Unique Structure and Function of Chloride Transporting CLC Proteins

Michael Pusch and Thomas J. Jentsch

***Abstract*—CLC proteins are a large structurally defined family of Cl^- ion channels and H^+/Cl^- antiporters with nine distinct genes in mammals. The membrane-embedded part of CLC proteins bears no obvious similarity to any other class of membrane proteins, while the cytoplasmic C-terminus of most eukaryotic and some prokaryotic CLCs contains two regions with homology to cystathionine beta synthase (CBS) domains that are found in other proteins as well. Different members serve a broad range of physiological roles, including stabilization of the membrane potential, transepithelial ion transport, and vesicular acidification. Their physiological importance is underscored by the causative involvement in at least four different human genetic diseases.**

From functional studies of the *Torpedo* homologue ClC-0, a homodimeric architecture with two physically separate ion conduction pathways was anticipated and fully confirmed by solving the crystal structure of prokaryotic CLC homologues. The structure revealed a complex fold of 18 α-helices per subunit with at least two Cl^- ions bound in the center of each protopore. A critical glutamic acid residue was identified whose side-chain seems to occupy a third Cl^- ion binding site in the closed state and that moves away to allow Cl^- binding. While the overall architecture and pore structure is certainly conserved from bacteria to humans, the bacterial proteins that were crystallized are actually not Cl^- ion channels, but coupled H^+/Cl^- antiporters. These recent breakthroughs will allow us to study in further detail the structure, function, and the physiological and pathophysiological role of CLC proteins.

***Index Terms*—Channelopathy, gating, ion channel, permeation.**

I. Introduction: The Double-Barreled *Torpedo* Channel

IN THE EARLY 1980s, Miller *et al.* described a curious "double-barreled" chloride channel from the electric organ of *Torpedo* fish reconstituted in planar lipid bilayers [1]. Single-channel openings occurred in "bursts" separated by long closures. A single burst was characterized by the presence of two open conductance levels of equal size and the gating (i.e., openings and closings) during a burst could be almost perfectly described as a superposition of two identical and independent conductances that switched between open and closed states with voltage-dependent rates α and β [2] (Fig. 1)

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M. Pusch is with the Institute of Biophysics, Italian Research Council, Genoa I-16149, Italy (e-mail: pusch@ge.ibf.cnr.it).

T. J. Jentsch is with the Center for Molecular Neurobiology (ZMNH), University of Hamburg, Hamburg D-20246, Germany (e-mail: jentsch@zmnh.uni-hamburg.de).

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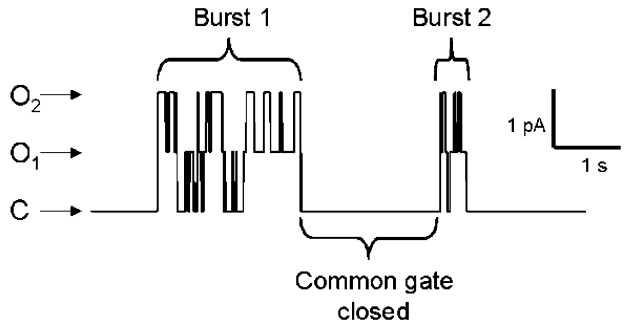

Fig. 1. Double-barreled ClC-0 channel. A simulated single-channel trace of ClC-0 is shown in which three conductance levels (C = closed; O_1, O_2 open conductance levels) are seen. Activity occurs in bursts, and during a burst the probability of observing one of the conductance levels is distributed binomial as if activity arises from two independent and identical pores. The slow common gate acts on both pores simultaneously.

These relatively fast openings and closing events gave rise to the name "fast gate" for these gating transitions during a burst. But how could it be excluded that these events just represent the presence of two identical channels in the bilayer? It was the presence of the long interburst closed events, during which no channel activity was observed, that demonstrated that the two "protopores" were tied together in a molecular complex and could be inactivated by the so-called slow gate. From these results, Miller formulated the "double-barreled" model, according to which the channel consisted of two physically distinct, identical protopores, each with a proper fast gate and an additional common gate that acts simultaneously on both protopores [3] (Fig. 1).

For a relatively long time, this double-barreled Cl^- channel remained a somewhat unique curiosity with little physiological relevance. This situation changed dramatically with the molecular cloning of the *Torpedo* channel by Jentsch *et al.* [4] in 1990, and immediately afterwards with the identification of mammalian homologues. Numerous novel physiological functions of the various CLC homologues were discovered [5]. The recent determination of the crystal structure of bacterial homologues marked the next breakthrough for the structure–function analysis of these proteins [6], [7]. A big surprise was then the recent discovery that the bacterial CLC homologue that was used for crystallization is not a Cl^- ion channel but a H^+/Cl^- antiporter [8].

