex} and likely competes with Cl^- for its occupancy. A Cl^- ion in S_{ex} has been observed crystallographically only when the side chain of Glu_{ex} has either been removed or constitutively protonated [22-23]. In contrast to what is seen in S_{cen}, a Cl^- in S_{ex} is only coordinated by backbone amides, rather than from side-chain atoms [22-23]. The nearly complete conservation of the ion-coordinating residues suggests that the molecular basis for ion binding and selectivity are also maintained.

All three of CLC-ec1's sites bind ions rather weakly, with millimolar affinity [23-24]. Independent measurements of Cl^- binding to WT and mutant CLC-ec1 have been carried out with X-ray crystallography, isothermal titration calorimetry (ITC) and saturation equilibrium dialysis [23-24]. Overall the three approaches yield comparable results and the small discrepancies are due to differences in the experimental approaches and conditions used. Cl^- binding is tightest to S_{cen}, with an affinity ~0.7-3 mM, intermediate to S_{ex}, with a K_d~ 0.9-3.9 mM, and weakest to S_{in}, with an affinity >20 mM. Surprisingly, multiple ions bind to the pathway with minimal interactions between them, even when separated by very small distances (~6 Å) [23-24]. This suggests that in CLC-ec1 the electrostatic repulsion between the ions is compensated by interactions with the protein. In contrast, multiple ions interact strongly in the pore of a CLC channel [25-26], and the uncompensated electrostatic repulsion promotes fast diffusion through these channels [22,27]. We speculate that the weak interactions between ions in the Cl^- permeation pathway of a transporter ensure slow down ion movement and that this is a key element differentiating the CLC channels from the transporters.

One surprising observation from the ITC measurements is that 2 Cl^- ions bind simultaneously to the E148A mutant at S_{ex} and S_{cen} with 10-60-fold higher affinity than to the isolated sites [24]. The structural bases of this increase in affinity are not clear: the structures of the WT and E148A proteins are nearly superimposable, aside from local changes at the mutation site [22]. This suggests that a large change in affinity can arise from subtle structural alterations, not currently detectable crystallographically. Alternatively, it could be that different conformations of the transporter protein are favored in solution and in the crystals. If so, these rearrangements might be sufficient to compensate and stabilize the electrostatic repulsion between Cl^- ions bound to the pathway.

Direct binding measurements have not been possible on other CLC family members because of the limited availability of purified protein. However, the apparent K_m for transport [24,28] or conduction [26,29-30] and the equilibrium K_d [23-24] are close. This strongly suggests that that the determinants for ion binding are largely conserved between the two branches of the CLC family.

Most CLC channels and transporters share a similar selectivity sequence of $SCN^->Cl^->Br^->NO_3^->I^-$ [26,31-33]. However, while the crystal structure of CLC-ec1 elucidated the molecular basis for ion binding to the CLC's our view of how these proteins select between different anions remained clouded. The selectivity in the CLC's seems to be determined by a single site, S_{cen}, while S_{ex} is non-selective and S_{in} exhibits a slight preference for SCN^- over Cl^- [24]. What makes S_{cen} selective? The obvious candidates are residues whose side chains directly coordinate the Cl^- ion and that when mutated drastically alter binding, like Glu_{ex} or Tyr_{cen}. However, mutations at these positions leave selectivity largely unchanged in both channels and transporters [24,32,34] ruling these two residues out as key determinants for selectivity. Early experiments showed that the third residue forming S_{cen}, Ser_{cen}, could play a role in selectivity, the S123T mutant of CLC-0 has increased I^- and Br^- permeability

[35-36]. The discovery that in atCLC-a, a NO_3^- selective H^+-coupled exchanger from *Arabidopsis thaliana*, Ser_{cen} is replaced by a Proline further cemented the role of this residue in determining selectivity [12]. Inspired by this observation several groups mutated Ser_{cen} to Proline in the CLC-5 and CLC-ec1 transporters and in the CLC-0 channel. The result was a striking conversion: the mutant proteins acquire the NO_3^- selectivity characteristic of atCLC-a [24,37-38] and the converse Proline to Serine mutation transforms atCLC-a into a Cl^-/H^+ exchanger [38]. These results show that inter-anionic selectivity in the CLC's is regulated by a single amino-acid side chain: a Proline confers NO_3^- specificity while a Serine guarantees Cl^- selectivity. A mechanism of selectivity based on the direct interaction between substrate and a single side chain is profoundly different and more flexible than that of other transport systems. The K^+, Na^+ and Ca^{2+} channels all share a similar pore structure but select for ions through different mechanisms. In the K^+ channels a dehydrated ion interacts with the backbone carbonyls of a highly conserved sequence [39-43] while in the Na^+ and Ca^{2+} channels long range electrostatic interactions with charged residues and other ions in the pore also greatly contribute to selectivity [40,44].

