Minireview

Structural basis for ion conduction and gating in ClC chloride channels

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Abstract **Members of the ClC family of voltage-gated chloride channels are found from bacteria to mammals with a considerable degree of conservation in the membrane-inserted, pore-forming region. The crystal structures of the ClC channels of *Escherichia coli* and *Salmonella typhimurium* provide a structural framework for the entire family. The ClC channels are homodimeric proteins with an overall rhombus-like shape. Each ClC dimer has two pores each contained within a single subunit. The ClC subunit consists of two roughly repeated halves that span the membrane with opposite orientations. This antiparallel architecture defines a chloride selectivity filter within the 15-Å neck of a hourglass-shaped pore. Three Cl^- binding sites within the selectivity filter stabilize ions by interactions with α-helix dipoles and by chemical interactions with nitrogen atoms and hydroxyl groups of residues in the protein. The Cl^- binding site nearest the extracellular solution can be occupied either by a Cl^- ion or by a glutamate carboxyl group. Mutations of this glutamate residue in *Torpedo* ray ClC channels alter gating in electrophysiological assays. These findings reveal a form of gating in which the glutamate carboxyl group closes the pore by mimicking a Cl^- ion.**

Key words: Chloride channel; Chloride selectivity; Gating

1. Introduction

Chloride is the major permeant anion in nature, its movement across membranes is the basis for important cellular processes. Chloride channels allow the conduction of Cl^- across the cell membrane down the electrochemical gradient. ClC channels are members of a large family of chloride-selective ion channels which are found from bacteria to man [1,2]. Nine isoforms of ClC channels in humans are involved in various physiological functions. They are expressed either in the plasma membrane or in the membranes of intracellular organelles. In the plasma membrane, certain ClC channels control the resting potential in muscle and certain neurons. Others are involved in the reabsorption of NaCl from the kidney into the bloodstream [1]. In intracellular organelles ClC channels are involved in acidification processes [1]. The importance of ClC channels is emphasized in several diseases associated with their malfunction. Mutations in ClC channels cause myotonias, certain forms of epilepsies, nephropathies and osteopetrosis [1].

ClC channels represent a unique family of ion channel proteins [3]. They are not related in sequence or structure to any of the other known families such as the members of the cation channel family (such as K^+, Na^+ and Ca^{2+} channels), the family of ligand-gated ion channels (such as acetylcholine, γ-aminobutyric acid and glycine receptors), or of cystic fibrosis transmembrane conductance regulator [4]. The recent elucidation of the molecular structure of two bacterial ClC channels provides the basis for the understanding of their functional properties.

2. The structure of the ClC channel

The breakthrough in the structure determination of ClC channels came from the discovery of bacterial homologues [5]. The overexpression of the ClC channels from *Escherichia coli* (EcClC) and *Salmonella typhimurium* (StClC) allowed an initial structural characterization by electron microscopy [6] and the structure determination by X-ray crystallography [7]. The first structures of EcClC and StClC were solved at 3.5 Å and 3.0 Å respectively [7]. In order to define the ion–protein interactions with accuracy and to investigate the structures of mutants, however, it was necessary to obtain data at higher resolution. This objective was accomplished by crystallizing EcClC in complex with a Fab fragment from a monoclonal antibody that binds to a structural epitope of the channel, an approach which has already been successfully used for other membrane proteins [8,9]. The overall structures of EcClC and StClC in different crystal forms and in complex with the Fab fragment were found to be very similar.

The structure of the EcClC dimer is shown in Fig. 1a [7,9]. EcClC contains two identical subunits, which are related by a two-fold axis of symmetry perpendicular to the membrane plane. Viewed along the two-fold axis from outside the cell, each subunit is triangular. The entire channel with two subunits is shaped like a rhombus with major and minor diagonals of 100 and 55 Å respectively. The two anion-selective pores of the dimer are indicated by bound ions (Fig. 1a). Each pore is completely contained within the center of the triangular subunit with a mutual distance between the two ion conduction pores of about 39 Å. With respect to the pore stoichiometry, the structure is in accordance with early electrophysiological work on ClC-0, which predicted the double-barreled architecture of ClC channels [10].