II. Overview of the Family of CLC Proteins

Expression cloning was used to isolate the first member of the CLC family, the *Torpedo* channel ClC-0 [4]. This channel is highly expressed in the electric organ of electric rays. It localizes to the noninnervated membrane of the electrocyte, where it

serves to stabilize the voltage across that membrane and to pass large currents that are generated by the depolarizing influx of sodium through acetylcholine receptor channels located in the opposite, innervated membrane.

The sequence of ClC-0 [4] revealed a protein of about 90 kDa with a large hydrophobic core that was predicted to be able to span the membrane at least ten times and a large predicted cytoplasmic tail. Of note, there was no significant homology to any other known ion channels, including the cyclic adenosine monophosphate (cAMP)-activated Cl^- channel called the cystic fibrosis transmembrane conductance regulator (CFTR) and the ligand-gated gamma amino butyric acid ($GABA_{A)}$ and glycine receptor Cl^- channels. Thus, ClC-0 represented an entirely new channel class.

The cloning of ClC-0 opened the gate for cloning an entire family of CLC chloride channels by homology. In mammals, this family comprises nine distinct members. They can be grouped into three branches by homology. Members of the first branch (ClC-1,-2, -Ka, and -Kb) perform their function in the plasma membrane, while the members of the two other branches (comprising ClC-3, -4, -5, and ClC-6 and -7, respectively) are predominantly found in membranes of intracellular vesicles, in particular the endosomal–lysosomal pathway.

The diverse and important physiological functions of these channels were impressively demonstrated by the pathologies resulting from their mutational inactivation in human genetic disease and in mouse models.

ClC-1 is the major skeletal muscle chloride channel [9]. Similar to ClC-0, to which it is closely related, its function is to stabilize the plasma membrane voltage of myocytes. Its disruption in a natural mouse mutant [10] and mutations in humans [11] lead to myotonia, a form of muscle stiffness. The lack of repolarizing current through ClC-1 leads to muscle hyperexcitability. Thus, stimuli that normally elicit just one action potential give rise to trains of action potentials, resulting in impairment of muscle relaxation. Interestingly, myotonia in humans can be inherited as a recessive or a dominant trait. In dominant myotonia, the mutant protein can still associate with WT ClC-1 subunits to form dimeric channels. It exerts a dominant negative effect by changing the voltage dependence of the dimer through the common gate [12], [13]. As expected, dominant myotonia is clinically less severe, as 25% of the channels are composed entirely of wild-type (WT) subunits in heterozygous patients.

In contrast to ClC-1, which is nearly exclusively in skeletal muscle, ClC-2 is broadly, if not ubiquitously, expressed [14]. Its currents may be activated by hyperpolarization, cell swelling, and moderate extracellular acidification. Many functions were attributed to ClC-2. Those roles, however, were not confirmed by a ClC-2 KO mouse, which unexpectedly revealed testicular and retinal degeneration that were attributed to defects in transepithelial transport across Sertoli cells and the retinal pigment epithelium, respectively [15]. Although ClC-2 might play some role in regulating the intracellular chloride in neurons [16] and thereby affect inhibitory neurotransmission, ClC-2 mice lacked signs of epilepsy [15]. However, in humans, heterozygous ClC-2 mutations were associated with epilepsy in a few families [17]. However, several key findings of the functional analysis of these mutations [17] could not be reproduced [18].

ClC-Ka and ClC-Kb are two highly homologous channels that are both expressed in certain epithelial cells of the kidney, as well as in epithelia of the inner ear [19]–[21]. It is now known that they need a small accessory β-subunit, barttin, for their transport to the plasma membrane [21]. Both channels are important for transepithelial transport in either organ. Mutational inactivation of ClC-Kb in humans leads to Bartter syndrome, a disease associated with severe renal salt wasting, because this channel plays an important role in NaCl reabsorption in a certain nephron segment (the thick ascending limb) [22]. The disruption of ClC-K1 in mice (equivalent to ClC-Ka in humans) leads to renal water loss, as its expression in the thin limb is important for the establishment of a high osmolarity in kidney medulla [23]. In humans, the loss of barttin, the common β-subunit, leads to deafness in addition to renal salt loss [24]. This has been attributed to a defect in potassium secretion by inner ear epithelia. In these cells, ClC-Ka/barttin and ClC-Kb/barttin are needed for the basolateral recycling of chloride that is taken up by a basolateral sodium–potassium–two-chloride cotransporter [21].

The roles of intracellular CLC chloride channels are best understood for ClC-5 and ClC-7. In humans, the mutational loss of ClC-5 leads to Dent's disease, an inherited kidney stone disorder which is also associated with the loss of proteins into the urine [25]. ClC-5 is predominantly expressed in the proximal tubule of the kidney, where it localizes to apical endosomes [26]. The knock-out of ClC-5 in mice has revealed that the lack of this channel impairs endocytosis of protein and fluid-phase markers [27]. Further, the loss of phosphate and calcium into the urine, which ultimately lead to kidney stones, may be explained by the decreased renal endocytosis and processing of calciotropic hormones [27]. The decrease in endocytosis is due to an impairment of the luminal acidification of endosomes [27], [28]. ClC-5 seems to be important to neutralize the electric current of the H^+-ATPase that acidifies these vesicles. Without such a neutralization, the voltage over the endosomal membrane would inhibit further H^+-pumping, severely limiting endosomal acidification.