In contrast, the selectivity mechanism of the CLC's appears to be ideally suited to fine-tune the transport specificity of a conserved pathway to different physiologically relevant substrates with minor alterations. It will be interesting to see whether the other amino-acid side chains that naturally replace Ser_{cen} in other CLC homologues lead to specific selectivity changes or if this is a Serine and Proline specific mechanism. Lastly, it is surprising to note how the principles of selectivity are conserved between the two branches of the CLC family despite a >1000-fold difference in rates at which channels and transporters mediate ion translocation.

The Cl^- transport pathway

The crystal structure of CLC-ec1 elucidated the arched conduit through which Cl^- ions cross the membrane [21-22]. Two distinct structural elements sterically regulate access ion movement in and out of the pathway: the side chain of Glu_{ex} gates ion access to and from the extracellular solution while Tyr_{cen} and Ser_{cen} pinch the pathway at its intracellular end and likely form the intracellular gate. During the transport cycle the two gates alternatively open to allow substrate exchange between the sites in the pathway with the intra– and extra-cellular solutions. In the crystal structure of the E148Q mutant of CLC-ec1, which is thought to mimic the protonated state of the gate, the mutated side chain rotates out of the Cl^- permeation pathway opening it to the extracellular side and a third Cl^- ion binds to S_{ex} [22]. Functionally, mutations at Glu_{ex} lead to CLC channels that are constitutively open [22,45-46] and transporters that mediate H^+-independent Cl^- transport corroborating its role as the extracellular gate [9-11, 22,32,47]. The nature of the intracellular gate is, on the other hand, less obvious [48-50]. The occlusion of the pathway by Tyr_{cen} and Ser_{cen} is obvious but incomplete; thermal agitation might allow enough side-chain flexibility to allow for unhindered ion movement [51-52]. However, several results cement the role of Tyr_{cen} and Ser_{cen} as part of the intracellular gate. Mutations that reduce the side-chain volume at Tyr_{cen}, i.e. to Alanine or Serine, open the pathway towards the intracellular side [48-49]. This destabilizes ion binding to S_{cen}, resulting in decreased ion occupancy of this site and abolish or degrade H^+-coupling [48-49]. Extensive mutagenesis has not been carried out on Ser_{cen}, so the role of this residue in ion binding and H^+-coupling is still unexplored. Mutations that reduce the steric hindrance at the intra- or extracellular gate do not lead to an increase in the absolute Cl^- transport rate [48-50] as could be expected from a permeation pathway that mediates free diffusion since it is gated only at one end. Only when both steric gates are simultaneously "removed", through the E148A/Y445S double mutation, a large increase (>20-fold) in the Cl^- transport rate is seen [50]. While remarkably high, the Cl^- transport rate of the doubly ungated mutant is only ~35000 ion s^{-1}, ~20-fold lower than the lowest reported conductance for a CLC channel [53]. Is this mutant an unusually fast transporter or a very slow channel? We don't have a definitive answer;

however several lines of evidence support the latter hypothesis. First, the crystal structure of a doubly ungated mutant of CLC-ec1 shows a very narrow and unbroken pathway that connects the intra– and extra-cellular solutions and that could support free Cl^- diffusion [50]. Second, a recent fluorine NMR study has shown that the transport cycle of CLC-ec1 involves a Cl^--dependent conformational change that propagates to the inter-subunit interface [54]. In the doubly ungated mutant this rearrangement is absent, suggesting that Cl^- transport has become uncoupled from the conformational cycle of the protein. Lastly, a recent study has suggested that ion unbinding is the rate limiting step for ion transport in the doubly ungated mutant [24]. So, the low rate of diffusion reflects the relatively high affinity (~1 mM) of Cl^- for the protein. In summary, Cl^- transport through the doubly de-gated mutant is passive, happens through a continuous transmembrane pathway, is uncoupled from conformational changes in the protein and is rate-limited by ion unbinding; all characteristics typical of a *bona fide* ion channel.