The topology of the EcClC subunit is shown in Fig. 1b [7]. The overall architecture of EcClC is remarkable: rather than

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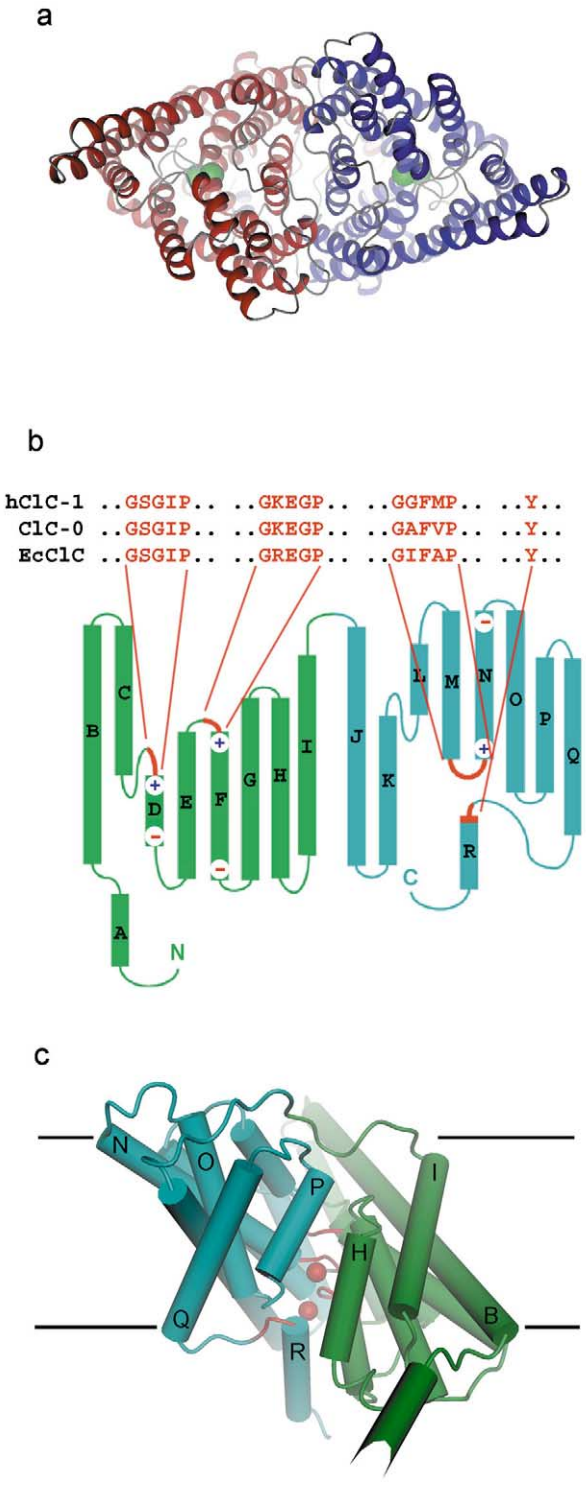
consisting of straight membrane-spanning helices of about equal size, the helices within each subunit vary in length. Some of them (e.g. helices B, N) are very long and are tilted by about 45° with respect to the membrane normal (Fig. 1c); others are short and penetrate the membrane only half way. The EcClC subunit consists of two topologically related domains, which span the membrane in opposite directions in an arrangement called an 'antiparallel architecture' (Fig. 1b) [7]. The two domains, despite their similarity in structure, show only weak conservation on the sequence level. Structurally the two domains are related by a pseudo two-fold axis of symmetry in the center of the membrane (Fig. 1c). This pseudo two-fold relationship makes it possible to bring together loops at the end of α-helices from different parts of the structure to form a selectivity filter for Cl^- ions. This 'antiparallel architecture' has previously been observed in aquaporins, a family of water-selective channels, which are otherwise completely unrelated to ClC channels [11].



The transmembrane structure is conserved within the entire ClC channel family. This is apparent when looking at the signature sequence of ClC channels, which is scattered over the entire subunit and is conserved from bacteria to mammals (Fig. 1b) [7]. The main differences between the bacterial channels of known structure and eukaryotic ClC channels are the presence of large intracellular domains attached to the C-terminus of most eukaryotic and some prokaryotic ClC channels which are absent in EcClC and StClC [12,13].