Vesicular acidification is apparently also the major role of ClC-3, a Cl^- channel expressed on synaptic vesicles in addition to endosomes [29]. The KO of ClC-3 in mice led to blindness and to a severe degeneration of the hippocampus [29]. The mechanism of this degeneration, however, is incompletely understood.

ClC-7 is the only mammalian CLC protein that is prominently expressed on lysosomes in addition to late endosomes [30]. Surprisingly, its disruption in mice led to a severe osteopetrotic phenotype [30]. It was shown that in bone-resorbing osteoclasts, ClC-7 can be inserted together with the H^+-ATPase into its ruffled border, a specialized plasma membrane that faces the resorption lacuna. An acidification of the resorption lacuna is necessary for the degradation of bone. ClC-7 KO osteoclasts failed to acidify the resorption lacuna, readily explaining the osteopetrotic phenotype of the mice [30]. This phenotype suggested that ClC-7 might also be mutated in human osteopetrosis. Indeed, ClC-7 mutations were found in autosomal recessive malignant infantile osteopetrosis [30] and later also in an autosomal

dominant form of the disease [31]. Like with myotonia due to ClC-1 mutations, autosomal dominant osteopetrosis is clinically more benign, since one-quarter of the ClC-7 dimers are expected to consist entirely of WT subunits.

ClC-4 and ClC-6 remain the only CLC channels for which no disease or mouse knockout (KO) phenotype has been described as yet. The physiological functions of CLC channels, and the pathologies resulting from their disruption, are nonetheless impressive and reveal the previously unsuspected importance of chloride channels. Furthermore, the biophysical effects of CLC mutations found in human disease have often been studied and have greatly increased our understanding of the structure–function relationship of this channel class.

III. Architecture of CLC Proteins

Several important questions regarding the double-barreled structure of CLC proteins could be answered using site-directed mutagenesis and functional electrophysiological analysis of mutated channels expressed in "heterologous" systems like frog oocytes, lipid bilayers, and mammalian cell lines. Early on, a multimeric structure was suggested on the basis of dominant negative disease-causing mutations of the muscle channel ClC-1 [12], [32]. Using mutations that altered fast gating, slow gating, and/or channel conductance of ClC-0 and combining altered and unaltered channel subunits, a homodimeric architecture could be established [33], [34]. It could also be shown that each pore of the double-barreled channel is formed entirely from one subunit [33], [35]. This architecture is fundamentally different from that found, e.g., in K^+ channels that are four-fold symmetric tetramers in which the pore is formed in the central symmetry axis [36]. The K^+ channel architecture poses restrictions on the topology of the pore: it has to be straight and perpendicular to the membrane. No such restrictions apply to CLC channels, and indeed the crystal structure of bacterial CLC homologues did not reveal a clear straight ion conducting pore [6] (see below). The crystal structure of two bacterial homologues, a *Salmonella* and an *E. coli* homologue, have been determined [6]. The two structures are very similar and, thus, only the *E. coli* CLC-ec1 structure, for which more recently a higher resolution was obtained [7], will be discussed here. The overall architecture of CLC proteins revealed by the crystal structure is strikingly consistent with the double-barreled shotgun cartoon: several nearby Cl^- ion binding sites were identified in each subunit, indicating probably the most selective permeation points, but the binding sites of the two subunits are far from each other ($>\sim$40 Å) consistent with the independence of the permeation process in the two pores.

Overall, the crystal structure displays a complex fold with 18 α-helices per subunit. Unexpectedly the structure revealed an internal pseudosymmetry within each subunit: The N-terminal half and the C-terminal half have a very similar fold and also some spurious sequence similarity, but are oppositely oriented in the membrane. The two halves of each subunit "sandwich" around the central Cl^- ion binding sites with helix ends pointing into the center of the membrane. Thus, CLC proteins have possibly evolved by gene duplication of an ancient protein with half of the length of the "modern" version and assembled as a homotetramer in a dimer-of-dimers arrangement (Fig. 2).