It is not clear how well the mechanistic picture established for CLC-ec1 is conserved in other CLC transporters and whether the corresponding point mutations would also lead to the functional transformation of the transporters into channels. While some key features, such as the role of Glu_{ex}, Glu_{in} and the 2:1 stoichiometry [9-12,32], [47,55], are conserved in all CLC transporters significant differences exist. The transport rate of CLC-4 and CLC-5 was measured with non-stationary noise analysis and found to be ~10^5 ion s^{-1}[28,56-57], surpassing by ~30-fold the human Cl^-/HCO_3^-exchanger as the fastest known transporter [58]. In other words, CLC-4/-5 appear to be ~100-fold faster than CLC-ec1 and mediate coupled H^+/Cl^- exchange ~10-fold faster than Cl^- diffuses through the channel-like mutant of CLC-ec1. It is not clear to us how appropriate is to use non-stationary noise analysis to deduce the “conductance” of a transporter. The parabolic dependence of the variance from the mean current relies on the assumption that the protein fluctuates between a conductive and a non-conductive state, concepts which are hard to define for a transporter. The steady state power spectra of CLC-4 and -5 currents decreases at higher frequencies and can be approximated with the sum of 1 to 3 Lorentzian functions [28,56-57]. This decrease is a hallmark of channel-mediated ion transport [59] and incompatible with simple carrier-type models [60-61]. This result was interpreted to indicate that these transporters undergo a channel-like transition between silent and active modes at very high frequency. However, the shape of the power spectra for a transporter depends on the assumed transport model and an unusual mechanism might lead to unexpected spectral properties. Lastly, the contribution of additional sources of noise, such as shot noise or $1/f^{\alpha}$ noise, to the power spectrum was not considered in the original analysis. In conclusion, this result is intriguing and puzzling at the same time. It is hard to envision what the conformational changes taking place in a CLC exchanger might be, when a complete transport cycle that exchanges 2 Cl^- for 1 H^+ takes place in ~ 10 μs, a time scale more suited to the thermal fluctuations of loops and side chains rather than the concerted movement required for active transport [62]. The localized nature of these fast conformational changes is also hard to reconcile with the recent findings that involve regions far from the ion binding sites in transport [55,63-64]. For example, two loops ~ 35 Å away from the permeation pathway play a key role in transport. Zn^{2+} inhibition of CLC-4 transport is mediated by a tri-His motif in the extracellular loop connecting helices N and O [64] and the B-C loop controls gating kinetics and MTS inhibition of CLC-3 [55]. It is possible that the conformational changes underlying transport in CLC-ec1 and CLC-3/-4/-5 are fundamentally different. The strong conservation of key residues for transport and similarities in the transport characteristics would suggest otherwise, but recent results hint that this might indeed be the case. Transport in CLC-ec1 remains coupled in the whole pH range tested, pH 3-7, and uncoupling by polyatomic anion is not a consequence of the conversion of the transporter into a channel [9,32-33]. In contrast it has been recently reported that the CLC-3 H^+/Cl^- exchanger becomes uncoupled at acidic pH’s [55] and that CLC-4 functions as an ion channel when Cl^- is replaced by SCN^- as the main charge carrier [57].

In conclusion, the CLC transporters mediate Cl^- transport through a pathway that is delimited by two steric gates, Glu_{ex} and Tyr_{cen}, whose coordinated movement during the transport cycle leads to alternate exposure of the binding sites. Ion binding to S_{ex} and S_{in} is mediated by backbone amides while two side chains contribute to S_{cen}. Selectivity is mostly determined by Scen and is critically dependent on the residue at Ser_{cen}. The determinants of ion binding and selectivity are conserved between the channels and transporters. Simultaneous removal of the two gates leads to a channel-like pore that is constitutively open and mediates slow diffusion of ions downhill the electrochemical gradient. These results reinforce the idea that the CLC channels have evolved from the CLC transporters and that similar basic principles regulate gating, permeation and selectivity in both protein sub-types. We still do not know what conformational changes lead to opening of the inner gate and we are only starting to glimpse how these rearrangements might extend beyond the immediate neighborhood of the ion binding region.

