CLC-0 and CFTR: Chloride Channels Evolved From Transporters

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Chen T-Y, Hwang T-C. CLC-0 and CFTR: Chloride Channels Evolved From Transporters. Physiol Rev 88: 351–387, 2008; doi:10.1152/physrev.00058.2006.—CLC-0 and cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channels play important roles in Cl− transport across cell membranes. These two proteins belong to, respectively, the CLC and ABC transport protein families whose members encompass both ion channels and transporters. Defective function of members in these two protein families causes various hereditary human diseases. Ion channels and transporters were traditionally viewed as distinct entities in membrane transport physiology, but recent discoveries have blurred the line between these two classes of membrane transport proteins. CLC-0 and CFTR can be considered operationally as ligand-gated channels, though binding of the activating ligands appears to be coupled to an irreversible gating cycle driven by an input of free energy. High-resolution crystallographic structures of bacterial CLC proteins and ABC transporters have led us to a better understanding of the gating properties for CLC and CFTR Cl− channels. Furthermore, the joined force between structural and functional studies of these two protein families has offered a unique opportunity to peek into the evolutionary link between ion channels and transporters. A promising byproduct of this exercise is a deeper mechanistic insight into how different transport proteins work at a fundamental level.
I. INTRODUCTION

Ion channels and transporters are integral membrane proteins that transport ions and small molecules across cell membranes. In doing so, they control many critical physiological processes, including determining the voltage across cell membranes, maintaining the cell volume, controlling the release of hormones and neurotransmitters, and regulating the secretion or absorption of important ions and substances, to name a few. Traditionally, the transport processes of these two classes of molecules were viewed from very different angles. Ion channels were thought to contain a water-filled pore through which ions diffuse down their electrochemical gradient. In contrast, molecules or ions moved by transporters were thought to first bind to the transporter molecules, with the substrate binding then inducing conformational changes that switch the binding site from one side of the membrane to the other. Many transport proteins “pump” substrates across cell membranes by coupling the substrate translocation to an energy releasing process (187, 303). It is generally believed that a large conformational change of the transporter molecule occurs during the transport process.

With the progress in the structural and functional studies of membrane transport in the last 10 years or so, the separation line between ion channels and transporters has become more and more murky. It was discovered in the mid 1990s that glutamate transporters can simultaneously work as chloride channels (83, 91, 267, 295, 325). Recent studies showed that some toxin molecules, when bound to a classical transporter molecule, Na⁺-K⁺-ATPase, can convert this transporter into an ATP-ordered translocation to an energy releasing process (187, 303). It is generally believed that a large conformational change of the transporter molecule occurs during the transport process.

Passive transport moves a solute across the membrane from a side of high electrochemical potential to the other side that is of low electrochemical potential. Two types of membrane proteins, facilitated transporters and ion channels, transport substrates across the membrane “passively.” However, the fundamental mechanism by which these two passive transport proteins work is thought to be very different. Binding of the substrate to a facilitated transporter from one side of the membrane induces conformational changes that expose the substrate to the other side of the membrane. The concentration gradient of the substrate thus drives the cycle of the conformational changes that is coupled to the movement of the substrate. Because of this coupling of substrate movement to a cycle of protein conformational changes, the transport rate for a facilitated transporter is relatively low. On the other hand, ion channels contain an “aqueous” pore that allows diffusion of substrates (in this case, permeant ions) to occur (Fig. 1A, left). Once the pore is opened (by a process called “gating”), ions diffuse through the opened pore at a very high rate, normally on the order of 10⁶/s or higher. Nevertheless, either passive process dissipates the electrochemical gradient across the membrane built up by the action of an active transporter that converts one form of energy (e.g., the chemical potential in ATP) to another (e.g., the electrochemical gradient across cell membranes). Contrary to passive transport mechanisms, an active transporter molecule can pump ions across the membrane against the electrochemical gradient. To do so, an input of free energy is required. For some transport molecules, such as Na⁺-K⁺-ATPase or members of the ABC transporter family of which CFTR is a member, ATP is hydrolyzed during the transport cycle, and the energy directly harvested from ATP hydrolysis is used to do the work. This type of transport mechanism is called primary active transport because the energy comes directly from ATP, which is considered the energy currency in cells (Fig. 1B). Another class of transport proteins mediates the net transfer of one solute against its electrochemical gradient by using the energy derived from the electrochemical gradient of CFTR Cl⁻ channels and their transporter partners from the same family. While delving into the evolutionary connection between ion channels and transporters, we hope to gain mechanistic insights into how these proteins carry out the essential function of membrane transport.

II. GENERAL MECHANISMS OF ION CHANNELS AND TRANSPORTERS

Membrane transport mechanisms have been classified thermodynamically based on their ability to mediate “passive” or “active” transports (126, 187, 303) (Fig. 1A). Passive transport moves a solute across the membrane from a side of high electrochemical potential to the other side that is of low electrochemical potential. Two types of membrane proteins, facilitated transporters and ion channels, transport substrates across the membrane “passively.” However, the fundamental mechanism by which these two passive transport proteins work is thought to be very different. Binding of the substrate to a facilitated transporter from one side of the membrane induces conformational changes that expose the substrate to the other side of the membrane. The concentration gradient of the substrate thus drives the cycle of the conformational changes that is coupled to the movement of the substrate. Because of this coupling of substrate movement to a cycle of protein conformational changes, the transport rate for a facilitated transporter is relatively low. On the other hand, ion channels contain an “aqueous” pore that allows diffusion of substrates (in this case, permeant ions) to occur (Fig. 1A, left). Once the pore is opened (by a process called “gating”), ions diffuse through the opened pore at a very high rate, normally on the order of 10⁶/s or higher. Nevertheless, either passive process dissipates the electrochemical gradient across the membrane built up by the action of an active transporter that converts one form of energy (e.g., the chemical potential in ATP) to another (e.g., the electrochemical gradient across cell membranes). Contrary to passive transport mechanisms, an active transporter molecule can pump ions across the membrane against the electrochemical gradient. To do so, an input of free energy is required. For some transport molecules, such as Na⁺-K⁺-ATPase or members of the ABC transporter family of which CFTR is a member, ATP is hydrolyzed during the transport cycle, and the energy directly harvested from ATP hydrolysis is used to do the work. This type of transport mechanism is called primary active transport because the energy comes directly from ATP, which is considered the energy currency in cells (Fig. 1B). Another class of transport proteins mediates the net transfer of one solute against its electrochemical gradient by using the energy derived from the electrochemical gradient of

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another solute. Thus solute A is transported against its electrochemical gradient at the expense of dissipating the electrochemical gradient of solute B. Two situations can occur. If solutes A and B are transported in the same direction, then the transport mechanism is called “cotransport,” or “symport.” If solutes A and B are transported in opposite directions, then the transport mechanism is called “countertransport,” or “antiport” (104) (Fig. 1C). The bacterial CLC protein, such as the one from Escherichia coli (CLC-ec1), is a Cl⁻-H⁺ antiporter (3).

Although passive and active transports are defined thermodynamically, such definitions have an implication for the structure of the substrate-transport pathway. For transporters, however, a water-filled, open pore as seen in the open state of an ion channel is not allowed because in such a situation substrate will move down the electrochemical gradient. In the past several decades, physiologists envisioned that the ion-conducting pathway of ion channels should be controlled by only one physical gate (Fig. 1A, left), while the substrate-transport pathway of any transporter must be guarded by at least two gates (Fig. 1A, right). Thus the opening of either gate at any moment will not create an open pore in transporters for substrate diffusion (104).

In this review, primary active transport and the antiport mechanism of secondary active transport will be the focus of discussions because homologs of CFTR and CLC Cl⁻ channels belong to these two categories of transport mechanisms, respectively. Schematic transport cycles for these two types of transport mechanisms are shown in Figure 1, B and C. For the primary active transport (Fig. 1B), ATP first binds to the transport molecule, which also serves as an enzyme to hydrolyze ATP. Hydrolysis of the bound ATP and subsequent release of the hydrolytic products, ADP and Pᵢ, complete the hydrolysis cycle that is coupled to the movement of the substrate across the membrane. In the antiporter mechanism shown in Figure 1C, the two transported molecules, “1” and “2,” are moved in opposite directions; when solute 1 binds to the transporter on one side of the membrane, the other solute (solute 2) must be released, and vice versa. The alternate binding of the two substrates is important to form a complete transport cycle. Investigators have thus speculated an outwardly facing and an inwardly facing structure for transporters as the two extreme states of a “rocking banana”-like motion shown in Figure 1C. One important feature in the antiporter mechanism is the exchange

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**Fig. 1.** A: ion channels versus transporters. The cartoon depicts the difference in the traditional concepts of ion channels and transporters. For ion channels (left), ions diffuse through the open pore, which is thought to be controlled by one gate. For transporters (right), because an open pore is not allowed at any time, the transport pathway must be guarded by at least two gates. The opening of the two gates is coordinated so that no open pore is allowed. The “1” represents the transported substrate that binds to the binding site in the transport pathway. B: primary active transport mechanism. Energy directly harvested from the hydrolysis of ATP (the energy currency in cells) is used to pump the substrate across the membrane against an electrochemical gradient. C: secondary active antiport mechanism. The two substrates, “1” and “2,” are transported in opposite directions. The direction of the transport cycle (“1” out and “2” in, or “1” in and “2” out) depends on the electrochemical gradients of these two substrates.
ratio between the two transported substances. If \( n \) molecules of substrate \( S \) are transported in exchange of \( m \) molecules of substrate \( C \), and if the charge carried by substrate \( S \) and \( C \) are \( \Delta Z_S \) and \( \Delta Z_C \), respectively, the net charge movement per transport cycle is \( n\Delta Z_S - m\Delta Z_C \). When this number is zero, the antiporter is not electrogenic, as in the \( \text{Cl}^- - \text{HCO}_3^- \) exchanger in red blood cells (95). In the cardiac \( \text{Na}^-\text{Ca}^{2+} \) exchanger, it has been shown that three \( \text{Na}^+ \) were transported in exchange for the transport of one \( \text{Ca}^{2+} \) in the opposite direction (38). Thus \( n\Delta Z_{\text{Na}} - m\Delta Z_{\text{Ca}} = 3 - 2 = 1 \), making this transport mechanism electrogenic. Because the transporter molecules in CLC family move \( \text{Cl}^- \) and \( \text{H}^+ \) in opposite directions, the \( \text{Cl}^- - \text{H}^+ \) antiporter activity is always an electrogenic process regardless of the exchange ratio.

CFTR and CLC-0 are bona fide \( \text{Cl}^- \) channels because \( \text{Cl}^- \) moves through the pore of these two molecules passively with a rate of \( 10^8 - 10^9 \) ions/s. Thus single-channel currents can be readily measured. Functional properties of these \( \text{Cl}^- \) channels, however, suggest that they may be viewed as evolutionary descendants of those transporters in the ABC and CLC protein families. For example, hydrolysis of ATP by CFTR is coupled to a closed-open-closed gating cycle (97), and the opening of the CLC channels is affected by \( \text{Cl}^- \) and \( \text{H}^+ \) (51, 211, 240). In the following discussions, we will first inspect the structure and function of CLC and CFTR \( \text{Cl}^- \) channels and those of their transporter counterparts. At the end of the paper, we will speculate on the potential evolutionary linkage between ion channels and transporters in these two families.