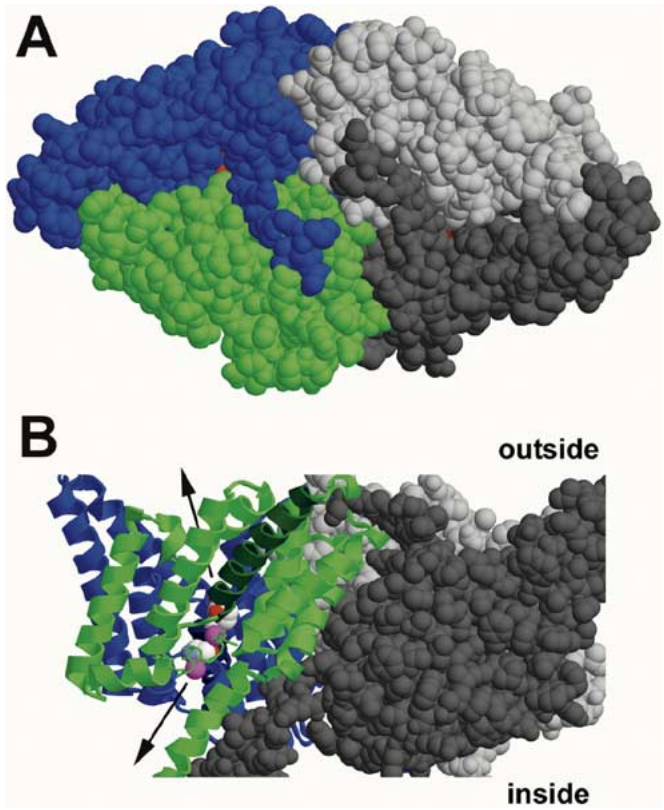

Fig. 2. Overall architecture of ClC-ec1. (A) The protein is viewed in spacefill representation from the extracellular side. Subunit 1 is drawn in gray, while subunit 2 is drawn in green and blue. The pseudotwofold internal symmetry for each subunit is highlighted with the different colors for each half-subunit. In the green–blue subunit, the critical glutamate E148 is colored red and can be seen only partially. (B) The protein is rotated such that the view is more lateral. The green–blue subunit is shown in cartoon with a small stretch of helix J shown as backbone-trace for clarity. Chloride ions are shown in magenta, and the amino acids S107 (between the two Cl^- ions) and E148 (above the top Cl^- ion) are shown in spacefill. Arrows indicate the probable extracellular and intracellular ion entry/exit pathways. This and the following figure were prepared with the RasTop program that is based on Rasmol [37] using the pdb-entry 1OTS from which water and Fab fragments have been removed for clarity.

Before describing in more detail the structure of CLC-ec1, basic functional properties of mammalian CLC channels will be discussed.

IV. Gating of Mammalian CLC Channels

As described above, animal CLC channels can functionally be divided into three classes. The first class consists of plasma-membrane channels (ClC-1, ClC-2, ClC-Ka, and ClC-Kb); the *Torpedo* channel ClC-0 also belongs to this class. These proteins are clearly Cl^- ion channels (in contrast to some bacterial homologues that appear to be H^+/Cl^- antiporters; see below). They have a directly measurable single-channel conductance ($>=$ 1 pS) [35], [38]–[40] (A. Picollo and M. Pusch, unpublished results) the reversal potential measured in Cl^- gradients follows the Nernstian prediction of passive diffusion [14], [41], [42]; and they all exhibit relatively slow macroscopic gating relaxations that depend on voltage, Cl^- concentration, and pH. These channels will be discussed in more detail below. The second class comprises the highly homologous proteins

ClC-3, ClC-4, and ClC-5. *In vivo* these proteins are mostly expressed in intracellular organelles, but at least some functional plasma membrane expression can be achieved in heterologous systems [43]–[45] allowing an electrophysiological characterization. Currents induced by these proteins are characterized by an extreme outward rectification—corresponding to movement of Cl^- ions from the extracellular side into the cytoplasm or correspondingly to a movement from the lumen of intracellular vesicles into the cytosol. Currents are measurable only for voltages $>\sim+20$–40 mV, such that a true reversal potential cannot be measured [43], [44]. Current activation at positive voltages has apparently two kinetic components. A minor component shows relatively slow kinetics while the major component of the current is associated with very fast activation and deactivation kinetics that cannot be resolved using standard electrophysiological techniques. These fast kinetics may actually represent an "instantaneous" activation, i.e., a rectification of the open pore. Noise analysis supports this hypothesis [46]; however, the single-channel properties of ClC-3, -4, or -5 are still unclear [46], [47], and it is fair to state that the basic mechanisms of function of these proteins are still relatively little understood. In particular the relationship of the double-barreled structure and the functional properties is unknown.

The third group of CLC channels is composed of ClC-6 and ClC-7. Both proteins are mostly found in membranes of intracellular organelles [30], [48]. No functional electrophysiological data have so far been obtained for these proteins, and it is, thus, not certain if they are Cl^- channels.