The H^+ transport pathway

The three anionic binding sites of CLC-ec1 let us visualize the Cl^- permeation pathway through the membrane. In contrast, the route taken by H^+ is still elusive. Early mutagenesis experiments showed that two glutamate residues, E148 and E203 in CLC-ec1 (we will refer to the latter as Glu_{in} in the remainder of the review), as the extra– and intra-cellular H^+ acceptors [9,65]. Their role was confirmed in other CLC transporters [10-11,56]. When Glu_{ex} and Glu_{in} are mutated to non-protonatable residues the CLC exchangers lose the ability to transport H^+ while Cl^- transport is retained. Neutralization of the other glutamates or aspartates only has minor effects on transport and coupling, at least in CLC-ec1 [65]. The specificity of the loss of H^+ transport associated to these mutations suggests that these residues directly line the H^+ transport pathway. Thus, there appears to be a functional symmetry between Glu_{in} and Glu_{ex}; however two fundamental differences exist. First, Glu_{ex} is conserved in both channels and transporters while Glu_{in} is strictly conserved only among the transporters [65]. In the channels a non-protonatable and hydrophobic residue such as valine or leucine replaces Glu_{in} [65]. The second difference is that Glu_{ex} regulates the flow of both Cl^- and H^+ ions while Glu_{in} appears to be little more than a H^+-shuttle between the intracellular milieu and the CLC-ec1 core [9,56,66]. Mutants where Glu_{in} is replaced with any protonatable residue still maintain significant H^+ transport [56,66].

The H^+ entry– and exit-points in the CLC transporters are separated by a ~14 Å long stretch lined by mostly hydrophobic residues. How a H^+ crosses this gap is not clear. Several mechanisms have been proposed and –in some cases– tested, some involve proteinaceous pathways others don't [48,56,67-69]. One early candidate H^+ acceptor was Tyr_{cen}. This residue is nearly equidistant between Glu_{in} and Glu_{ex} and its hydroxyl moiety is one of the few protonatable sites in the hydrophobic core of the protein [48]. However, the Y445F mutant of CLC-ec1 transports H^+ as efficiently as the WT protein [48] ruling out a direct involvement of Tyr_{cen} in the H^+ pathway. The second hypothesis was that the Cl^- ion bound to S_{cen} [48] functions as the intermediate H^+ acceptor site. This proposal was spurred by the observation that impaired anion occupancy of S_{cen}, as a consequence of mutagenesis or of the replacement of Cl^- with polyatomic anions such as SCN^- [33,37,48,55,57], directly correlates with a degradation of H^+ coupling. This hypothesis predicts that an HCl molecule is transiently formed in S_{cen}, so that reduced Cl^- occupancy of this site directly translates in diminished H^+ transport. Since the Cl^- ion in S_{cen} is thought to be mostly or completely dehydrated [22] the formation of an HCl molecule in S_{cen} is less outlandish than it could appear at a first glance. However, direct evidence supporting (or disproving) this idea is still missing and the resolution of the available crystal structures is not sufficient to determine the hydration state of the Cl^- ion in S_{cen}. The third and most recent suggestion is that, following a side-chain rotation of Glu_{in}, a chain of hydrogen-bonded water molecules is formed inside the hydrophobic core of CLC-ec1,

transiently connecting Glu_{in} and Glu_{ex} and allowing H^+ conduction [69]. This intriguing hypothesis however lacks direct supporting or contrasting evidence. Higher resolution crystal structures are needed to determine whether water molecules can indeed bind to the apparently hydrophobic core of CLC-ec1 and whether the proposed rotation of Glu_{in} does indeed take place.

In conclusion, Glu_{ex} regulates Cl^- and H^+ access to and from the extracellular solution, while H^+ exchange with the intracellular milieu is regulated by Glu_{in}. Thus, the trajectories described by the ions through the CLC transporters converge extracellularly and diverge intracellularly. It is yet unclear how H^+'s move between Glu_{in} and Glu_{ex} and how H^+ and Cl^- binding and transport are coupled.