The EcClC structure was used as a template for the structure of the transmembrane part of eukaryotic ClC channels. Several mutants, identified in various channelopathies in different ClC channels, can be located on the EcClC structure [14]. More recent studies made use of the structure to guide experiments. The EcClC structure served as a model for the identification of the binding site of inhibitors to ClC-1 [15] and to study the electrostatic influence of charged residues in the channel vestibule on ion conduction [16,17]. It was concluded that the EcClC structure matches the pore of ClC-0 even in the absence of the large cytoplasmic domains [16].

3. The selectivity filter and the pore

The ClC channel pore is located at the interface between the two domains in the center of the triangular ClC subunit (Fig. 1a,c) [7,9]. The pore has an hourglass-like shape with two aqueous vestibules coming from the extracellular and intracellular side of the membrane, which narrow down at the selectivity filter (Fig. 2a). The selectivity filter is a narrow 15 Å long region in the center of the membrane around the pseudo two-fold axis of the ClC subunit. This narrow selectivity filter contains three ion binding sites (named S_{int}, S_{cen} and S_{ext}) in which chloride ions are aligned in a single file to bridge the two channel vestibules (Fig. 2b,c) [9]. The three ion binding sites are formed by conserved stretches of residues, which are all located on loop regions on the N-terminal end of α-helices D, F, N and R (Figs. 1b and 2a). In this arrange-

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Fig. 1. EcClC structure. a: View of a ribbon representation of the EcClC dimer from the extracellular side. The two subunits are colored in blue and red. Ions bound to the selectivity filter are colored green. b: Topology of the EcClC subunit. The α-helices are drawn as cylinders and labeled A–R. The two domains within the subunit are colored green and cyan. Stretches of residues encoding the selectivity filter are colored in red and are shown aligned in single letter code above. The partial charges at the end of helices involved in Cl^- binding are indicated by + and −. c: View of the EcClC subunit from the dimer interface along the pseudo two-fold axis. α-Helices are drawn as cylinders, loop regions as cords, the Cl^- ions as spheres, the coloring scheme of b is maintained. The membrane is indicated by black lines. For b and c the extracellular side is at the top, the cytoplasmic side at the bottom. Figs. 1–3 were prepared with DINO (http://www.dino3d.org).