III. CLC TRANSPORT PROTEIN FAMILY

The CLC family encompasses members widely distributed in various species ranging from bacteria to humans. These protein molecules play a variety of biological roles, including maintenance of membrane potential, regulation of transepithelial \( \text{Cl}^- \) transport, and control of intravesicular pH (142, 145, 146, 197, 326). The importance of the CLC family members is best illustrated by numerous hereditary diseases caused by their defects, such as myotonia congenita (101, 143, 156, 163, 351, 352), Dent’s disease (100, 183, 184), Bartter syndrome (123, 160, 309), osteopetrosis (161), and idiopathic epilepsy (118), and also from several gene knock-out studies in mice (36, 161, 235, 300, 328). The first member of the CLC gene family, CLC-0, was identified and cloned from the electric organ of the Torpedo ray (147, 227). Homology cloning efforts in the 1990s identified nine members in mammalian species (CLC-1 to CLC-7, CLC-Ka and CLC-Kb), constituting three subfamilies (142, 197). CLC-0 and its mammalian homologs, CLC-1, CLC-2, CLC-Ka, and CLC-Kb form one subfamily; they share \( 50-60\% \) amino acid identity in the primary sequence. These members are \( \text{Cl}^- \) channels, and they are all present on the cell membrane to control \( \text{Cl}^- \) flux and membrane potential. The second branch of the family includes CLC-3, CLC-4, and CLC-5. These proteins are located on the membrane of intracellular vesicles and are thought to be important in maintaining the pH of these vesicles (114, 217, 300), consistent with their functional properties as \( \text{Cl}^- - \text{H}^+ \) antiporters. However, recent work argued that CLC-3 is a double-barreled \( \text{Cl}^- \) channel, residing on the postsynaptic plasma membrane of certain neurons (330). That CLC-3 is a \( \text{Cl}^- \) channel on the cell membrane was also suggested by the functional role of CLC-3 in regulating cell volume (74, 75, 343), although this issue has been controversial (200, 335). The third branch of the family contains CLC-6 and CLC-7, which are expressed in various tissues. These two proteins cannot be functionally expressed in heterologous expression systems (37). It has been reported that they may be present also in the intracellular organelles to regulate the vesicular pH (161).

Besides the CLC members cloned from mammalian species, several members in bacteria (140, 198, 239), yeast (99, 124, 282) and Caenorhabditis elegans (31, 222, 279, 301) have also been identified by searching the genomic databases. The discovery of bacterial CLC members is particularly important as the large quantity of bacterial CLC proteins allow a direct structural approach. Although the amino acid sequence identity between the bacterial CLCs and those of the mammalian CLCs is only \( \sim 20\% \), studies of mammalian CLC channels have shown that they should adopt an overall similar structural architecture (81, 86, 175, 252). Thus high-resolution crystal structures from bacterial CLCs provide a useful framework to understand functional data of both bacterial and mammalian CLCs.

IV. BACTERIAL CLC PROTEIN: A CHLORIDE-PROTON ANTIPORTER

A. Structure of CLC-ec1

The E. coli CLC molecule (CLC-ec1) consists of \( \sim 400 \) amino acids (77), which correspond to the NH\(_2\)-terminal half of the vertebrate CLCs that contain \( \sim 800-1,000 \) amino acids (147, 227, 299, 311). The NH\(_2\)-terminal portion of the vertebrate CLC proteins is embedded in the lipid bilayer and forms the ion-transport pathway (77). The large quantity of CLC proteins purified from E. coli renders it possible to grow two- and three-dimensional crystals for structural analyses (77, 78, 216). These structural studies showed that a CLC functional unit consists of two identical subunits, each containing an anion-transport pathway related to each other by a twofold symmetry perpendicular to the membrane plane (see Fig. 2A), con-
Even though Cl\textsuperscript{−} the backbone amide group from Gly-106 and Ser-107. A to R, from the NH\textsubscript{2} and COOH terminus of the protein. Some of these α-helices run halfway through the lipid membrane, with the positively charged end of the helix dipole coordinating Cl\textsuperscript{−} in the middle of the cell membrane (77, 78). In the crystal structure, two anion-binding sites are identified in the ion-transport pathway of the wild-type protein. They are, respectively, named Scen and Sint to reflect their locations (central, internal) in the pore (Fig. 2A). The Cl\textsuperscript{−} at Scen interacts with the side-chain hydroxyl of Ser-107 in helix D and that of Tyr-445 in helix R, and also the main-chain nitrogen atoms from Ile-356 and Phe-357 of the helix N (77, 78). The Cl\textsuperscript{−} binding site Sint is located ~6–7 Å intracellular to Scen, approximately at the junction where the intracellular aqueous solution meets the pore. The Cl\textsuperscript{−} at this position is coordinated by the backbone amide group from Gly-106 and Ser-107. Even though Cl\textsuperscript{−} at Sint may appear to contact water molecules on the intracellular side, the bound Cl\textsuperscript{−} at Scen is hardly visible when the molecule is viewed from the intracellular entrance of the Cl\textsuperscript{−}-transport pathway, mostly due to a constriction of the pathway near Ser-107. However, a cysteine introduced at Tyr-445 in CLC-ec1 or at the corresponding Tyr-512 position in CLC-0 is accessible to bulky methane thiosulfonate reagents applied from the intracellular side (175, 205, 353), suggesting that the constriction near Ser-107 does not completely obstruct the ion-transport pathway.

The ion-transport pathway in the bacterial CLC molecules is also constricted on the extracellular end by the NH\textsubscript{2} termini of helix F and helix N. A glutamate residue (Glu-148) projects its side chain into the pore, and the Cl\textsuperscript{−} exit pathway is occluded by this negatively charged side chain at a location ~4 Å external to Scen (Fig. 2A). When this glutamate is mutated to a non-charged amino acid, a Cl\textsuperscript{−} is seen at this position (named Sext because this ion-binding site is external to Scen) (78). This negatively charged glutamate residue is conserved throughout the CLC family members except for CLC-K. As will be described later, the glutamate residue is critical for the transporter function of the bacterial CLC molecules. The corresponding glutamate residue also plays an important role in the gating properties of CLC channels.

The bacterial CLC structure reveals that Cl\textsuperscript{−} at these three binding sites do not make direct contact with positively charged amino acids. Rather, they may be stabilized by the positive end (NH\textsubscript{2} terminus) of helix dipoles and by favorable, long-range, electrostatic interactions (92). Whether all three binding sites can be simultaneously occupied by anions was recently addressed by Lobet and Dutzler (185) by titrating the anion concentrations used in growing the bacterial CLC protein crystals whose structures were later solved. Their data suggested that all three binding sites can be occupied simultaneously by anions (185).

In summary, the CLC-ec1 structure shows multiple Cl\textsuperscript{−} binding sites, and the transport pathway appears to be disrupted by the side chain of Glu-148 and Ser-107 at
the extracellular and intracellular end of the transport pathway, respectively. Because the structure of the wild-type protein did not show an apparent open pore, it was speculated that the structure might represent a closed-state channel. With hindsight, however, the multiple constrictions in the ion-transport pathway may have already suggested that this bacterial molecule does not contain a freely diffusible pathway for Cl\(^{-}\) and is likely to be a transporter. More recent functional studies, as described below, indeed provide evidence that the bacterial CLC proteins are Cl\(^{-}\)-H\(^{+}\) antiporters.

**B. Mechanistic Function of CLC-ec1**

The bacterial CLC molecules were discovered in the 1990s. The only functional study prior to the crystallographic era was performed using a flux measurement. With this crude assay, and several biochemical approaches, Maduke et al. (198) first revealed the homodimeric arrangement of the two subunits and the preference for various anions in the *E. coli* CLC protein. Under the false assertion that the bacterial CLC was an ion channel, the anion preference was thought to be the channel permeability sequence, which is roughly similar to those of vertebrate CLC channels. Recently, a more rigorous functional assay, the lipid bilayer recording, was performed on *E. coli* CLC molecules (1, 3). These electrophysiological measurements showed that the reversal potential of the current is determined by both the Cl\(^{-}\) and H\(^{+}\) gradients across the membrane and that a diffusion model based on the conventional Goldman-Hodgkin-Katz equation cannot explain the experimentally determined reversal potential. On the other hand, the calculation based on a Cl\(^{-}\)-H\(^{+}\) antiporter model with a coupling transport ratio of 2Cl\(^{-}\):1H\(^{+}\) closely predicted the experimental results. It was thus proposed that the bacterial CLC was a Cl\(^{-}\)-H\(^{+}\) antiporter but not an ion channel (3).

The discovery that CLC-ec1 is a Cl\(^{-}\)-H\(^{+}\) antiporter immediately raised an interesting question: do vertebrate or mammalian CLCs also function as transporters? For nearly two decades, it was thought that the recorded currents from eukaryotic CLC proteins were carried by Cl\(^{-}\) through ion-diffusion pores. In the muscle-type CLC channels (such as CLC-0 and CLC-1), the reversal potentials measured from these channels follow faithfully the imposed Cl\(^{-}\) gradients, and are not altered by external or internal pH (47, 116, 268, 269). In addition, these channels showed sizable single-channel conductance, from \(\sim 1\) to 10 pS, in physiological Cl\(^{-}\) solutions. Conductance in this range corresponds to \(\sim 10^{8}\) to \(10^{7}\) ions/s turnover in the pore with a physiologically pertinent driving force, values too large for the slow turnover rate of a transporter. However, it was unclear whether members of the other two subbranches of CLC family might function as transporters.

Except for the muscle-type CLC channels, mammalian CLC proteins are known to reside in the membrane of intracellular organelles. By overexpressing these proteins in heterologous expression systems, they can appear on the plasma membrane. However, so far, no convincing single-channel recordings have been obtained in these members (cf. Ref. 330). Does the current generated from these mammalian CLCs result from the Cl\(^{-}\)-H\(^{+}\) antiporter activity? Using H\(^{+}\)-sensitive microelectrodes, Picollo and Pusch (232) showed that the pH on the extracellular side of the membrane was changed upon activation of CLC-4 and CLC-5, while the same assay failed to detect pH changes when CLC-0 and CLC-2 were over-expressed.

Jentsch’s group (275) employed H\(^{+}\)-sensitive dyes to detect the intracellular pH change using imaging methods. They reached a similar conclusion that some mammalian CLCs, including CLC-4 and CLC-5, indeed are able to transfer protons across the cell membrane. The ability of these mammalian CLCs to transport protons may well explain their functions in the regulation of the intravesicular pH (114, 161, 171, 217, 300), a physiological role similar to that played by bacterial CLC proteins in maintaining the normal pH within *E. coli* (140). Interestingly, a CLC member from *Arabidopsis thaliana* CLCa (AtCLCa) is able to accumulate nitrate in the vacuole, and this CLC molecule behaves as an NO\(_{3}^{-}\)-H\(^{+}\) exchanger (67). The antiporter mechanism of this plant CLC protein is directly connected with its physiological role for nitrogen fixation. Thus it seems that both animals and plants have borrowed the functional architecture of the primordial CLC protein to perform anion-H\(^{+}\) exchange reactions.

The structural basis of the Cl\(^{-}\)-H\(^{+}\) antiporter function has attracted attention ever since the bacterial CLC molecule was known to be a transporter. Because of the intriguing structural implication, the functional role of the key glutamate residue, Glu-148, was examined first. Using lipid bilayer recordings, Accardi and colleagues (1, 3) showed that the coupled transporter behavior described above disappeared when this glutamate residue facing the extracellular side (Glu\(_{\text{ex}}\)) was mutated to alanine. In the E148A mutant, H\(^{+}\) was hardly transported through the mutant protein, and the reversal potential of the mutated CLC-ec1 closely followed the equilibrium potential of Cl\(^{-}\) calculated from the Nernst equation. Thus the mutant protein became a Cl\(^{-}\) channel or a Cl\(^{-}\) uniporter. Accardi et al. (5) went further to identify Glu-203 of CLC-ec1 as a H\(^{+}\)-binding site facing the intracellular medium (Glu\(_{\text{in}}\)) (5). Glu\(_{\text{in}}\) is conserved among CLC family members in an interesting way. In CLC members that are thought to be Cl\(^{-}\)-H\(^{+}\) antiporter, the corresponding residue is always a glutamate. For those members that are thought to be Cl\(^{-}\) channels, the negatively charged glutamate is not conserved. These studies suggest that Glu-203 and Glu-148 are two protonation sites in the H\(^{+}\) transport pathway. The Cl\(^{-}\) transport in CLC-ec1 likely follows the path-
way defined by the three observed Cl\(^{-}\)-binding sites in the crystal structure. Thus the Cl\(^{-}\)- and H\(^{+}\)-transport pathways of CLC-ec1 converge at the extracellular side, but diverge at the intracellular side, as depicted in Figure 2A (5).