A degraded H^+ pathway exists in the CLC channels

The evidence discussed so far suggests that there is a strong evolutionary link connecting the CLC channels and transporters. Both sub-classes achieve the same end result, Cl^- transport, by adapting a similar structural scaffold to their respective, and thermodynamically opposite, mechanisms. The residues that line the ion permeation pathway, form the ion binding sites and regulate Cl^- access to the pathway are highly conserved between the two sub-groups. In this context of overarching similarities it is perhaps unsurprising that opening and closing of the CLC channels is dictated by H^+ and Cl^- binding in a manner that is reminiscent of a broken transporter's conformational cycle. The unexpected discovery however, was that gating of CLC-0 is coupled to H^+ transport, not just binding. The first hint of this came from a curious phenomenological observation made by Richard and Miller [70] who noticed a temporal asymmetry in the steady-state gating behavior of CLC-0. A single CLC-0 channel intercalates long periods of silence and short bursts of activity where it rapidly flickers between the closed state and two equidistant open states. The long closures are due to a cooperative process involving both subunits in the CLC dimer and their cytoplasmic domains [71-72], while the fast flickers reflect the independent opening and closing of each individual pore [35,71,73]. Richard and Miller noticed that the probability of entering into or and exiting from a long silent period depends on the state of the single-pore gates: the common gate closes more frequently with only one pore open and conversely is more likely to open with two open single pores [70]. This asymmetry implies that the three states are not at thermodynamic equilibrium and that an energy source is needed to sustain this non-equilibrium gating. Their proposal was that gating of the CLC-0 channel is linked to dissipation of the Cl^- electrochemical gradient [70]. More recently, the discovery of the H^+/Cl^- exchanger branch of the CLC family [9-13] spurred a re-evaluation of the original conclusion. A more accurate analysis of the single channel behavior showed that the gating asymmetry of CLC-0 is indeed coupled to substrate permeation, but not of Cl^- as originally proposed but of H^+ [74-75] and that a H^+ gradient can be used to invert the conformational cycle of CLC-0. This implies that for every complete cycle of the slow gate in CLC-0 a H^+ is transported across the membrane. Thus, a remnant of the H^+ pathway of the CLC transporters is also found in CLC-0. In hindsight it is striking how this form of gating is unique among ion channels but is normal and required for a secondary active transporter. Thus CLC-0 functions as an extreme example of a degraded H^+-coupled Cl^- transporter: several millions Cl^- ions permeate through CLC-0 for each H^+ transported. It is worth noting, however, that in CLC-0 transport of H^+ and Cl^- is passive; both ions flow downhill their respective electrochemical gradients. Thus, CLC-0 retains one essential feature of a secondary active transporter, the energy driving the protein's conformational change comes from substrate binding, but has lost another essential characteristic, it is not able to harness the energy resulting from the dissipation of a H^+ gradient to drive Cl^- accumulation or *vice versa*.

These results show that CLC-0, a Cl^- channel, also mediates H^+ transport. Where is the H^+ pathway? In CLC-0 Glu_{in} is replaced by a non-protonatable Valine. Thus, either the channels have evolved an alternate intracellular entry point into the H^+ pathway or a completely different transport pathway has evolved. Current data is not sufficient to choose one hypothesis over the other; rather there is data supporting both. It has been proposed that a conserved Glutamate residue next to Glu_{in}, serves as the intracellular H^+ acceptor in CLC channels [75-76]. When this residue, E291 in CLC-1, is mutated to Aspartate or Glutamine there is a drastic reduction in the open probability of the channel and a loss of sensitivity of the currents to the intracellular H^+ concentration [76]. While these results do not prove that E291 acts as a Glu_{in} surrogate they are at least consistent with this hypothesis. In contrast, two separate lines of evidence support the idea that a second and separate H^+ pathway exists in CLC-0. In CLC-0 Glu_{ex} is directly accessible to intracellular H^+ in a voltage dependent fashion [77] as well as to extracellular H^+ [78]. Thus, Glu_{ex} can be protonated from both sides of the membrane suggesting that the Cl^- permeation pathway of CLC-0 is also capable of conducting H^+. How can a positively charged H^+ permeate through an anion selective pore? A possible answer to this conundrum came from an elegant series of experiments by Zifarelli and Pusch [79]. They found that the closing rather than the opening rate of CLC-0 depends on $[H^+]_{in}$. This result is inconsistent with the idea that a protonation event of a single residue, such as Glu_{ex}, directly or indirectly leads to channel opening. They propose an alternative mechanism: a neutral water molecule penetrates into the permeation pathway and binds to an unoccupied S_{cen} site and dissociates into a H^+-OH^- couple. The H^+ then protonates Glu_{ex}, opening the channel, and the OH^- binds to S_{cen} and blocks permeation until it is competed away by an incoming Cl^- ion. This model is qualitatively and quantitatively consistent with the drastic reduction in the channel opening rate seen when H_2O is substituted with the harder to dissociate D_2O and the voltage dependent inhibition of the E166A mutant currents by OH^- at high pH [79]. It remains to be seen whether this mechanism of H^+ transport can also account for the non-equilibrium gating properties of CLC-0 or if the two represent independent and unrelated H^+-transport mechanisms through the this channel.