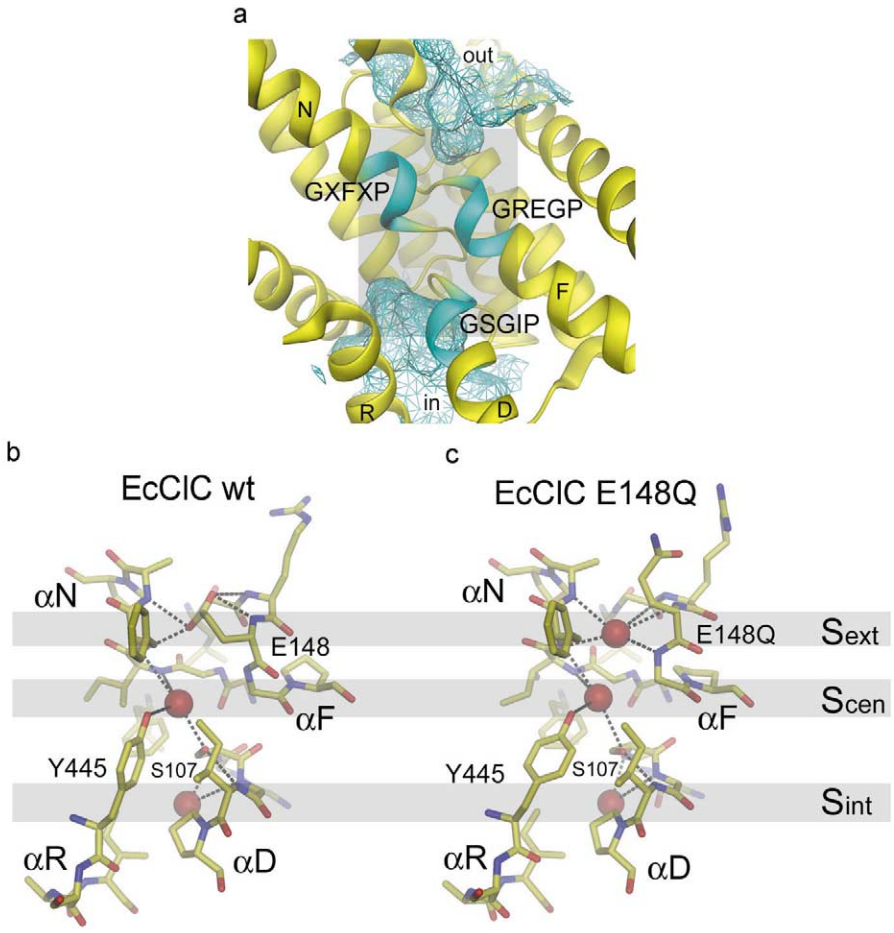

Fig. 2. Structure of the pore and the selectivity filter. a: Structure of the ion conduction pore in a view from the dimer interface along the pseudo two-fold axis. Front α-helices are removed for clarity. The protein is drawn as a ribbon. Aqueous cavities, which approach the selectivity filter from the extracellular solution (out) and the cytoplasm (in), are shown as a cyan mesh. N-termini of α-helices involved in ion binding are colored cyan, the conserved stretches of residues are labeled. The region of the selectivity filter is emphasized (gray). b: Structure of the selectivity filter of wild-type (wt) EcClC. Selected residues in the vicinity of the bound Cl^- ions (red spheres) are shown. Hydrogen bonds between the Cl^- ions and the protein and between Glu148 and the rest of the protein are shown as dashed lines. c: Structure of the selectivity filter of EcClC mutant E148Q. Atoms are represented as in b. Cl^- ion binding sites are labeled (S_{ext}, S_{cen} and S_{int}).

ment the positive end of the helix dipole points towards the ions and stabilizes them by electrostatic interactions (Fig. 2a–c) [7].

Two of the three ion binding sites (S_{int} and S_{cen}) are occupied in the wild-type (wt) channel (Fig. 2b) [9]. The Cl^- ion bound to S_{int} is located at the interface between the aqueous vestibule and the selectivity filter. The ion is partly desolvated and still hydrated where it is exposed to the intracellular vestibule. It interacts with the free backbone amide groups on the loop preceding α-helix D and is otherwise surrounded by side chains of hydrophobic residues (Fig. 2b).

At a distance of 6.5 Å towards the extracellular side, another Cl^- ion is bound to site S_{cen}. This ion has completely shed its solvation shell and instead interacts with partial positive charges from backbone amide and hydroxyl groups of residues of the selectivity filter. The interactions include the free backbone amide nitrogen groups preceding α-helix N and the side chain hydroxyls of Ser107, which is part of the conserved GSGIP motif, and of Tyr445 (Figs. 1b and 2a). The conserved residue Tyr445 is located at the beginning of α-helix R at the C-terminus of EcClC. In eukaryotic ClC channels this helix precedes the large soluble domains of currently unknown function. It is conceivable that Tyr445 provides the domains a way of directly regulating the selectivity filter.

Extracellular to binding site S_{cen}, the pore is blocked by the side chain of Glu148 (Fig. 2b). Glu148 is part of the GK/REGP sequence motif which is conserved among ClC channels with the exception of the kidney channels ClC-Ka,b (Fig. 1c). The position of Glu148 is remarkable not only because it blocks the exit of the Cl^- ion towards the extracellular side, but also because, assuming that the glutamate is not protonated, it places a negative charge very close (~4 Å) to the Cl^- ion in S_{cen}. Its location, however, is reasonable from an electrostatic point of view, since it bridges the two partial positive helix end charges of the pseudo symmetry-related α-helices N and F which would otherwise repel each other [7,9].