One question arises with the identification of Glu-148 and Glu-203 being the protonation sites in the H\(^{+}\)-transport pathway of CLC-ec1: How is H\(^{+}\) transported between these two sites? An examination of the CLC-ec1 structure suggests that the conserved residue Tyr-445, whose side chain directly coordinates the Cl\(^{-}\) at S\(_{\text{cen}}\), may be a midway station for H\(^{+}\) transport because this residue is located halfway between Glu-148 and Glu-203, and because numerous examples of tyrosine hydroxyl groups are known to participate in H\(^{+}\) transfer in various proteins (137, 173). Accordingly, Accardi et al. (2) mutated this conserved tyrosine residue and found that the H\(^{+}\) transfer ability of CLC-ec1 was not lost in Y445F or Y445W mutants, indicating that the protonation of the tyrosine hydroxyl is not required for the H\(^{+}\) transport. In the same series of experiments, however, they found a striking correlation between the anion occupancy at S\(_{\text{cen}}\) and the Cl\(^{-}\)-H\(^{+}\) coupled transport by replacing Tyr-445 with various amino acids. An independent experimental maneuver, in which the coupled transport of H\(^{+}\) with various anions in the wild-type CLC-ec1 was measured, also revealed a similar correlation between the anion occupancy at S\(_{\text{cen}}\) and the coupled transport (223). In light of these results, Miller and colleagues proposed that the central Cl\(^{-}\) itself provides a transient protonation site necessary for coupling H\(^{+}\) and Cl\(^{-}\) in the transport cycle (2, 223). It has been suggested from a theoretical study that the anion occupancy of the Cl\(^{-}\)-binding sites in CLC-ec1 renders the intrinsic potential of S\(_{\text{cen}}\) more negative (345), a condition that may be required for H\(^{+}\) to pass the energy barrier in the transport pathway. The proposal that the Cl\(^{-}\) itself provides a transient protonation site is still amazing because HCl is an extremely strong acid in aqueous solutions. However, the low dielectric constant inside a protein, and the pore electrostatic potential from many surrounding charged residues, may make this hypothesis tenable as argued by Miller and colleagues (2).

That H\(^{+}\) transport requires anion occupancy at S\(_{\text{cen}}\) may suggest the synergistic binding of the transported H\(^{+}\) and anions at around Tyr-445. The Cl\(^{-}\) and H\(^{+}\) transport pathways diverge from this coupling site toward the intracellular medium. Such a transport mechanism appears to be very different from the alternating-site antiport mechanism described in Figure 1C, because no synergism between the bindings of two substrates is allowed in the classical antiport model. Further studies will be necessary to reveal the detailed mechanism of Cl\(^{-}\)-H\(^{+}\) antiporter mechanism in the bacterial CLC protein.

V. CLC-0: A PROTOTYPE CHANNEL IN THE CLC FAMILY

A. Introduction

CLC-0 consists of two identical subunits, each containing 804 amino acids (209, 210). Identical channel behaviors can be obtained from patch-clamp recordings of the expressed channels or from bilayer recordings of the purified channel protein (209, 210), suggesting that no auxiliary subunits are needed to generate functional CLC-0 channels. The primary amino acid sequence of all CLC molecules of vertebrate origin shows a molecular mass of approximately twofold that of the bacterial CLC proteins. The extra 400–500 amino acids form the large cytoplasmic domain. Thus the NH\(_{2}\)-terminal half of vertebrate CLC proteins is membrane-embedded and forms ion-transport pathways. Several mutational studies as well as experiments using substituted cysteine accessibility method (SCAM) have indicated that the overall structure of the pore-lining portion of CLC-0 should be similar to the anion transport pathway of bacterial CLC proteins (48, 78, 81, 174, 175, 196). The crystal structure of the COOH-terminal half of CLC-0, a component absent in bacterial CLC structure, was recently solved by Meyer and Dutzler (208). This COOH-terminal cytoplasmic domain contains a folded core of two tightly interacting cystathionine \(\beta\)-synthetase (CBS) subdomains, and this COOH-terminal domain from each subunit may form a dimer (208). If the assembly of the NH\(_{2}\)-terminal and the COOH-terminal halves of CLC-0 does not distort the individual structure, the overall CLC-0 structure may be similar to that shown in Figure 2B (208). This overall structural architecture may also represent the structures of other vertebrate CLC proteins.

Although various CLC Cl\(^{-}\) channels may have a similar structure, they exhibit very different functional properties. CLC-0 and CLC-1 are present in the muscle-related tissues. CLC-1 is the principal Cl\(^{-}\) channel that contributes to a major part of the resting conductance of the mammalian skeletal muscle, while CLC-0 exists in the Torpedo electric organ, an organ that derived evolutionarily from skeletal muscles. These two Cl\(^{-}\) channels control the membrane potential critical for the excitability of the cells (muscle cells and Torpedo electroplaex, respectively). Therefore, depolarization-activated opening is important for their physiological roles, to repolarize the membrane potential after excitation. On the other hand, CLC-Ka and CLC-Kb are expressed on the cell membrane of renal tubular cells to control the electrolyte balance (293, 317), and in the organ of Corti to regulate the ion...
transport across the stria vascularis (84, 247). Thus a continuous opening of such a CLC channel to support the transepithelial Cl\(^-\) transport may be more suitable for their primary physiological roles. Compared with the limited expression of other CLC channels, CLC-2 is widely distributed in almost all tissues, including neuronal and cardiac tissues, and this channel is opened by membrane hyperpolarization. A physiological role of CLC-2 in mediating the paradoxical excitation produced by GABA receptor activation in selected neuronal populations has been suggested (55, 297, 298).

Among these CLC Cl\(^-\) channels, only the gating function of CLC-0 has been studied in detail, and the properties potentially related to transporter functions (i.e., the regulation of gating by Cl\(^-\) and H\(^+\) ions) have been well documented. Although still speculative, comparing the structure-function relation between CLC Cl\(^-\) channels and CLC Cl\(^-\)/H\(^+\) antiporters may provide the opportunity to understand the evolutionary link between the two categories of protein functions in the CLC family. In the following section, we will only discuss the structure-function relationship of CLC-0, the prototype Cl\(^-\) channel in the CLC family. Readers who are interested in CLC-1, CLC-2, and CLC-K channels are referred to several review articles (18, 51, 146, 317).

**B. Channel Conductance and Ion Permeation of CLC-0**

Eukaryotic CLC channels are relatively nonselective among various monovalent anions: Cl\(^-\), Br\(^-\), NO\(_3\), or even larger anions such as I\(^-\) and SCN\(^-\) are permeant (197). The permeability ratios of these anions are usually within a factor of 10 (87, 90, 122, 193, 270, 353), with a relative permeability sequence: SCN\(^-\) > NO\(_3\) > Cl\(^-\) > Br\(^-\) > I\(^-\). Measuring the single-channel conductance of CLC channels has not been an easy task because of the low conductance of most of CLC channels (197). Fortunately, CLC-0 has a conductance of \(~10\) pS in physiological Cl\(^-\) concentrations, a conductance large enough for single-channel recording experiments. In comparison, the single-channel conductance values for CLC-1 and CLC-2 are, respectively, approximately eight- and threefold smaller than that of CLC-0 (245, 274, 332), making the single-channel studies of these two mammalian channels quite challenging. For molecules that are thought to be Cl\(^-\)/H\(^+\) antiporters, such as CLC-4 and -5, no convincing single-channel traces have yet been obtained, consistent with the idea that they may function as transporters.

Although the ion-permeation pathway of various CLC channels may be similar to that suggested from the bacterial CLC protein structure, the ion permeation mechanisms in different channels exhibit different features. For example, the single-channel current-voltage (I-V) relationship of CLC-0 is linear (176, 209, 210), but that of CLC-1 is known to be inwardly rectifying as deduced from the instantaneous I-V curve (87, 90, 122, 193, 270). Another more extreme example is the difference between CLC-0 and CLC-ec1. The critical residues lining the ion-transport pathway are conserved between these two molecules (81, 86, 175), yet the former is a Cl\(^-\) channel, while the latter is a transporter. The structural determinants in CLC-ec1 that prevent the Cl\(^-\)-transport pathway from becoming a Cl\(^-\)-diffusion pathway is not yet known.

CLC-0 is an ion channel, so the ion-transport pathway is open as an ion-diffusion pore as long as the side chain of Glu-166 (which corresponds to Glu-148 or Glu Ec1 of CLC-ec1, and is probably the only gate in CLC-0) moves out of the pore. Several theoretical calculations have been conducted to understand the ion permeation mechanisms of CLC channels, using the atomic coordinates from bacterial CLC proteins (56, 57, 92, 215, 345). One study suggested a potentially important role of residue 318 of bacterial CLCs, which corresponds to G352 and E417 in CLC-0 and CLC-1, respectively. Brownian dynamic simulations showed that the charge at this position may determine the Cl\(^-\) exit rate towards the extracellular solution, thus explaining the differences between CLC-0 and CLC-1 in single-channel conductance as well as in the rectification property of I-V curves (57). Another study calculated the source of the stabilizing energy of the bound Cl\(^-\) and predicted that the positive charge of the residues Lys-131 and Arg-340 (corresponding to residues 149 and 401 of CLC-0) stabilizes Cl\(^-\) binding in the ion-transport pathway (92). These computational studies have a major limitation in that the bacterial CLC molecules are Cl\(^-\)/H\(^+\) antiporters but not ion channels. Because the fundamental principles of ion transport between ion channels and transporters are so different, it is not known if any conclusions from these computational studies of bacterial CLC proteins are indeed valid for CLC channels. To examine the theoretical predictions, Zhang et al. (353) tested the functional importance of the corresponding charged residues in CLC-0 and CLC-1. The results suggested that some (e.g., Lys-149 of CLC-0 corresponding to Lys-131 of CLC-ec1) but not all of the predicted residues assume a functional role in determining the pore functions of CLC-0. The results, however, do not rule out potential functional roles of these residues in the transporter function. The role of Lys-149 in CLC-0 functions was further elaborated by the recent work of Engh et al. (80).

Besides residues suggested from theoretical calculations, mutagenesis approaches for more than a decade have identified several important charged residues that contribute electrostatic energy to the pore (48, 174, 209). Being an anion channel, the ion-binding sites in the CLC-0 pore must be associated with an intrinsic positive poten-
tial, a feature that has been experimentally revealed by modifying the introduced cysteine using charged methane thiosulfonate reagents (175). The positive intrinsic potential in the pore of CLC-0 might help select anions versus cations. The same positive intrinsic potential then disfavors the movement of $\text{H}^+$ to the pore to protonate the side chain of Glu-166, a process thought to be critical for the gating mechanism of CLC-0. Therefore, the occupancy of $\text{Cl}^-$ may be important for the protonation of pore residues, as suggested by theoretical calculations. The computational study also suggested several potential pathways for $\text{H}^+$ permeation through the bacterial CLC protein, which may or may not exist in CLC-0 (345).