The picture emerging from these studies is that the “ideally selective” CLC Cl^- channels are actually quite porous to H^+’s. In addition to the Cl^- pore one or more paths for H^+ permeation exist in the CLC channels. Whether these H^+ pathways bear any resemblance to the H^+ transport pathway of the CLC transporters or not remains to be seen.

Structure and functional role of the cytoplasmic domains

The structure of CLC-ec1 revealed the three dimensional organization of the transmembrane region of CLC channel and transporters [21]. However, all eukaryotic CLC’s and ~50% of the prokaryotic ones have large cytoplasmic domains that play key roles in modulating the trafficking and function of these proteins. Mutations in these domains lead to genetically inherited disorders such as dominantly inherited myotonia (Thomsen’s disease) [80-82], Dent’s disease [83-85], infantile malignant osteopetrosis [16-17] and Bartter’s syndrome [86-87]. In some CLC’s truncation of the cytoplasmic domains leads to retention of the transmembrane region in the ER [88-89] while in others it leads to inactive proteins but does not impair trafficking [90]. In all cases correct targeting and protein function could be restored by co-transfecting separate plasmids encoding for the split domains [88,90] or by injecting the purified cytoplasmic domain together with a plasmid encoding for the transmembrane domain [89]. The C-terminal domains also play a key role in regulating the activity of CLC channels and transporters [46,82,91-92] and in at least one case channel opening is associated to a large conformational change in these domains[72].

While the sequence conservation of these regions is rather poor they all contain two cystathionine β-synthetase (CBS) domains, CBS1 and CBS2, each ~50 amino acids long and

separated by linkers of variable length and hydrophobicity [82]. Recently the crystal structures of the isolated cytoplasmic domains from the CLC-0 and CLC-Ka channels and from the CLC-5 transporter have been solved [93-95] (Fig. 1B). The structure of the cytoplasmic domain of the CLC-Ka channel revealed that this domain is organized as a dimer where the two monomers are arranged along a 2-fold axis of symmetry. The large interaction surface (>2000 $Å^2$) is formed by the two CBS2 motifs and the narrow channel crossing the interfaces is lined by water molecules [95]. Mutagenesis, crosslinking, and analytical ultracentrifugation experiments demonstrated that this interaction surface is also preserved in the CLC-0 channel, which however only crystallized in monomeric form [95]. The structure of the cytoplasmic domain from CLC-5 revealed that this basic structural arrangement is also conserved in the transporter sub-branch of the CLC family [94]. These structures have been used as templates to build homology models for the cytoplasmic domains of other CLC's, such as CLC-1 and AtCLC-a [96-97] and extensive mutagenesis experiments validated these *in silico* models. This suggests that the overall fold and dimeric arrangement are shared and conserved features of the cytoplasmic domains of CLC proteins [96-97].

Nucleotide binding and modulation of CLC function

A recent and exciting discovery has been that adenosine nucleotides, such as ATP, bind to the cytoplasmic domain of some CLC's to modulate ionic transport [94,96-102]. This result directly connects CLC function to the metabolic state of a cell and opens new questions on the physiological roles of these proteins.

Intracellular ATP directly modulates both flavors of CLC proteins: it inhibits the CLC-1 channel [94,96,99-100]. [101] and atCLC-a transporter [97], and activates the CLC-5 transporter [102]. Despite the opposite sign of the modulation these interactions share the same basic characteristics: the nucleotides bind with an affinity in the high micromolar to millimolar range and there is little selectivity between nucleotides sharing the same base. The cytoplasmic domain of CLC-5 was crystallized in the ATP– and ADP-bound forms and established the structural basis of this modulation [94]. Nucleotides bind to a crevice at the interface between the two CBS domains in a head-in configuration: the adenine base is inserted deep into a greasy pocket and interacts with residues from both CBS domains. The phosphate tail, on the other hand, is partly solvated and interacts weakly with the protein. ATP and ADP bind to the CLC-5 cytoplasmic domain with nearly identical conformations and binding affinities, ~100 μM. The ATP binding site of CLC-5 profoundly differs from the catalytic sites of most enzymes which interact tightly with the phosphate groups, bind ATP in a Mg^{2+}-dependent fashion and are formed by the conserved Walker A and B motifs [103]. These differences underscore the non-catalytic role of this binding site. These characteristics account for and are mirrored by the indiscriminate effects of ATP, ADP, AMP and adenosine on CLC-1 and CLC-5, these nucleotides have similar half saturation concentrations, ~1 mM, and elicit comparable maximal responses. In contrast adenine, IMP and GTP are virtually inert and cAMP binds weakly confirming that the nucleotide head is critical for specificity.