The plasma membrane localized channels ClC-1 and ClC-2, and in particular the "prototype" ClC-0 channel, have been studied most extensively from a functional point of view. A fundamental difference between classical voltage-gated cation channels and the CLC Cl^- channels regards their voltage dependence. Both type of gates (i.e., the single protopore gate and the common gate) of the *Torpedo* channel ClC-0 are voltage dependent. The apparent gating valence of the protopore gate is around one and that of the common gate around two, while they exhibit an opposite voltage dependence [2], [49], [50]. However, no clear "voltage sensor" such as the S4-segment of K^+ and Na^+ channels is evident from the primary or from the three-dimensional (3-D) structure. In fact the voltage-dependence of the fast gate arises indirectly from the coupling of a transmembrane movement of the permeant anion to channel opening [49], [51]. Lowering the extracellular Cl^- concentration "shifts" the voltage dependence of the open probability to more positive voltages, i.e., renders opening more difficult [49]. Also intracellular Cl^- affects the open probability of the protopore gate mainly by altering the closing rate constant [41], [51]. This strong coupling of gating and "downhill" permeation renders the gating an intrinsically irreversible process. Furthermore, it implies that altering the permeation process (e.g., by mutation) also affects channel gating and *vice versa*. In fact, many results from mutagenesis studies and attempts to establish a transmembrane topology have been difficult to interpret in the absence of a crystal structure (see [5] for review). An irreversible coupling of the single protopore gate and the common gate of ClC-0 has been observed by Richard and Miller at the single-channel level: bursts of channel activity (see Fig. 1) started more often with both protopore gates open and ended more likely with only one of the protopore gates open [52]. This imbalance, which on principal grounds requires an external energy input, was indeed stronger in stronger electrochemical gradients, demonstrating a coupling of permeation and gating and a coupling of the two types of gates [52]. Based on a large temperature dependence of the kinetics of the common gate, it has been suggested that it is accompanied by a substantial conformational rearrangement [50]. This is consistent with the finding that mutations in many protein regions affect the common gate [13], [33], [41], [53]–[60]. However, the molecular mechanism of the common gate is still obscure.

Gating of ClC-1 largely resembles that of ClC-0 [57], [61], [62]; however, its small single-channel conductance [40] renders a detailed analysis difficult. Qualitatively, also ClC-1 shows a double-barreled appearance [13]. However, the two gates seem to be more strongly coupled in this channel compared to ClC-0 [62]. ClC-2 shows a quite complicated and slow gating behavior that, in addition, depends on the expression system and will not be discussed here in detail [14], [63]–[68].

A pair of highly homologous kidney and inner ear specific channels (ClC-K1 and ClC-K2 in rodents; ClC-Ka and ClC-Kb in humans) was cloned more than a decade ago [19], [20], [69]; however, these channels expressed only little in heterologous systems despite their sequence similarity to ClC-0. The low expression was also surprising because *in vivo* immunocytochemistry showed a plasma membrane localization [70], [71]. Furthermore, a basolateral plasma membrane localization of ClC-Kb was strongly suggested by the disease phenotype of Bartter's syndrome [22]. The lack of expression rendered a detailed biophysical analysis impossible. The human channel ClC-Kb is of particular interest because of its involvement in the salt-wasting nephropathy, Bartter's syndrome type III [22]. Only recently it became clear that ClC-K channels need the associated small transmembrane protein barttin for efficient plasma membrane expression [21]. The barttin gene was identified by positional cloning of the locus leading to a particular type of Bartter's syndrome associated with deafness [24]. Interestingly, ClC-K activity can be markedly regulated by the extracellular pH and by the extracellular Ca^{2+} concentration in the millimolar concentration range [21], [70], [72]. Since the fast protopore gate is probably mostly open (see below), the pH and Ca^{2+} regulation could act via the common gate, but the underlying mechanisms remain to be identified.

V. Permeation of Mammalian CLC Channels

Cl^- channels do not generally discriminate strongly among halides, except for fluoride, that is quite impermeant in most Cl^- channels probably due to its strong hydration. Based on anomalous mole fraction effects, it has been suggested that CLC pores can accommodate more than one ion at a time [42], [49] in agreement with the presence of multiple Cl^- ions in the crystal of ClC-ec1 [7]. Ion permeation has been most extensively studied in the muscle channel ClC-1 at the macroscopic level. The channel exhibits an almost perfect