C. Gating Properties of CLC-0

The gating of CLC-0 consists of multiple mechanisms, which are summarized in Figure 3. Based on the kinetics of the channel opening, the gating of CLC-0 includes “fast” and “slow” gating, which operate in the time scale of milliseconds and seconds, respectively. Shown in Figure 3A is a single-channel recording trace of CLC-0. The long, nonconducting states between bursts of channel activities (indicated by arrows in Fig. 3A) represent the closure of the slow-gate. When the slow-gate is open, a burst of channel activity appears, and three current levels (the nonconducting level, the middle level, and the fully open level) can be observed. The current fluctuation within a burst is called fast-gating because the gating mechanism operates at a much faster time scale (milliseconds) than that of slow-gating (seconds to minutes). Relaxation of the macroscopic current of CLC-0 provides ways to measure the fast-gating and the slow-gating kinetics from ensemble of channels (Fig. 3, B and C).

The characteristics of fast-gating in single-channel recordings, three equally spaced current levels and the binomial distribution of the probabilities of these three current states, have been observed for more than two

![Gating functions of CLC-0.](http://physrev.physiology.org/)

**Fig. 3.** Gating functions of CLC-0. **A**: single-channel behaviors of CLC-0. Top panel shows a 14-s recording for CLC-0 at $-60$ mV under a symmetrical $\text{Cl}^-$ concentration of 120 mM. The dotted line represents the zero-current level. The recording was obtained from an inside-out membrane patch excised from a *Xenopus* oocyte expressing wild-type CLC-0. Arrows indicate the long inactivation events corresponding to the closure of the slow-gate. The activities between the inactivation events represent the fast-gating transitions, which can be seen better with the expanded trace on the left. The fast-gate activity fluctuates among three equidistant current levels, and the probabilities of these three current levels follow a binomial distribution. The expanded trace on the right shows a transition from the slow-gate, open state to the slow-gate, closed state. **B**: fast-gating at the macroscopic current level. The recording was made from the excised inside-out membrane patch of the human embryonic kidney (HEK) 293 cell expressing the inactivation-suppressed mutant C212S. The prepulse voltage is 100 mV, while the tail voltage is $-100$ mV. The tested voltage is from $+100$ to $-160$ mV in $-20$ mV steps. Notice that the time constant of the current relaxations upon voltage change is $\approx 10$ ms. **C**: the slow-gating process of the wild-type CLC-0 examined by macroscopic current recordings from an excised inside-out membrane patch. Left panel shows original recording traces under voltage clamp from the holding voltage of 0 mV to $+40$ mV for 50 ms. One pulse was given every 6 s to monitor the relatively slow time course as shown in the right panel (temperature = 23.5°C). Circles in the right panel represent the current measured from the end of the voltage pulse (averaged from the traces between two vertical lines in the left panel). For **B** and **C**, the patches were recorded in symmetrical 140 mM $\text{Cl}^-$ solutions, and dotted lines represent the zero-current level.
decades. Such a unique property prompted Miller and co-workers (116, 212, 213) to argue that a single CLC-0 channel contains two ion-permeation pores. The three equidistant current levels with binomial distribution are also found in CLC-1 (274). In addition, when heterodimeric channels were generated by coexpressing wild-type and mutant CLC-0 channel subunits, or by linking subunits of CLC-0 and CLC-1, CLC-0 and CLC-2, or CLC-1 and CLC-2, single channels with two nonidentical but independent pores were observed (174, 196, 209, 332). Thus the “double-barreled” model of CLC channels had been firmly established even before the crystallographic era. From the now available high-resolution bacterial CLC structures, CLC-0 should consist of a membrane portion that forms the ion-conducting pore linked with a COOH-terminal cytoplasmic portion whose structure has also been solved recently (208).

1. Slow-gating function of CLC-0

Slow-gating of CLC-0 is controlled by membrane potential. Membrane depolarization favors the nonconducting state of the slow-gate; the closed state of the slow-gate at depolarized potential normally lasts for seconds or tens of seconds, mimicking the inactivation of the voltage-gated cation channels. Thus slow-gating of CLC-0 is sometimes called “inactivation gating.” In addition to membrane potential, slow-gating of CLC-0 is also controlled by temperature, chloride ion concentration, and pH. High extracellular pH, low extracellular Cl−, and high temperature favor a nonconducting (inactivation) state of the slow-gate (47, 50, 53, 214, 243, 336). A recent study suggests that slow-gating may also be modulated by the redox state of the environment (172). These studies, however, have not provided mechanistic information regarding the molecular mechanism of slow-gating. An experiment in the early 1990s showed an interesting phenomenon that the operation of the slow-gate is through a nonequilibrium gating cycle, in which the closure of the slow-gate predominantly succeeds a state in which one of the two fast-gates is open, while the slow-gate opens to a state in which the fast-gates of both pores are open (262). This nonequilibrium gating is more prominent with a larger Cl− gradient across the channel pore imposed by the membrane voltage (262). Recent unpublished data, however, suggest that it may be the H+ gradient across the cell membrane that provides the energy for the nonequilibrium gating cycle (M. Maduke, personal communication). From a retrospective view, although the mechanisms underlying these effects are not known, the nonequilibrium gating cycle suggests that the CLC-0 slow-gating may be coupled to the fast-gating, and the gating mechanisms are linked to the Cl−/H+ gradients across the cell membrane. However, without knowing the physical nature of the slow-gate, the coupling mechanism remains elusive.

Electrophysiological measurements have shown that the open probability ($P_o$) of the slow-gate is reduced upon increasing temperature. Because this temperature dependence is extremely high, with a $Q_{10}$ of $\sim 40$ (50, 243), a large protein conformational change is thought to be associated with the slow-gating transition. Extracellular Zn$^{2+}$ is known to facilitate the inactivation process, thus inhibiting CLC-0 (50). The effect of extracellular Zn$^{2+}$ on the slow-gating process is supported by the observation that a mutant C212S, whose slow-gate is locked in the open state, loses its sensitivity to Zn$^{2+}$ inhibition (176). Although the slow-gating of CLC-0 can be affected by numerous point mutations, including C213G, C480S (176), P522G, and L524I (243), the mechanism underlying the mutational effects is largely unknown. Manipulations that disrupt the COOH-terminal intracellular domain have been shown to affect the kinetics of slow-gating, suggesting that, in addition to the membrane-spanning part of the protein, the COOH-terminal half of the channel is also important for slow-gating (85, 94, 120, 121, 132, 199, 277, 342).

Sequence comparisons and a recent crystallographic study of the COOH-terminal domain of CLC-0 have revealed that ATP-binding CBS domains exist in the COOH terminus of CLC channels (85, 203, 208). The binding of ATP to the COOH terminus of CLC-1 has been demonstrated to affect channel gating (28). One observation from the crystallographic study of CLC-0 is that the two COOH-terminal domains of CLC-0 form a dimer in solutions, but the dimeric structure is not present when the proteins form a crystal (208). Thus the strength of this dimeric interaction is probably weak, raising the possibility that the interaction of the two COOH termini may be dynamically associated with channel gating. Recent experiments using fluorescence resonance energy transfer (FRET) techniques showed that the COOH terminus of the two CLC-0 subunits may undergo a large movement relative to each other (39), reminiscent of the speculation by Miller more than two decades ago (213). Experiments in CLC-2 and C. elegans CLC channels also showed that point mutants, deletion mutants, or splice variants of the cytoplasmic COOH terminus alter not only the slow-gating but also the accessibility of the pore to extracellularly applied methane thiosulfonate reagents (70, 120, 347). These results may suggest that alterations in the cytoplasmic domain lead to a conformational change of the outer pore vestibule and the associated glutamate gate. Perhaps the relative movement of the COOH-terminal domains (11) is transduced to the two Cl−-permeation pores through the last alpha helix (helix R) of the transmembrane domains, because Cl− and pH-dependent fluorescence changes from fluorophores labeled at helix R of CLC-ec1 have been observed, suggesting a gating-associ-
ated movement of this helix (27). However, direct structural studies of CLC-ec1 offer no such conformational change for helix R in various Cl\textsuperscript{−} and pH conditions (185). Thus a unified picture in the gating-associated conformational change of CLC channels has not yet been settled.

2. The fast-gate opening mechanism of CLC-0

In single-channel recordings, the transitions within the burst represent the fast-gating activities of CLC-0. The opening of this gate depends on the membrane potential and is regulated by pH and Cl\textsuperscript{−}. At physiological pH and Cl\textsuperscript{−} concentrations (for example, symmetrical 150 mM Cl\textsuperscript{−} on both sides of the membrane), the fast-gate \( P_o \) is higher at more depolarized membrane potentials (53, 116, 176, 210, 212, 213, 244). For example, at −120, −90, and −60 mV, the fast-gate \( P_o \) is 0.3, 0.5, and 0.7, respectively. The slope of the \( P_o \)-\( V \) curve corresponds to an e-fold change in \( P_o \) per ~25 mV, which, according to the conventional idea of voltage dependence, may imply that the total gating charge movement is equivalent to the movement of one unit charge across the entire membrane electric field.

Single-channel studies revealed that the slope of the \( P_o \)-\( V \) curve came from two sources: the voltage dependence of the fast-gate opening rate (which has a positive slope with a gating charge of ~0.7) and that of the closing rate (which has a negative slope with a gating charge of ~0.3) (53). Experimental evidence suggests that the opening and the closing processes of the fast-gate may not be the reverse of each other and may have to be considered separately. The positive slope of the voltage dependence of the opening rate indicates that opening of the fast-gate is favored by membrane depolarization. However, this is only a simplified description of fast-gate opening. When the membrane potential is hyperpolarized to a very negative range (for example, more negative than −150 mV), the depolarization-activated channel activity of CLC-0 is very small, but the channel’s \( P_o \) does not approach zero. Instead, in this very hyperpolarized voltage range, the fast-gate opening rate is paradoxically increased by membrane hyperpolarization (53). Thus, besides a depolarization-activated opening mechanism (in the more depolarized voltage range), there is a hyperpolarization-activated opening mechanism for the fast-gate of CLC-0; the fast-gate opening process may have multiple components with opposite voltage dependence. On the other hand, the closing rate appears to depend on the membrane voltage monotonically, with a fixed slope of approximately −0.3 throughout the voltage range that has been examined (47, 52, 53).

The control of fast-gate opening of CLC-0 by Cl\textsuperscript{−} and pH provides a first glimpse for a possible relationship between this gating process and the Cl\textsuperscript{−}-H\textsuperscript{+} antiporter activity. It is the depolarization-activated, fast-gate opening that is regulated by [Cl\textsuperscript{−}]\textsubscript{o}; reducing [Cl\textsuperscript{−}]\textsubscript{o} shifts the opening rate curve to the depolarization direction, thus making the fast-gate more difficult to open. The closing rate, however, is not significantly affected by altering [Cl\textsuperscript{−}]\textsubscript{i} (47, 52, 53). Therefore, the overall effect of reducing [Cl\textsuperscript{−}]\textsubscript{o} is a parallel shift of the steady-state \( P_o \)-\( V \) curve of the fast-gate towards a more depolarized direction. Such an effect can also be achieved by reducing the intracellular proton concentration ([H\textsuperscript{+}]\textsubscript{i}). As revealed by Hanke and Miller (116) more than two decades ago, increasing the pH of the intracellular solution by 1 unit (10-fold reduction in [H\textsuperscript{+}]\textsubscript{i}) shifts the \( P_o \)-\( V \) curve to the right by ~60 mV. Thus altering [Cl\textsuperscript{−}]\textsubscript{i} and [H\textsuperscript{+}]\textsubscript{i} appears to exert, at least at the phenomenological level, a similar effect on the fast-gate \( P_o \)-\( V \) curve.