ATP binding to the cytoplasmic domain of CLC-1 inhibits the channel by promoting closure of the common gate [96]. This inhibition is enhanced at low intracellular pH's and reduced by oxidation [99-101]. This inhibitory effect could be physiologically relevant in a fatigued muscle fiber where the intracellular pH decreases [104-105], the redox state is altered [106] and in the case of intense activity or ischemia the ATP is converted to IMP [107]. These changes would relieve inhibition of CLC-1 thereby increasing its contribution to membrane conductance. This would in turn reduce muscle excitability and thus promote fatigue.

The mechanism of action and physiological role of ATP activation of CLC-5 is less clear [94,98,102]. CLC-5 currents are potentiated by intracellular pH, a ~2-fold maximum increase

at pH 6, and by intracellular ATP, a ~2-fold maximum increase at 10 mM ATP. Interestingly the effects of ATP and $[H^+]_{in}$ are not additive: the current potentiation induced by the simultaneous application of saturating $[H^+]_{in}$ and ATP is only ~2-fold [37,102]. It has been proposed that this lack of additivity indicates that H^+_{in} and ATP do not act independently [102]. However, if H^+_{in} and ATP act through different and independent pathways to maximally activate CLC-5 then no further activation is possible when either one of them is present at saturating concentrations. Further experiments are needed to elucidate this point. In contrast to what is seen in CLC-1 ATP activation of CLC-5 does not depend on oxidation [102]. Lastly, mutagenesis experiments based on the crystal structure of the CLC-5 cytoplasmic domain confirmed that interactions with the adenine and ribose moieties are essential for ATP binding and current potentiation while protein interactions with the a-phosphate are not important. H^+_{in} still potentiate the currents of the ATP-insensitive mutants, suggesting that these intracellular ligands activate CLC-5 through two independent pathways [102]. The physiological role of the non-selective modulation of CLC-5 is still unclear and will require further testing.

The best insights on the physiological role of ATP modulation of a CLC transporter come from a recent study on atCLC-a, the NO_3^-/H^+ exchanger from *Arabidopsis thaliana* [97]. Saturating concentrations of intracellular ATP reduce transport activity by atCLC-a ~2-fold. In contrast to what is seen for CLC-1 and CLC-5, atCLC-a discriminates between adenosine nucleotides: ATP binds and inhibits transport, ADP appears to be completely inert and AMP competes with ATP for the binding site and diminishes its inhibitory effect but it does not modulate atCLC-a activity by itself [97]. The molecular bases of this selectivity are not clear since most of the key residues that determine nucleotide binding are conserved between CLC-5 and atCLC-a [97]. Thus atCLC-a activity and vacuolar NO_3^- uptake [12] are exquisitely sensitive to the ATP/AMP ratio in the cytosol. The daytime increase of this ratio which occurs during photosynthesis would then result in reduced NO_3^- uptake while the nightly decrease of the ATP/AMP ratio would relieve atCLC-a inhibition and thus promote NO_3^- accumulation in the vacuole [97].

Conclusion and outlook

Three discoveries highlighted the last decade of research on the CLC protein family. First, the crystal structure of CLC-ec1 revealed the complex topology of the transmembrane region of these proteins. Without direct structural information translating of the available mutagenesis into a serviceable molecular model was nearly impossible. Second, the unexpected discovery that the CLC family equally split between Cl^- channels and H^+/Cl^- exchangers stimulated the biophysicists and physiologists alike. The former found a powerful tool to probe the gray area separating active and passive transport mechanisms, while the latter have been re-evaluating the physiological roles of these proteins in intracellular compartments. Third, the newly discovered modulation of CLC function by adenosine nucleotides created a novel link between the electrical and metabolic states of a cell.

These discoveries have brought new questions and exciting challenges to the front. Only the crystal structure of a *real* CLC channel will tell us how different or how similar the CLC channels and transporters look like. The structures of a full length CLC protein in the apo and/or ATP bound forms are needed to understand how the cytoplasmic and transmembrane domains communicate and interact. This vital structural information will be translated into a working model for a CLC transporter or channel only with additional knowledge on the conformational dynamics of the protein. Lastly, the contribution of the CLC transporters to H^+ homeostasis in intracellular compartments and bacteria is still unknown. This in our opinion is the central problem in understanding the physiological necessity for the functional split in the CLC family.