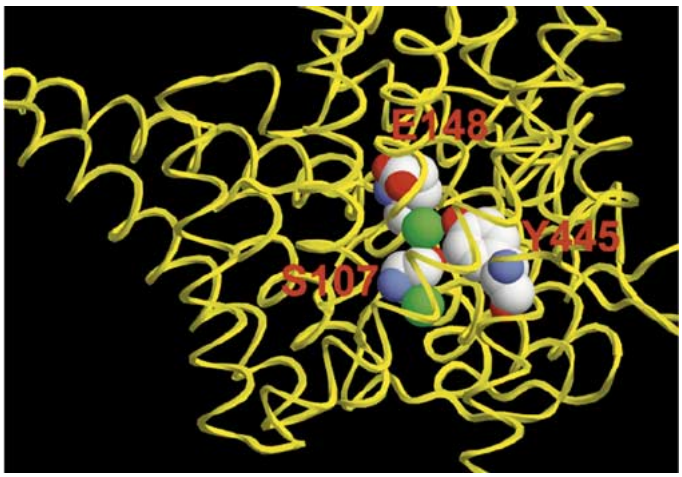

Fig. 3. The Cl^- binding sites in ClC-ec1. Only one subunit is shown with the intracellular side at the bottom. Cl^- ions (in green) and the amino acids S107 (between the two Cl^- ions), E148 (above the top Cl^- ion) and the coordinating Y445 are shown in spacefill.

selectivity of anions over cations [42]. Among various anions tested the permeability sequence obtained from reversal potential measurements was $SCN^- \sim ClO4^- > Cl^- > Br^- > NO3^- \sim ClO3^- > I^- \gg BrO_3^- > HCO_3^- \sim F^- \gg$ methanesulfonate $\sim$ cyclamate $\sim$ glutamate, where the latter three organic anions are practically impermeable [42]. Furthermore, ClC-1 is blocked in a voltage-dependent manner by various organic anions, and permeation and block appear to involve strong hydrophobic interactions [42], [73]. Recently, the permeation properties of ClC-0 and ClC-1 have been modeled using Brownian dynamics based on the crystal structure of ClC-ec1 [74]. Overall these results predict a "knockoff" mechanism of permeation in which two relatively strongly bound Cl^- ions are destabilized by the arrival of a third ion. The predictions of these simulations remain to be tested experimentally.

VI. X-Ray Structure and Its Functional Implication: A Pivot Glutamate Controls the Protopore Gate

The X-ray structure of ClC-ec1 marked a breakthrough for the structure–function analysis of CLC proteins [6], [7]. As shown in Fig. 3, in each subunit two Cl^- ions could be resolved. The central binding site (S_{cent}) is completely buried in the protein. Major direct protein–ion interactions involve the amide NH groups of some amino acids and the side-chains of two residues (Y445 and S107). The directly Cl^- interacting residues are well conserved among CLC proteins, and most of them are located in loops connecting two helices. In fact, several helices have their N-terminal end within the membrane. This topology reflects the pseudosymmetrical sandwich structure described above (Fig. 2). It is interesting to note that none of the directly interacting residues is positively charged. Direct salt bridging of a Cl^- ion would probably lead to a too-strong binding and less efficient conduction. However, simulation studies have shown that several more distantly located positively charged residues make significant contributions to the free energy of Cl^- ion binding [74]–[77]. The more intracellularly located binding site S_{in} is in direct contact with the intracellular solution (Fig. 3) indicating the most likely intracellular pore entrance. In agreement with this, and confirming that the ClC-ec1 crystal structure is relevant for eukaryotic CLC channels, mutations in many residues that delineate the inner pore entrance alter typical pore properties like rectification and single-channel conductance in an expected way [33], [34], [41], [49], [61], [65], [78]–[80].

In contrast, the exit of the central Cl^- ion toward the intracellular side is impeded by the highly conserved serine 107 that is located between S_{in} and S_{cent} (Fig. 3) [74], [75]. Probably some conformational rearrangement of the intracellular entrance has to occur for an efficient Cl^- conduction. In agreement with the necessity of a conformational change of the inner pore during channel opening, strong state-dependent binding of small organic inhibitors from the inside has been described [59], [78], [81]. The exit of the central Cl^- ion toward the extracellular side is clearly impeded by the negatively charged side-chain of a conserved glutamate (E148) (Fig. 3) [6]. In order to study the role of E148, Dutzler *et al.* solved the structure of ClC-ec1 in which the glutamate was mutated to alanine (E148A) or glutamine (E148Q). Since at that time direct functional analysis of ClC-ec1 was not yet possible [82], Dutzler *et al.* used the well-studied *Torpedo* ClC-0 as a model to investigate the functional effect of the mutations [7]. The mutant structures were almost identical to that of WT ClC-ec1 with a single difference: where the negatively charged E148 side-chain in WT CLC-ec1 blocked ion movement was now sitting a third crystallographically identified Cl^- ion Cl_{ext}^- [7]. This structure appears to be an opened confirmation, at least concerning movement of the central Cl^- toward the outside. Consistent with this simple structural result, both ClC-0 mutants, E166A and E166Q, showed a constitutively open phenotype: they appeared to have lost the voltage and Cl^-dependence of the open probability [7], [59]. Interestingly, WT ClC-0 can also be opened by lowering the extracellular pH, probably by protonation of the corresponding glutamate [7], mimicking the E166Q mutation. These findings clearly establish E148 as a major player in the gating of CLC proteins. In this respect, it is noteworthy that ClC-K channels are only little voltage dependent showing almost constitutive activity at all voltages [21], [83]. The wimpy voltage dependence is related to the fact that ClC-K channels, and only these, carry a valine residue at the glutamate-position. In fact, mutating the valine to glutamate confers marked voltage-dependent gating to ClC-K channels [21], [72]. Also for the strongly outwardly rectifying channels, ClC-3–ClC-5, the conserved glutamate is of prime importance: mutating E211 in ClC-5 (or its equivalents in ClC-3 or ClC-4) to alanine leads to a loss of the strong rectification and to an almost linear current–voltage relationship [44], [84].