On the other hand, the hyperpolarization-activated fast-gate opening mechanism is not regulated by [Cl\textsuperscript{−}]\textsubscript{i} or [H\textsuperscript{+}]\textsubscript{i}. It is the extracellular H\textsuperscript{+} ([H\textsuperscript{+}]\textsubscript{o}) that helps activate the fast-gate opening at the hyperpolarized membrane potential (47). The overall effect of increasing [H\textsuperscript{+}]\textsubscript{i} (or reducing extracellular pH) is an elevation of the minimal \( P_o \) of the fast-gate \( P_o \)-\( V \) (47, 269), an effect very different from the shift of the \( P_o \)-\( V \) curve along the voltage axis in response to the change of [Cl\textsuperscript{−}]\textsubscript{i} (47, 52, 53). Thus the regulations of fast-gating by Cl\textsuperscript{−}, H\textsuperscript{+}, and membrane potential appear to be very complicated. A unified theory is urgently needed to explain all these gating phenomena. A successful model will promote our understanding of the structure-function relationship of CLC-0 as well as the linkage of CLC channels and transporters.

D. Mechanistic Models for CLC-0 Fast-Gating

In the last decade, several mechanistic models have been developed for the fast-gating mechanism of CLC-0. In the first class that could be called “conventional” theory, the fast-gate of CLC-0 was proposed to be opened by extracellular Cl\textsuperscript{−}. The idea first came from an observation that Cl\textsuperscript{−} and various permeable anions can shift the \( P_o \)-\( V \) curve of the CLC-0 fast-gate (244). In the original model, it was proposed that the gate is linked with an anion-binding site in the pore, and the opening of the fast-gate may come from a direct activation by the binding of extracellular Cl\textsuperscript{−} to this binding site located within the membrane electric field. Because Cl\textsuperscript{−} carries a negative charge, the voltage dependence of the fast-gate opening could then come from the voltage-dependent binding step (244). A slightly modified model was later proposed by suggesting that Cl\textsuperscript{−} binding to the pore is not a voltage-dependent process (53; but see Ref. 79). Rather, Cl\textsuperscript{−} may first bind to a site outside the membrane electric field, and the Cl\textsuperscript{−}-bound channel then undergoes a conformational change, which translocates the bound Cl\textsuperscript{−} to an inner site within the membrane electric field and opens the gate (53;
Inward movement of Cl\textsuperscript{−} indicated that a H\textsuperscript{+} binding to the pore may permit the transport of H\textsuperscript{+} to reach the side chain of Glu-166 (2, 223, 345).

The degraded transporter model for fast-gating of CLC-0 can also explain the extracellular H\textsuperscript{+} effect. At very negative membrane voltages, outward movement of intracellular H\textsuperscript{+} is not favored, but the inward movement of H\textsuperscript{+} from the extracellular side to Glu-166 may instead replace the outward H\textsuperscript{+} movement to open the fast-gate (211). The transport of H\textsuperscript{+} from the intracellular and the extracellular solutions to Glu-166 should be voltage dependent. The experimental observation that the depolarization-activated opening (due to movement of H\textsuperscript{+} from intracellular solution to Glu-166) is associated with a gating charge of 0.7, while the hyperpolarization-activated opening (favored by external H\textsuperscript{+}) has a gating charge of −0.3 is consistent with such a picture. A recent functional study of the E166D mutant provided further evidence that both the extracellular and internal H\textsuperscript{+} can reach this residue (316). Thus, if H\textsuperscript{+} indeed can reach the side chain of Glu\textsubscript{x} to control the fast-gate opening, H\textsuperscript{+} must be transported across CLC-0. Unlike the traditional model in which Cl\textsuperscript{−} is the activating ligand, this new model suggests that H\textsuperscript{+} is the activating ligand, and the gating charge for the voltage-dependent opening of the CLC-0 fast-gate is H\textsuperscript{+} but not Cl\textsuperscript{−} (211)! However, if H\textsuperscript{+} is solely responsible for the closing rate, one might expect that the closing rate will also be increased by the membrane depolarization if H\textsuperscript{+} is transported from Glu\textsubscript{x} to the external solution during the fast-gate closing process. This prediction on the closing rate apparently contradicts the experimental observation.

The degraded transporter model does not require a concerted exchange of H\textsuperscript{+} with Cl\textsuperscript{−}, like the coupled Cl\textsuperscript{−}-H\textsuperscript{+} exchange shown in CLC-ec1. In essence, therefore, the model presents CLC-0 as an H\textsuperscript{+}-gated Cl\textsuperscript{−} channel, with Cl\textsuperscript{−} being an allosteric modulator. A model that more closely links the gating of CLC-0 with the Cl\textsuperscript{−}-H\textsuperscript{+} antiporter activity in CLC-0 may also be possible; for example, the opening and closing of the gate of CLC-0 is strictly coupled to a Cl\textsuperscript{−}-H\textsuperscript{+} antiporter activity such that one transport cycle is associated with an open-close transition for channel activities. Such a model may be called a “coupled transporter” model. In this case, the observed current in CLC-0 may come from two parts: the ionic current through the CLC-0 pore and the transporter cur-
rent through a $\text{Cl}^- - \text{H}^+$ antiporter cycle. However, this antiporter current would be difficult to detect by the reversal potential measurement because the $\text{Cl}^- - \text{H}^+$ antiporter activity in CLC-0, if it exists, would be four to five orders of magnitude smaller than the current carried by the $\text{Cl}^-$ flux through the CLC-0 pore.

The transporter models, especially the feature of $\text{H}^+$ activation of CLC-0, help explain the variable gating phenomena among different CLC channels and their mutants. As described above, the opening of CLC-2 is favored by membrane hyperpolarization (108, 225, 242, 311, 320, 361). In addition, various manipulations of channel proteins, including single-point mutations, in CLC-0 and CLC-1, convert the channel into a hyperpolarization-activated channel (88, 89, 194, 199, 277, 331, 352). It is interesting to point out that the hyperpolarization-activated opening mechanism of CLC-2, and the artificially created hyperpolarization-induced channel opening in CLC-0 and CLC-1 mutants, are sensitive to external pH (242). Perhaps in hyperpolarization-induced channel gating, the $\text{H}^+$ transport pathway from $\text{Glu}_\text{in}$ to $\text{Glu}_\text{ex}$ is disrupted, and therefore, the depolarization-activated opening mechanism is not functional, leaving only the hyperpolarization-activated opening mechanism which results from protonation of $\text{Glu}_\text{ex}$ by extracellular $\text{H}^+$.

**E. Unsettled Issues and Future Directions**

Each of the aforementioned models describes a specific aspect of the fast-gating mechanism, but falls short in explaining other gating properties. Overall, these models may be grouped into two general categories: those which propose that opening of the gate is via the competition between $\text{Cl}^-$ and the channel gate ($\text{Cl}^-$ is the activating ligand, the conventional models), and those that propose that the opening of the gate is somewhat related to the $\text{Cl}^- - \text{H}^+$ antiporter activity ($\text{H}^+$ as the activating ligand, the transporter model). In conventional models, the fast-gate opening by $\text{Cl}^-$ is thought to result from a mechanism whereby $\text{Cl}^-$ occupancy at $S_{\text{ext}}$ repels the negatively charged side chain of $\text{Glu}_\text{ex}$. However, single-channel studies in CLC-0 have suggested that the competition between $\text{Cl}^-$ and the side chain of the glutamate residue may be used to explain the effect of $[\text{Cl}^-]$, on the closure of fast-gate; fast-gate closure of CLC-0 is reduced by an increase of $[\text{Cl}^-]$, (49, 52–54), a “foot-in-the-door” effect first described in cation channels (304). The internal and external $\text{Cl}^-$ effects on fast-gating of CLC-0 are very different. For example, altering $[\text{Cl}^-]$, mainly affects the opening rate, while changing $[\text{Cl}^-]$, alters the closing rate but has little effect on the opening rate of the fast-gate (52, 53). The apparent $\text{Cl}^-$ affinities in modulating the opening rate and the closing rate by $[\text{Cl}^-]$, and $[\text{Cl}^-]$, respectively, differ by $>20$-fold, suggesting that the $\text{Cl}^-$-binding sites involved in the opening and closing mechanisms may be different (52, 53). In addition, the fast-gate opening and closing mechanisms are differentially affected by point mutations. For mutations at the external pore entrance of CLC-0, for example, K165, the effect is on the opening rate but not the closing rate (174). On the other hand, for a mutation at the intracellular pore entrance of CLC-0, K519, the effect is more on the closing rate but not the opening rate (52). Taken together, these results strongly suggest that these two aspects of the fast-gating mechanism, the opening and closing processes, may not be reversible processes of each other. If the foot-in-the-door effect of $\text{Cl}^-$ in the pore, that is, the competition of $\text{Cl}^-$ with the negatively charged side chain of Glu-166 for occupying $S_{\text{ext}}$, is the mechanism underlying the $\text{Cl}^-$ effect on the closing rate, it seems difficult to use the same mechanism to explain the external $\text{Cl}^-$ effect on the fast-gate. In fact, it was questioned previously if the competition of $\text{Cl}^-$ with the Glu-166 side chain could reflect the fast-gate opening mechanism because the opening rate and the closing rate of the fast-gate are separately controlled by external and internal $\text{Cl}^-$ in very different ways (49).