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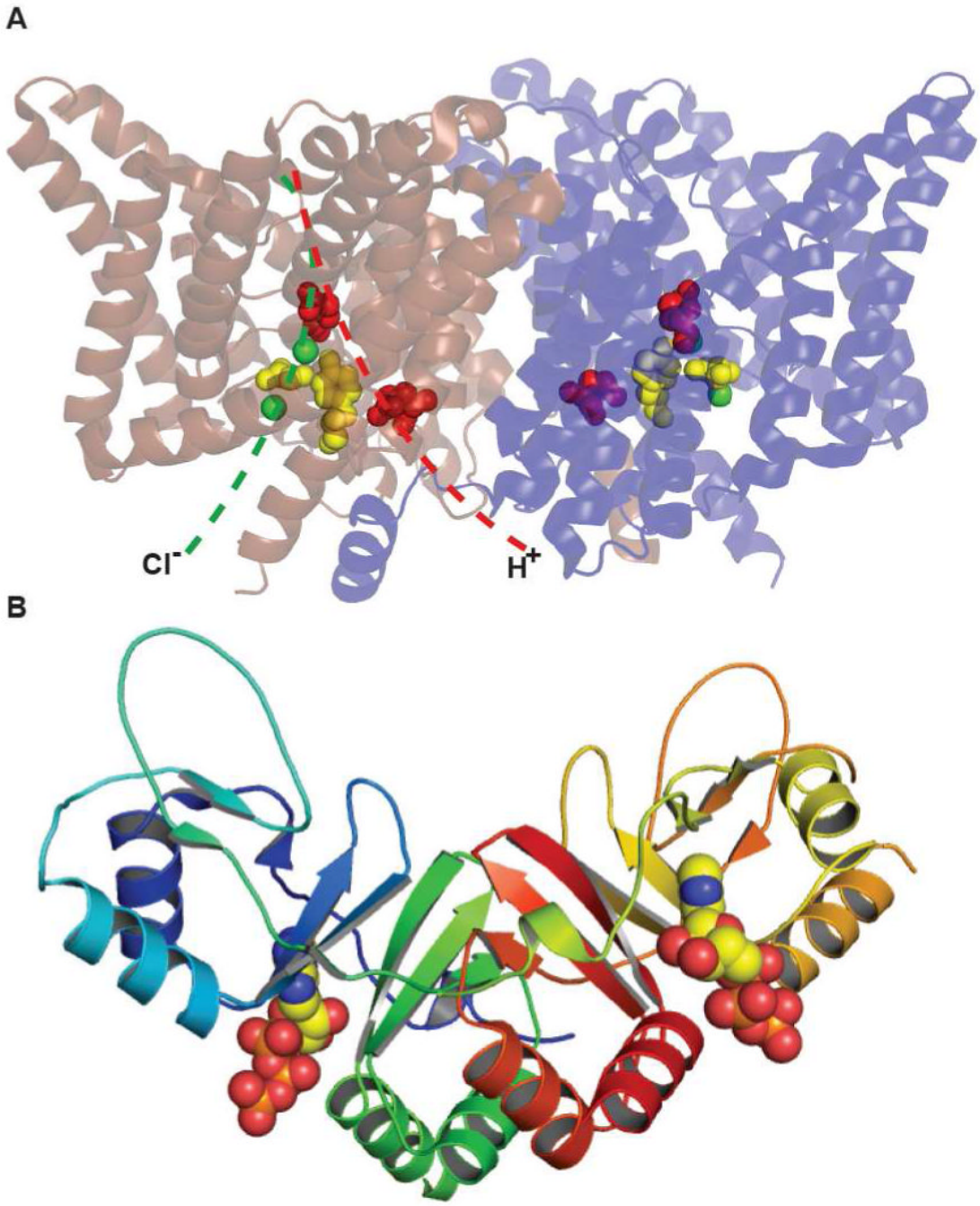

Figure 1.

CLC structures. A) The CLC-ec1 dimer is viewed from the plane of the membrane in cartoon representation. Glu_{in} and Glu_{ex} are shown in red spacefilling models, Tyr_{cen} and Ser_{cen} are shown in yellow spacefilling models. Green spheres represent bound Cl^- ions. The green and red dashed lines respectively represent the putative Cl^- and H^+ pathways through CLC-ec1. B) Ribbon representation of the CLC-5 cytoplasmic domain in the ATP bound form. The ATP molecule is shown as a CPK model.