Since the path of the chloride towards the cell exterior is blocked, it was hypothesized that the EcClC structure represents a closed, non-conducting conformation and that in a conducting open conformation the glutamate side chain would have to move out of the way to open up another ion binding site [7]. This hypothesis was confirmed on a structural level in two mutants, which replaced the Glu148 residue with Ala (E148A) and Gln (E148Q) [9]. In both mutants a Cl^- ion

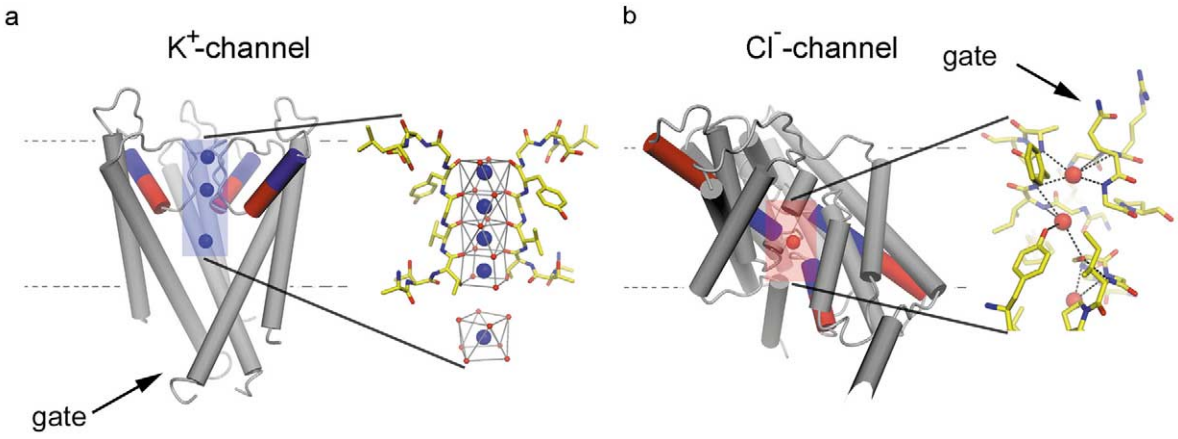

Fig. 3. Comparison of K^+ and Cl^- channels. a: Structure of the KCSA K^+ channel. The front subunit is removed for clarity. The helices are shown as cylinders. The amino-terminal and carboxy-terminal halves of the pore helices are colored red and blue respectively. The structure of the selectivity filter is shown enlarged. K^+ ions are shown as blue spheres, H bonds as gray lines. The extracellular space is at the top, the cytoplasm at the bottom. The membrane is indicated by dashed lines. b: Structure of the EcClC subunit. The protein is shown in the same view as in Fig. 1c. The colors are as in panel a. The structure of the selectivity filter of the E148Q mutant is shown enlarged. Cl^- ions are shown as red spheres, H bonds as dashed lines.

is bound to the site, which is occupied by Glu148 in the wt structure. In the E148Q mutant the glutamine side chain is visible in a conformation pointing towards the extracellular vestibule (Fig. 2c). The Cl^- ion in the new ion binding site S_{ext} is bound at the interface between the two N-termini of α-helices N and F, only coordinated by the free backbone amide NH groups (Fig. 2c). S_{ext} is accessible to the extracellular vestibule. The overall structures of both mutants show only minor changes compared to the EcClC wild type.

In the two mutants all three ion binding sites are occupied by Cl^- ions. The ions bridge the intra- and extracellular vestibules in a single file as expected for an open channel. It was therefore assumed that the structure of both mutants resembles an open channel conformation [9].

4. ClC channel gating

Gating is a property of ion channels which allows them to open and close their permeation pathway in response to external stimuli. ClC channels exhibit a complex gating behavior, where gating is tightly coupled to ion permeation [2,18]. The best characterized system in that respect is ClC-0, which has been extensively studied on a macroscopic and on a single channel level [2]. Two different gating modes allow ClC-0 to open and close either the individual pores on a millisecond timescale (fast gating) or both pores at once in the order of seconds (slow gating) [19]. Whereas the slow gating process is currently not well understood, we have ample information for the fast gating process, to which I will refer from now on as gating.

Gating of ClC-0 is voltage-dependent, the channels are open at (inside) positive voltages and close when the membrane potential changes to negative voltages [20,21]. In addition to voltage, other parameters also influence gating. Extracellular chloride is known to open the channel [20,21]. Thus ClC-0 acts like a ligand-gated channel where the ligand is chloride itself. Also extracellular pH strongly influences gating such that a decrease in pH causes an opening of the channel [22]. In addition to the dominating extracellular effects, intracellular chloride and pH also affect gating [21,23].