The “transporter” model of the fast-gating mechanism is not without flaws. First, the strictly coupled transporter model appears to be less convincing because of a dichotomy in the mutational results of the Tyr to Ala mutation at the center of the transport pathway. In CLC-ec1, Y445A mutation significantly reduces the $\text{Cl}^-$ occupancy at $S_{\text{cen}}$ and therefore severely uncouples the $\text{Cl}^- - \text{H}^+$ transport (2). However, the corresponding mutation of Y512A in CLC-0 is almost without functional consequence (4). Thus, if a $\text{H}^+$-transport pathway exists in CLC-0, it must differ from that in CLC-ec1, and the $\text{Cl}^-$ and $\text{H}^+$ transports may not be tightly coupled, the essence of the degraded transporter model. Even with the degraded transporter model, however, several problems remain unsettled. For example, how does $\text{Cl}^-$ help $\text{H}^+$ in opening the fast-gate? Previous studies have shown that only anions that are permeable through the pore or are able to block the pore can increase the fast-gate opening of CLC channels (244, 270). Thus the binding sites for these anions to exert their effects on fast-gating likely reside in the pore, and the occupancy of anions in the pore must be critical for $\text{H}^+$ to reach $\text{Glu}_\text{ex}$. However, although computational studies suggest the presence of such anion binding sites (92, 345), the crystal structure of CLC-ec1 provides no evidence of anion-binding sites extracellular to the gate. Recent studies in CLC-ec1 showed that the occupancy of an anion at $S_{\text{cen}}$ is critical for $\text{H}^+$ transfer from $\text{Glu}_\text{in}$ to $\text{Glu}_\text{ex}$ (223). However, $S_{\text{cen}}$ is intracellular to the side chain of $\text{Glu}_\text{ex}$. When the gate is closed, how does a $\text{Cl}^-$ in the extracellular medium reach $S_{\text{cen}}$? Another unknown in the transporter model is the intracellular protonation site. If $\text{H}^+$ is the activating ligand
of CLC-0, and if fast-gating of CLC-0 indeed bears the
lineament of the Cl⁻/H⁺ antiporter activity, one may ex-
pect that the protonatable residues are conserved. Glu_{ex}
is indeed conserved throughout the CLC family except for
CLC-K, a channel without prominent gating kinetics.
However, the intracellular Glu (Glu_{in}) is conserved only in
those members that are thought to function as Cl⁻/H⁺
antiporters (5). In CLC-0, the residue corresponding to
Glu-203 of CLC-ec1 is a valine, a residue that cannot be
titrated by H⁺. If the H⁺ transport pathway exists in
CLC-0 to allow H⁺ movement from intracellular solution
to Glu_{ex}, where is the initial intracellular protonation site
responsible for the internal H⁺ regulation of CLC-0 fast-
gating? The degraded transporter model may argue that
the requirement of a conserved titratable residue facing
intracellular medium may not be necessary (211). How-
ever, a computational study suggested that the negative
potential near the residue corresponding to Glu-203 of
CLC-ec1 is absent in the CLC-0 homology model (345); the
lack of this negative potential would prevent proton trans-
fer in this region. If this is the case, how does internal H⁺
reach to Glu_{ex}? In all proposed gating mechanisms of CLC-0 de-
scribed in this review, the interaction of Cl⁻ and/or H⁺
with the gate may provide the evolutionary link between
channels and transporters in this family. Detailed Cl⁻ and
H⁺ pathways and the Cl⁻- and H⁺-binding sites on the
CLC-0 channel are also critical for understanding the
gating mechanisms. Therefore, identification of the inter-
nal protonation site(s) responsible for the internal pH
effect on fast-gating should be an important problem to
tackle in the future. The CLC-ec1 structure suggests a
Cl⁻-permeation pathway that is evolutionarily preserved
in CLC-0, but whether there is a similar H⁺ transport
pathway in CLC-0 remains an outstanding question. The
conventional models propose that the gate is opened by
Cl⁻. Where Cl⁻ binds in the pore before it opens the gate
is still unsettled. All these questions are simply asking:
where are the binding sites for the activating ligands in
CLC-0, and where do these ligands go after they interact
with those sites? Finally, although the major portion of
the above discussion centers on the relation between the
antiporter activity and the fast-gating of CLC-0, the rela-
tion of the antiporter activity with the operation of the
CLC-0 slow-gating may also need to be contemplated in
light of the reports that the nonequilibrium slow-gating
cycles are fueled by the Cl⁻ gradient (262), and perhaps
the H⁺ gradient (M. Maduke, personal communication)
across cell membranes.
As mentioned at the beginning, CLC-0 can be viewed
as a ligand-gated channel, and the biophysical studies
have suggested that the ligands may be Cl⁻ and/or H⁺.
The special feature that is thought to distinguish this
channel from the classical neurotransmitter-gated chan-
nel (e.g., acetylcholine receptor or the GABA-gated Cl⁻
channel) is that the ligand binding is stoichiometrically
coupled to one closed-open-closed gating cycle. At this
point, however, without a firmly documented trans-chan-
nel movement of Cl⁻ or H⁺, the idea of an irreversible
gating cycle for CLC-0 channels, although conceptually
invigorating, remains somewhat hypothetical. In the fol-
lowing discussions, we will present CFTR, a Cl⁻ channel
gated by ATP. Being a member of the ABC transporter
category, CFTR is known to hydrolyze ATP (170). An ines-
capable inference here is an irreversible gating cycle
driven by the input of free energy from ATP hydrolysis.
From a wealth of structure-function studies of ABC trans-
porters, we will try to catch a glimpse of the relationship
between ion channels and transporters using CFTR as a
model system.

VI. ABC TRANSPORTER FAMILY

The ABC transporters, one of the largest families of
homologous proteins, consist of integral membrane proteins
that share unique topological characteristics, namely, two
membrane-spanning domains (MSD) and two nucleotide-
binding domains (NBD) (125, 127). Nearly all the mem-
ers of the ABC superfamily perform active transport of
substrates against cellular concentration gradients, utiliz-
ing ATP hydrolysis as the source of the free energy. ABC
proteins are found in all prokaryotic and eukaryotic cells.
This gene superfamily is so prevalent in prokaryotic cells
that 3–5% of the bacterial genome sequenced to date
encodes ABC proteins (258). In the human genome, on the
other hand, 48 ABC transporters have been identified (68).
Based on phylogenetic analysis, the human ABC super-
family has been classified into seven subfamilies (ABCA–
ABCG). The importance of ABC proteins in human phys-
iology is attested by the fact that 17 of the 48 human ABC
proteins are linked to genetic diseases. These include
Tangier disease (ABCA1) (159), Stargardt disease (ABCA4)
(157), Dubin-Johnson syndrome (ABCC2) (164), familial per-
sistent hyperinsulinemic hypoglycemia of infancy (ABCC8)
(312), and cystic fibrosis (ABCC7) (263). In addition to
causing these diseases, multidrug resistance empower-
ed by ABC transporters that export antibiotics or
chemotherapeutic reagents poses significant clinical
hurdles for the treatment of bacterial infection (165,
233) and cancer (13).

The functional diversity of members of this super-
family is suggested by the fact that human ABC proteins
have a wide tissue distribution (69). In addition, the sub-
strates for different ABC proteins cover a broad spectrum
of substances, including sugars, amino acids, ions, drugs,
polysaccharides, and proteins. It seems logical to imagine
that once a primordial but effective “pumping machine”
employing the basic modular design of the ABC trans-
porter (see sect. vii) emerged, more ABC proteins evolved
simply through alterations of the transport pathway to meet the need of accommodating different substrates (291). This scene of evolutionary relatedness in all ABC proteins can be sensed not only from the structure-biological point of view (discussed in sect. vii), but also from their functional similarities. For example, CFTR is a member of the ABCC subfamily. While CFTR is a genuine ion channel, carrying out the function of passive diffusion of negatively charged chloride ions, many related members of this subfamily, e.g., ABCB1, a leukotriene transporter (166, 188), actively transport various organic anions. While it is obligatory for these active transporters to possess a binding site for their anionic substrates, interestingly, the presence of a binding site for organic anions in the ion permeation pathway of CFTR has been repeatedly demonstrated (6, 177–179, 206, 280, 289, 321, 354–356). Does this binding site for organic anions in CFTR represent an evolutionary vestige when an active transporter of the ABC family is converted to an ion channel? This question will be revisited after we discuss the structure and function of ABC transporters and CFTR.

VII. STRUCTURE/FUNCTION OF ABC TRANSPORTERS

A. Structural Characteristics of the Nucleotide Binding Domain in ABC Transporters

The core domains (2 MSDs and 2 NBDs) of eukaryotic ABC transporters are usually encoded by a single gene. Thus a single polypeptide contains these four core domains. For many of the prokaryotic ABC proteins, however, individual domains are encoded by different genes, and the resulting polypeptides are assembled into multidomain proteins. Among different members of the ABC superfamily, the MSDs are highly divergent in both amino acid sequence and topology, whereas the NBDs share significant sequence and structural homology. This is perhaps not surprising since the MSDs likely contain substrate binding sites that must be different in different transporters. In addition, while the NBDs serve as the common engine to harvest the free energy of ATP hydrolysis, the MSDs will perform different functions depending on the physiological roles a particular ABC transporter assumes.

Like other ATP binding proteins, the ABC transporters’ NBDs contain the characteristic Walker A (GxxGxGKS/T, x represents any amino acid) and Walker B (hhhhhD, h represents a hydrophobic amino acid) nucleotide binding motifs (327). Unique to the ABC superfamily is the “signature sequence” (LSGGQ) whose function remains unclear in spite of early speculation that it serves to transduce signals from ATP binding/hydrolysis to the conformational changes in other domains of the ABC transporter during a transport cycle (14). In 1998, Hung et al. (135) reported the first crystal structure of the ATP-NBD complex from the histidine permease (HisP) of Salmonella typhimurium.

As seen in other nucleotide binding proteins, the Walker A motif (also called P-loop) in the structure of HisP is heavily involved in coordinating β- and γ-phosphates of ATP. The bound ATP is further stabilized by a tyrosine residue (Y16) that interacts with the adenine ring of ATP. The aspartate residue (D178) in the Walker B motif interacts with a water molecule, which probably takes the position of the magnesium ion that normally serves as a cofactor for ATP hydrolysis. In addition, a glutamate residue (E179) immediately following the Walker B aspartate and a glutamine residue (Q100) in the Q-loop region, both highly conserved, form hydrogen bonds with a water molecule (H2O 437), which interacts with the γ-phosphate through a hydrogen bond that is nearly parallel to the PγOβ bond. Since this PγOβ bond breaks upon hydrolysis, it is speculated that water 437 is the most likely candidate for the “attacking” water during ATP hydrolysis and either Q100 or E179 could be the activating residue. This structure not only gives us a first glance of the molecular interactions between ATP and its binding pocket, importantly, it also provides structural explanations for many genetic and biochemical data obtained in vivo for the histidine transporter (292). Furthermore, the importance of the glutamate adjacent to the Walker B motif in ATP hydrolysis is confirmed for numerous other ABC proteins (218, 238, 302, 315), although the exact chemical mechanism by which this glutamate catalyzes ATP hydrolysis remains controversial (82, 115).

In less than 10 years, the structures of more than 10 other NBDs have been solved at an atomic level (45, 71, 98, 152, 167, 168, 250, 278, 296, 322, 346, 348). Although the overall sequence homology among NBDs from different ABC proteins is not very high except in those conserved motifs, interestingly however, the basic architecture is remarkably similar even between classical ABC transporters and somewhat distantly related proteins such as Rad50, a DNA repairing enzyme (130). The prototypical structure of an NBD consists of an F1-type core subdomain containing the nucleotide binding motifs (Walker A and B), which is sandwiched by the anti-parallel β-sheet subdomain usually containing an aromatic amino acid that interacts with the adenine ring of ATP (12), and the α-helical subdomain containing the signature sequence LSGGQ.

B. NBDs Form Dimeric Structures

Although some early reports show that the NBDs are crystallized as a monomer (152, 346), it is now generally accepted that there is a dynamic process for two NBDs to
associate/dissociate (i.e., dimerization). Despite a high structural conservation among NBD monomers, the first three crystal structures of NBDs show different dimeric configurations (71, 130, 135). This dispute had been resolved after more dimeric structures emerged. More recently solved structures of NBD dimers show a head-to-tail configuration (45, 296, 348). Once an NBD dimer is formed, the two ATP-binding sites are buried at the dimer interface. The bound nucleotides as well as many amino acid residues from each NBD participating in nucleotide interactions are intimately involved in forming a stable dimer. Because most of the inter-NBD interactions occur via the ATP molecules, these are often referred to in the literature as “glues.” This new dimer structure places the signature motif (LSGGQ) that defines the ABC transporter family at the dimer interface involved in interactions with ATP (especially with the γ-phosphate of ATP). Thus, once the NBDs dimerize, the ATP binding pocket is composed of the Walker A and B motifs from one NBD and the signature motif from the partner NBD. This assembly of the ATP binding pocket then satisfactorily explains the involvement of the signature sequence in ATP binding and hydrolysis from mutagenesis studies (23, 170, 259, 276, 307, 313) as well as photocleavage and cross-linking studies implicating the close proximity between the signature sequence and the opposing Walker A motif (93, 191). Most importantly, this head-to-tail assembly of NBD dimers is consistent with the dimeric configuration found in the holoenzyme structures of several bacterial ABC proteins (Fig. 4), including the vitamin B$_{12}$ transporter BtuCD from *E. coli* (186); a putative molybdate transporter, ModBC, from *Archaeoglobus fulgidus* (129); a metal-chelate transporter, HI1470/1, from *Haemophilus influenzae* (234); and a drug exporter, Sav 1866, from *Staphylococcus aureus* (65).