VII. Function as a H^+/Cl^- Antiporter

Accardi and Miller succeeded recently in obtaining a high-yield and extremely pure protein preparation of ClC-ec1 that allowed them to study macroscopic currents of protein reconstituted in lipid bilayers [85]. Currents were voltage-independent but activated by low pH; the apparent single-channel conductance was too low to be reliably estimated even from noise analysis [85]. Currents carried by the glutamate mutant

E148A were independent of pH [85], in agreement with analogous behavior for ClC-0. A puzzling finding was, however, that the reversal potential measured for WT ClC-ec1 did not follow the Nernstian prediction for a purely Cl^- selective channel but was significantly smaller, indicating the permeation of some cationic species [8], [85]. After detailed analysis, Accardi and Miller clearly demonstrated that ClC-ec1 is actually not a diffusive Cl^-selective channel, but instead a secondary active strictly coupled H^+/Cl^- exchanger [8]. The apparent stoichiometry was found to be an exchange of 2 Cl^- ions for each transported H^+ [8]. Given the opposite charges of Cl^- and H^+, this transporter is highly electrogenic with a net transfer of three elementary charges per transport cycle.

This result came as a big surprise for the ClC-field, as no previous piece of evidence, and in particular not even the atomic resolution structure of the protein, had indicated such a function. The basic mechanism of function of a coupled transporter is *a priori* quite different from that of a passively diffusive channel, and it will be very interesting to decipher the mechanism of this transport in the future. These results do not imply that all CLC proteins are H^+/Cl^- antiporters. Certain well-known ClC-proteins like, e.g., ClC-0, ClC-1, and ClC-2 are clearly Cl^- ion channels: they have a rather large conductance and they show almost perfect Nernstian $[Cl^-]$ dependence of the reversal potential. Their gating, however, depends on pH_{int} and pH_{ext} [86], and this dependence is likely related to the transport mechanism of ClC-ec1. In contrast, the function of several intracellular ClC-proteins, like ClC-3–ClC-7, has, however, not yet been clearly related to Cl^- ion channel activity, and the possibility that these proteins act as H^+/Cl^- exchangers will be interesting to study.

VIII. Pharmacology

Small organic molecules and peptides that interact with ion channel proteins have been extremely useful tools for the studying of voltage-gated cation channels and ligand-gated channels [87]. In this respect, it suffices to recall the potent neurotoxins α-bungarotoxin or tetrodotoxin that act on the nicotinic acetylcholine receptor and the voltage-gated Na^+ channel, respectively, with high affinity [87]. Also many medically useful drugs act on various types of ion channels. Unfortunately, the situation is worse for Cl^- channels in general and for CLC-proteins in particular, even though some progress has been made recently. Typical, "classical," Cl^- channel blockers include molecules like 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid (DIDS), 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid (SITS), 9-anthracenecarboxylic acid (9-AC), niflumic acid (NFA), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and others. Most of these drugs block (i.e., reduce the currents) of many Cl^- channels. They are, thus, quite unspecific. Furthermore, block occurs often with a low affinity $< (100\ \mu M)^{-1}$. There are, however, exceptions. For example, some CLC proteins are particularly resistant to any blocker: no sub-100-μM organic blocker of ClC-2 and no blocker at all of ClC-5 has been identified so far [88]. On the other hand, the muscle ClC-1 channel is so far the most "sensitive" ClC-protein. It is blocked by 9-AC and by derivatives of p-chloro-phenoxy propionic acid (collectively abbreviated as CPP) at concentrations below 10 μM [89]–[91]. The block of ClC-0 and ClC-1 by these classes of substances (i.e., 9-AC and CPP) has been studied in considerable detail, and they have proved to be useful tools to investigate the structure–function relationship of these channels. First, it has been found that both types of drugs can directly access their binding site only from the intracellular side and that they bind to the pore region [81], [88], [92]. Their action is strongly state dependent in that they bind much more tightly to the closed state than to the open state of the channel leading to a marked apparent voltage dependence of block [78], [81], [88], [93]. Starting from a critical amino acid identified independently from the crystal structure, Estévez *et al.* then used the bacterial structure as a guide to identify the crucial amino acids involved in blocking the muscle ClC-1 channel by 9-AC and CPP [92]. When mapped onto the bacterial structure, these critical amino acids clustered in a region around the central Cl^- ion binding site [92]. On the one hand, this rough identification of the binding site opens the way for further analysis of the interaction of these molecules with ClC-0 and ClC-1. On the other hand, the results of Estévez *et al.* demonstrate that the overall structure of ClC-0 and ClC-1 must be very similar to that of the bacterial ClC-ec1 and that, despite the quite different functional properties, the bacterial crystal structure is a quite good guide for the mammalian homologues.