It is clearly interesting to relate the structures of the selectivity filter in wt EcClC and in the site-directed mutants E148A and E148Q (Fig. 2b,c) to the fast gating process in ClC-0. The mutation of the corresponding residue Glu166 in ClC-0 to a neutral residue indeed abolishes voltage-dependent gating on a macroscopic and single channel level [9,24]. A similar result has been obtained for ClC-1 and ClC-2 [15,25,26]. These observations suggest that the respective Glu residue also serves as a gate for the fast gating mechanism in ClC-0, and probably in all other ClC channels which have a glutamate in this position. The structural and mechanistic details of fast gating in ClC-0 will have to be refined: some results suggest a conformational change in the intracellular part of the selectivity filter upon channel opening [24,27], and recent electrophysiological recordings of EcClC did not reveal any voltage dependence of gating and instead showed some unique properties in its ion permeation [32]. Additional experiments are required to clarify the structural basis for these observations.

5. Comparison of ClC channels with K^+ channels

With the availability of detailed structural information for two ion-selective channels, K^+ channels and Cl^- channels, it is possible to compare how nature developed independent solutions for two similar problems, the rapid permeation of K^+ and Cl^- across the membrane [7–9,28]. At first glance the two classes of ion channels could not look more different. The channels are not related in sequence and do not resemble each other on a structural level (Fig. 3). When looking at the ion–protein interactions, however, parallels become apparent. Both channels use the partial charges at the end of α-helices to stabilize ions in the center of the membrane (Fig. 3). They do that in the framework of two different architectures: in a parallel architecture in K^+ channels and an antiparallel architecture in Cl^- channels. K^+ channels use the negative partial charge at the C-terminus of the pore helix to stabilize ions in the aqueous cavity in the center of the membrane (Fig. 3a) [28]. The four helices are related by the four-fold axis of symmetry and point at the ions from the same side of the

membrane. In ClC channels the helices, which are related by the pseudo two-fold symmetry within the ClC subunit, stabilize the ions coming from opposite sides of the membrane (Fig. 3b) [7]. Both architectures satisfy the same energetic constraint that it is costly to bury the polar ends of α-helices in the low dielectric of the membrane. Consequently, the opposite end of the helix, which is not stabilized by the ion in the center of the membrane, is exposed to the aqueous solution.

In the selectivity filters of both channels we find multiple ion binding sites in which the ions, once they shed their hydration shell, are stabilized by partial charges of the protein. In K^+ channels the backbone carbonyls of the selectivity filter coordinate the ions similar to their coordination in solution (Fig. 3a) [8]. In Cl^- channels the ions interact with free amide NH and side chain hydroxyl groups (Fig. 3b) [9]. The multiple ion occupancy of the selectivity filter of K^+ channels is probably also a property of Cl^- channels. In both cases interaction with partial charges rather than the full charges of ionizable residues as well as multiple ion occupancy prevents too strong binding, which would hamper rapid ion conduction.

Unlike for ion conduction, the mechanisms of gating in the two ion channels are very different. In potassium channels selectivity filter and gate are separated at opposite sides of the membrane. The selectivity filter is located at the extracellular entrance of the pore, while the gate is at the bundle crossing of four helices at the intracellular side of the membrane (Fig. 3a) [29]. This separation ensures the conformational stability of the selectivity filter upon closing and opening of the channel, which is essential for efficient ion conduction. In ClC channels it has long been known that gating and ion conduction are coupled [18]. Indeed, the selectivity filter itself is located at the center of the membrane and the gate is the side chain of a glutamate residue, which binds to the extracellular end of the selectivity filter [9]. This way the glutamate competes with the permeating ion for the same binding site. The two different modes of gating in potassium channels and chloride channels allow for different ways of regulation. While gating by the glutamate side chain in the ClC channel directly responds to local changes in pH and ion concentration in the vicinity of the gate [9], gating in potassium channels occurs via protein domains coupled to the gate which then regulate the opening either by the interaction with ligands or by the sensing of the transmembrane voltage [30,31]. There are other mechanisms of gating in ClC channels, which involve the common gate and which are currently not well understood. It might well be that, on a structural level, those gating modes resemble gating in potassium channels.

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