NBD dimerization has also been demonstrated with biochemical assays. Using an analytic gel filtration technique, Moody et al. (218) showed that MJ0796 and MJ1267, two bacterial ABC transporters’ NBDs, can form a dimer in the presence of ATP. Neither ADP nor AMP-PNP can substitute for ATP in the dimerization reaction, suggesting a critical role of the γ-phosphate in catalyzing dimer formation. Since the dimer is only seen in mutants whose ATPase activity is abolished (by mutating the glutamate residue adjacent to the Walker B aspartate), it is suggested that ATP hydrolysis by wild-type dimers provides the energy to break the stable dimer formation (218, 296). Thus, during the normal ATP hydrolysis cycle, the NBD dimer is short-lived. This speedy dissociation of NBD dimers is an essential element for an efficient transport cycle, in which the presence of an absorptive state is not allowed. Chen et al. (45) showed crystallographically that MalK, the NBD of bacterial maltose transporters, can assume two different NBD dimeric structures depending on whether the structures contain ATP. In the absence of bound ATP, the structure shows a wide gap (>10 Å) between two NBDs. On the other hand, the gap is closed in the structure with bound ATP molecules. Based on these snapshots of NBD dimers, these investigators (45) proposed a tweezer-like motion upon ATP binding to NBDs for maltose transporters. This hypothesis is strongly supported by a recent demonstration that the crystal structure

![Fig. 4. Cartoon representations of the four full-length structures for ABC transporters. From left to right, structures are BtuCD (pdb 1L7V), a vitamin B$_{12}$ importer; SAV1866 (pdb 2HYD), a bacterial drug exporter; HI1470/1 (pdb 2NQ2), a metal-chelate-type ABC transporter; and ModBC (pdb 2ONK), a molybdate transporter, respectively. The two membrane-spanning domains are colored in yellow and magenta. The two nucleotide binding domains are colored in green and pink, except in SAV1866, where they are colored in their correspondent membrane-spanning domains. Bound ligands in the nucleotide binding domains are shown in ball-and-stick model and colored by their atom types. No ligand is present in the structure of HI1470/1. Cyclotetranodanate, ADP, and phosphate are ligands for the NBDs of BtuCD, SAV1866, and ModBC, respectively. It should be noted that the assembly of two MSDs, as well as the relationship between NBDs and MSDs in Sav1866, is fundamentally different from other structures.](www.prv.org)
of MalK in the posthydrolysis state shows a disengagement of ADP-bound Walker A and B motifs from the signature sequence of the partner NBD (192). The numerous structural and biochemical studies described above have generated a popular hypothesis that ATP binding-triggered NBD dimerization may serve as the power stroke to do mechanical work; hydrolysis of the bound ATP drives the dissociation of the NBD dimer, and subsequent release of the hydrolytic products, ADP and Pi, completes the transport cycle (125, 219).

By comparing NBD structures with or without ligands and structures in the presence of different nucleotides, one can learn about possible conformational changes induced by ATP binding and hydrolysis. Karpowich et al. (152) reported that ATP binding to the Walker A motif likely induces conformational changes involving the conserved D-, Q-, and H-loops, regions participating in interactions with the γ-phosphate of ATP. It is suggested that this induced-fit mechanism probably also plays a role in facilitating NBD dimer formation. The same report also showed an outward rotation of the LSGGQ-containing α-helical subdomain of the NBD in ADP-bound monomeric structure of MJ0796. Since the α-helical domain interacts directly with MSDs of ABC transporters (see sect. viIC), if we accept the idea that this ADP-bound monomer represents a posthydrolysis structure of NBD, this inward movement by ATP binding and outward movement following ATP hydrolysis of the α-helical subdomain may well be part of the signaling transmission mechanism relating NBD machine and the transport pathway in MSDs. While these structural/biochemical studies of isolated NBDs have provided important mechanistic insights into the molecular motion of NBDs upon ATP binding/hydrolysis, one apparent caveat of these approaches is the absence of MSDs, which could affect the conformational freedom of NBDs.

C. Crystal Structures and Working Model of ABC Proteins

Recent breakthroughs in solving the crystal structures of several ABC transporter holoenzymes (65, 66, 129, 136, 186, 234) not only confirm the long-speculated domain organization of the ABC proteins, but also allow a glimpse at the atomic level of the details of individual domains and their interactions (Fig. 4). Although these structures show similar, if not identical, arrangement of the two NBDs, the transmembrane domain folds exhibit a high degree of structural variability. For importers that transport its substrate into the cell such as BtuCD, HI1470/1, and ModBC, not only is the number of transmembrane segments different, the fold of individual MSD also shows striking diversity. However, one common feature of the importer structures with nucleotide-free NBDs is the presence of an inward-facing, central cavity that is accessible to the cytoplasm (third and fourth structures in Fig. 4), a likely candidate for the substrate translocation pathway. In contrast, the structure of BtuCD (first in Fig. 4) with bound cyclotetravanadate in its NBDs contains an outward-facing, central pathway. It is interesting that these pathways are only access to either the cytoplasm or the external medium as if they are “gated” on one end or the other by the respective transmembrane segments.

Some surprising findings emerge from the crystal structure of an exporter, Sav 1866 (second structure in Fig. 4). Compared with the domain arrangement seen in all the importers shown in Figure 4, the relationship between MSDs and NBDs in Sav 1866 takes a new twist. While the NBD dimeric structure of Sav 1866 is conserved, each NBD interacts not only with the corresponding MSD, but also with the MSD of the partner subunit. Thus each MSD straddles on both NBDs via its two intracellular loops (Fig. 4). This arrangement between MSDs and NBDs makes it difficult to envision a simple translational movement of the two subunits of an ABC transporter and necessitates a twisting motion during a transport cycle (65). Equally surprising is the observation that the NBD dimer interface of Sav 1866 with two bound ADP molecules (therefore suggesting a posthydrolytic conformation) is extremely similar to that of hydrolysis-defective MJ0796 with bound ATP, suggesting a limited separation of two NBDs during the cycle of ATP binding/hydrolysis as proposed by Jones and George (150). However, it is lately proposed that this ADP-bound structure of Sav 1866 actually represents a prehydrolytic ATP-bound conformation, since a similar overall structure of Sav 1866 was observed when the NBDs are occupied by the nonhydrolytic ATP analog AMP-PNP (66). This latter scenario assumes that AMP-PNP binding to NBDs can trigger NBD dimerization, although, at least for isolated NBDs of MJ0796 and Md11P, nonhydrolyzable ATP analogs fail to dimerize NBDs (141, 218). Nevertheless, the presence of an outward-facing pathway in Sav 1866 prompts Locher and colleagues (128) to propose a common mechanism of coupling ATP binding/hydrolysis to an alternating substrate binding site for both importers and exporters. In their model, MSDs flip from the inward-facing to the outward-facing configuration following NBD dimerization upon ATP binding. ATP hydrolysis and dissociation of ADP and phosphate reset the system to the original conformation. This model of an alternating substrate binding site for ABC transporters bears remarkable resemblance to the one proposed for the lactose permease (109) and the “rocking banana” cartoon depicted in Figure 1. The difference between an importer and an exporter is that while an importer translocates the substrate by ATP binding and subsequent NBD dimerization, the translocation of the substrate across MSDs of an
exporter is accomplished by ATP hydrolysis and dissociation of the hydrolytic products.

Close inspections of the interface between NBDs and MSDs also reveal intriguing structural features that may function in a hydrolytic cycle and the transport cycle remains debatable for HisP, when one of the two ATP sites is occupied by the vanadate-ADP product, e.g., ADP, can prevent ATP binding to this particular NBD. Alternative, at the end of an ATP hydrolysis cycle by one NBD, retaining the hydrolytic product, e.g., ADP, can prevent ATP binding to this particular NBD. Therefore, there are multiple ways to make two symmetrical ATP binding sites function asymetrically (162). Molecular dynamic simulations also show that docking and subsequent occlusion of ATP at one catalytic site of BtuCD is coupled to loosening of the nucleotide binding pocket at the second site (230; cf. Ref. 229). Furthermore, in a P-gp mutant where both catalytic glutamate residues are mutated to alanine, ATP was found occluded in just one site (315), consistent with earlier reports that in wild-type P-gp, one active site is empty when the other site is occupied by the vanadate-ADP complex (248, 319). More kinetic studies using crystal

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structures as a guide are needed to elucidate the sequence of molecular events during a transport cycle.

VIII. CFTR CHLORIDE CHANNEL

A. Introduction

CFTR (ABCC7; Ref. 264) is one of the very few members of the ABC proteins that does not function as an active transporter (one other analogous member is sulfonamide receptor, SUR or ABCC8). Unlike SUR, which acts as a regulatory subunit for K<sub>ATP</sub> channels (reviewed in Ref. 224), CFTR by itself functions as a chloride channel as demonstrated by Bear et al. (26) with purified CFTR in reconstituted lipid bilayers. Mutations that diminish the CFTR channel activity cause cystic fibrosis (CF), the most common, lethal genetic disease in Caucasians that affects 1 in ~2,500 newborns in the United States (334). In CF patients, normally Cl<sup>-</sup>-permeant epithelial cells in airways, pancreas, and other tissues become Cl<sup>-</sup> impermeable, causing defective salt, water, and protein transport. On the other hand, increased activity of the CFTR Cl<sup>-</sup> channel, usually caused by bacterial toxins, results in secretory diarrhea that incapacitates millions of people each year in developing countries (30). Therefore, understanding how the CFTR channel is gated will have a direct impact on the development of novel therapeutics for treatment of CF and secretory diarrhea.

As a member of the ABC superfamily, CFTR contains the characteristic architecture of two MSDs and two NBDs (NBD1 and NBD2) (Fig. 5). There is strong evidence that CFTR's two NBDs form a head-to-tail dimer similar to those found in other ABC transporters (323). The two ATP binding pockets (ABP) for CFTR are defined as follows: ABP1, formed by the Walker A and B motifs of NBD1 and the signature sequence of NBD2; ABP2, formed by the Walker A and B motifs of NBD2 and the signature sequence of NBD1. Similar to other members of the ABCC subfamily, the amino acid sequences of CFTR's two NBDs show significant differences even in those conserved motifs. For example, the glutamate residue adjacent to the Walker B motif is replaced by a serine residue in NBD1. A histidine residue that has been shown to play an important role in ATP hydrolysis in other ABC proteins (e.g., HlyB) (348) is also replaced by a serine in NBD1. In addition, the signature sequence in CFTR's NBD2 is somewhat degenerate (LSHGH instead of LSGGQ). This structural asymmetry of the two NBDs in CFTR likely accounts for the observation that only ABP2, but not ABP1, hydrolyzes ATP at an appreciable rate (9, 24, 302).

In addition to the canonical MSDs and NBDs seen with other ABC proteins, CFTR contains a unique regulatory (R) domain located between the NH<sub>2</sub>-terminal NBD (or NBD1) and the second MSD (Fig. 5). Phosphorylation of many of the consensus serine residues in the R domain is a prerequisite for CFTR to function. After the channel is phosphorylated by protein kinase A (PKA) and ATP, opening and closing (i.e., gating) of phosphorylated channels is controlled by ATP. Numerous mutagenesis studies have indicated that phosphorylation affects CFTR activity in a very complicated manner. Not only is the activity of CFTR incrementally regulated by differential phosphorylation of the PKA consensus sites, protein kinase C-dependent phosphorylation may also modulate CFTR. Interested readers are referred to several recent articles that have extensively reviewed this subject (96, 117, 284, 290, 358). Here we focus on ATP-dependent gating, which is likely to have an evolutionary relationship to the transport function for other ABC proteins.