A different blocker binding site was identified for certain kidney ClC-K channels. Some derivatives of CPP with two phenyl-groups (abbreviated here as 3-phenyl-CPP) and also DIDS were found to block ClC-K1 and CLC-Ka channels but not ClC-Kb and not other CLC channels [83], [91], [94]. ClC-K1, ClC-Ka, and ClC-Kb are about 80% identical to each other, and block by 3-phenyl-CPP and DIDS occurs from the extracellular side in competition with Cl^- ions [94]. Comparing the primary sequence between these channels, and using the ClC-ec1 crystal structure as a guide, two amino acids could be identified that are critically important for the differences in inhibitor sensitivity [83]. Both residues (N68/D68 and G72/E72 in CLC-Ka/CLC-Kb, respectively) are located in helix B, and in ClC-ec1 the corresponding side-chains point toward the extracellular channel entrance. The N/D68 residue is the most sensitive residue involved in drug binding and it is highly conserved: practically all CLC proteins, including ClC-ec1, present a negatively charged amino acid at the corresponding position. Mutating this residue in ClC-0 or ClC-1 leads to profound alterations in the gating behavior [53], [95]. Also the ClC-Kb D68N mutation drastically alters the gating behavior [83]. The precise role of this residue will be interesting to study in the future.

IX. cystathionine Beta Synthase (CBS) Domains

The crystallized bacterial CLC homologues have short cytoplasmic N- and C-termini. In contrast eukaryotic CLCs have longer N- and especially C-termini. These long C-termini are of significant functional importance [96], and they contain two conserved CBS domains [97]. Probably the two CBS domains of each subunit interact, and they seem to play a role in regulating the common gate [60]. However, none of the CBS domains is strictly essential for channel function [60], [98] and

their precise role is still unclear (see [99] for review). A possibly important clue about the role of CBS domains is that (as isolated peptides) they bind intracellular nucleotides like AMP and ATP [100]. Some disease-causing mutations in CBS domains diminish the binding [100], suggesting that it plays an important functional role. Among these mutations, the amino acid change G715E that is located between CBS1 and CBS2 in ClC-2 reduced the affinity for ATP tenfold [100]. This amino acid change was reported to cause epilepsy in humans and to alter the $[Cl^-]_{int}$ sensitivity of the channel [17]. These gating effects could not, however, be reproduced [18]. Nevertheless, Niemeyer *et al.* found that the G715E-mutation altered a small kinetic effect of intracellular AMP on the gating [18]. The role of intracellular nucleotides in the regulation of CLC activity and the involvement of the CBS domains need further study to allow firm conclusions to be drawn.

X. Conclusion

CLC proteins have been full of surprises right from their first revelation as funny double-barreled, irreversibly gating Cl^- channels. They challenge the classical distinction of ion channel and active transporter. Unexpected physiological roles have been discovered. They are involved in many human genetic diseases. They display an extremely complex 3-D structure, with a two-pore architecture of unknown relevance. These fascinating proteins will certainly occupy scientists from disciplines ranging from molecular medicine to molecular dynamics for several years.

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Michael Pusch received the Diploma degree and the Ph.D. degree in physics from Göttingen University, Göttingen, Germany, in 1987 and 1990, respectively.

He held postdoctoral positions at the University of Genoa, Genoa, Italy, and at the Center for Molecular Neurobiology (ZMNH), Hamburg University, Hamburg, Germany. He is currently a Research Scientist at the Institute of Biophysics, Italian Research Council, Genoa. His major interest is the experimental study of biophysical mechanisms of ion channels and transporters, including CLC proteins and voltage-gated cation channels.

Thomas J. Jentsch received the Ph.D. degree in physics and the M.D. degree from the Free University of Berlin, Berlin, Germany, in 1982 and 1984, respectively.

He held a postdoctoral position at the Institute for Clinical Physiology, Berlin, and at the Whitehead Institute for Biomedical Research, Cambridge, MA. He then established a junior research group at the Center for Molecular Neurobiology (ZMNH) of Hamburg University, Hamburg, Germany, where since 1993 he has headed his own Institute as Professor of Molecular Neuropathology. He has identified the gene family of CLC chloride channels and has characterized many of its members, with a recent focus on mouse models and the role of CLC genes in human disease. He is also studying KCNQ potassium channels and KCC potassium-chloride cotransporters.