B. Crystal Structures of CFTR’s NBD1

Before we discuss CFTR gating function and its mechanism, we first review recent advances in solving the crystal structure of human and mouse CFTR’s NBD1 by Lewis et al. (167, 168) and Thibodeau et al. (310; also see Ref. 250 for the NBD structure of another member of the
ABCC subfamily). Not surprisingly, the basic architecture of these two solved NBD1s is very similar to known NBD structures. All three characteristic subdomains described above are conserved. Also as seen in NBDs from other members of the ABC proteins, the bound MgATP is coordinated by the Walker A motif (K464 and S465 in human CFTR) and the Walker B aspartate (D572). However, as described above, two critical residues (Walker B glutamate and histidine in the "switch" region) that form hydrogen bonds directly or indirectly with the γ-phosphate in NBDs of other ABC proteins (135, 296) are replaced by serine. This likely explains why biochemical studies demonstrate that ATP is hydrolyzed in ABP2 but not in ABP1 (9, 24, 302). This structural/biochemical asymmetry, as described above, is an interesting feature of the ABCC subfamily (as well as many ABC transporters in bacteria, yeasts and fungi, see Ref. 151). The functional implications of this structural asymmetry will be discussed below.

Another interesting feature revealed by the structure of mouse CFTR’s NBD1 is a nonclassical interaction between ATP and the binding pocket. Instead of a trans configuration for the backbone γ torsion angle between the ribose and the nucleotide base usually seen in other ABC proteins (e.g., Ref. 296), the ATP molecule in mouse CFTR’s NBD1 assumes an unusual gauche + configuration. Unlike a classical ring-ring stacking interaction between ATP and an aromatic amino acid (e.g., tyrosine-11 in the previously mentioned crystal structure of MJ0796), the nucleotide base in the structure of mouse NBD1 (167) seems to be gripped by three hydrophobic amino acids (W401, L409, and F430). However, in the structure of modified human NBD1 (168), a canonical stacking interaction between the nucleotide base and the W401 residue is observed. Although the reason for this discrepancy remains unclear, it should be noted that each crystal structure only reflects a snapshot of the protein. Furthermore, some of these amino acids (e.g., F409 and F430) that interact with the nucleotide base are located in a region that may be structurally dynamic (as implicated by the elevated B-factors in the crystallographic data set). It is possible that these two structures of NBD1 represent NBD1 conformations appropriate for different states of the channel or the mutations made in human NBD1 to facilitate crystallization could potentially change the structure. Alternatively, the unusual ATP-W401 interaction in the first CFTR NBD1 crystal is caused by crystal lattice interactions that influence side-chain rotamers and configurations (310).

Also unique to CFTR’s NBD1 is the presence of a regulatory insertion region (amino acids 404–435) at the NH₂ terminus and a regulatory extension (amino acids 639–670) at the COOH terminus. It is interesting that both regions contain consensus serines for PKA-dependent phosphorylation (S422 in the regulatory insertion and S660 and S670 in the regulatory extension). However, only S660 and S670, but not S422, have been shown to be phosphorylated in vitro or in vivo (96). While the structural details of the 30-amino acid insertion is not completely solved in the crystal structure, implicating flexibility of that part of the molecule, the physical location of the regulatory extension may impart functional implications. According to sequence analysis, the regulatory extension, an α-helix in the crystal structure, belongs to the R domain. This helix, if present at the same location of the intact CFTR protein, would hinder NBD1/NBD2 dimer formation because of its location at the presumed dimer interface. Indeed, fitting NBD1 and modeled NBD2 into the MJ0796 dimer results in significant steric clashes (167, 220; Zou and Hwang, unpublished observations). This raises an interesting possibility that the role of this region together with other parts of the R domain is to prevent dimer formation; phosphorylation of the R domain may result in a conformation change that enables NBDs to dimerize. It should be noted that removal of this segment does not result in PKA-independent activity, indicating that this helix is not required for preventing dimer formation in nonphosphorylated CFTR (59). On the other hand, removing a larger part, or all, of the R domain renders the channel activity phosphorylation independent (33, 60, 261). Recent studies using cross-linking strategies for assessing NBD dimerization in CFTR also showed that PKA phosphorylation indeed promotes dimerization of CFTR’s two NBDs (207). However, it is important to note that the functional role of the R domain is more than a simple inhibition of CFTR opening (see details below).

While these NBD1 structures of CFTR have served as a guide for functional studies of the roles of the NBDs in channel gating (323, 360), the field still awaits high-resolution structures of the whole CFTR protein. Because of the technical difficulties in obtaining a large quantity of purified CFTR proteins, crystallization of CFTR remains a mounting challenge. Nevertheless, Ford and colleagues (21, 265) have made the first leap in solving the low-resolution (~20 Å) structures of CFTR from two-dimensional crystals. The overall shape and size are similar to those of the low-resolution structure of P-gp solved earlier (266). Since the two-dimensional crystal of CFTR was grown in the presence of AMP-PNP, a nonhydrolyzable ATP analog that can keep the channel in the open state (113, 138), it is inferred that one of the observed structures represents an open-channel conformation. It should be noted, however, that AMP-PNP by itself is a poor ligand to open CFTR channels (324; cf. Ref. 7). In addition, even the hydrolysis-deficient mutant E1371S-CFTR seldom assumes a stable open state in the presence of AMP-PNP alone (Cho and Hwang, unpublished data). These data underscore the importance of the exact configuration of the γ-phosphate in determining the structure of NBD dimers (150). Therefore, more studies are needed.
to ascertain the functional state of those low-resolution structures.

C. CFTR Function

Functional properties of CFTR have been extensively investigated ever since the gene was cloned in 1989 (264). Since the goal of the current review is to explore the structural/functional relationship between transporters and ion channels, we will confine our discussion to ATP-dependent gating, which is likely to be the analogous behavior of the transport function for other ABC proteins. The permeation properties of the CFTR pore and the cell biological aspects of CFTR function are discussed at length in Dawson et al. (64) and Guggino and Stanton (111), respectively. The technical aspects of gating studies of CFTR chloride channels, covered by other reviews (236, 358), are not repeated in the current article.

Several years before the CFTR gene was cloned, Paul Quinton’s elegant electrophysiological work on isolated sweat ducts has implicated a defective chloride conductive pathway as the fundamental pathophysiological mechanism for CF (249; cf. Ref. 154). It was found that the transepithelial potential of sweat ducts isolated from CF patients is significantly more negative than that of normal controls. This abnormal bioelectric potential in CF sweat ducts can be simulated in normal control ducts simply by replacing chloride ions with impermeant anions, indicating an involvement of a chloride permeation pathway that is defective in CF. This defective chloride conductive pathway then accounts for the elevated sweat [chloride], a diagnostic hallmark in CF. When the CFTR gene was cloned in 1989, it was somewhat puzzling initially why the CFTR protein shows a characteristic topology of an active transporter (264). This mystery was quickly solved by the demonstration that, in excised, inside-out membrane patches, hydrolyzable nucleoside triphosphates, such as ATP, GTP, ITP, CTP, and UTP, are required to activate CFTR chloride currents following PKA-dependent phosphorylation in a heterologous expression system that does not express native CFTR (15). Since single-channel chloride currents can be readily discerned, the most likely explanation for these observations is that CFTR itself encodes a chloride channel whose function is controlled by PKA-dependent phosphorylation and the presence of ATP. Several other studies solidify this conclusion. First, Anderson et al. (16) demonstrated that mutating amino acid residues in the putative pore-forming domain of CFTR alters the anion selectivity. Second, Rich et al. (261) show that partial removal of the R domain renders the CFTR channel phosphorylation independent (261). Lastly and most importantly, Bear et al. (26) painstakingly purified CFTR and incorporated the protein into reconstituted lipid bilayers. A chloride channel with all the characteristic biophysical properties as the channels found in native epithelia (105) or cells heterologously expressing CFTR was observed. This latter study leaves no doubt that CFTR itself is a chloride channel. It should be noted, however, the fact that CFTR itself is a chloride channel does not rule out the possibility that CFTR may carry out other functions (158, 283).

D. Roles of ATP Hydrolysis in CFTR Gating

The involvement of ATP hydrolysis in CFTR gating was implicated even in the earliest studies. Anderson et al. (15) and Nagel et al. (221) reported that ATP, not AMP-PNP or ATPγS (at 0.5–1 mM), can open PKA phosphorylated CFTR. This observation was taken as evidence for an involvement of ATP hydrolysis in opening the phosphorylated CFTR. Further support for this idea was the observation that Mg2+, a cofactor for ATP hydrolysis, is required for normal channel opening by ATP (15, 72, 170). Since AMP-PNP, when applied together with ATP, locks the channel in a stable open state that can last for minutes, it was proposed that ATP hydrolysis also controls channel closing (42, 113, 138). This AMP-PNP-induced, locked-open state is simulated by mutating the Walker A lysine at NBD2 (112), an amino acid residue that is critical for ATP hydrolysis (251). Therefore, it was proposed that hydrolysis by NBD2 closes the channel. Furthermore, since mutating the equivalent lysine at NBD1 (i.e., K464) decreases the opening rate (42, 112; cf.Refs. 237, 251), it was hypothesized that ATP hydrolysis at NBD1 controls channel opening. Thus two hydrolysis events at CFTR’s NBD1 and NBD2 were thought to be distinctly coupled to opening and closing of the channel (72, 350).

While this once popular model for CFTR gating can explain many early functional data, as more results emerged, however, its validity became questionable. Aleksandrov et al. (8) first demonstrated that 5 mM AMP-PNP or ATPγS can open phosphorylated CFTR in lipid bilayers. This observation was later confirmed in excised, inside-out patches by Vergani et al. (324), although the results with AMP-PNP in these two studies are quite different quantitatively. These studies cast serious doubt on the early notion that ATP hydrolysis is obligatory for channel opening (112, 138). It could well be that AMP-PNP is simply a poor agonist, an idea supported by many structural/biochemical studies of ABC proteins described in section IV.B, but sequence analysis and structural studies (167, 168) described above also suggest that CFTR’s NBD1 lacks the essential catalytic base for ATP hydrolysis. Moreover, photolabeling experiments with 8-azido-ATP, a hydrolyzable ATP analog that can sustain CFTR
E. Coupling of NBD Dimerization to Gating Transitions

As discussed in section vii, the two NBDs of the ABC proteins form a head-to-tail dimer upon ATP binding. In most cases, this dimer formation is observed when ATP hydrolysis is abolished by mutations (296). It is thus speculated that for the wild-type NBDs, ATP hydrolysis causes fast separation of the two NBDs. This rapid dissociation of NBDs ensures an effective transport cycle. That CFTR’s two NBDs may assume a prototypical head-to-tail configuration was elegantly demonstrated by Vergani et al. (323). Through statistical analysis of more than 20,000 NBD sequences, they identified a pair of amino acids (one, R555, in NBD1 and the other, T1246, in NBD2) that likely coevolved as a pair to maintain the ability of forming a hydrogen bond at the dimer interface. Indeed, in several known structures of NBD dimers, these two amino acids are rightfully oriented to form a hydrogen bond (45, 296, 349). After carrying out a series of well-designed mutant cycle analyses, Vergani et al. (323) made an insightful conclusion that these two amino acids (R555 in the signature sequence of NBD1 and T1246 in the Walker A motif of NBD2) do not interact when the channel is in the unliganded or liganded closed state, but they do interact when the channel is open or in the transition state. Thus one can envisage that NBD1 and NBD2 undergo a dynamic association/dissociation that is coupled to opening/closing of the channel. It is interesting to note that the functional importance of this hydrogen bond pair in CFTR is attested by the fact that mutations of either amino acid are associated with CF (CF Consortium, http://www.genet.sickkids.on.ca/cftr/). More recently, Mense et al. (207) engineered a series of cysteine pairs (one at NBD1 and the other at NBD2) based on the modeled NBD dimer structure of CFTR. Cross-linking experiments with sulfhydryl reagents not only confirmed that CFTR’s two NBDs form a dimer with a conserved interface, but also provided evidence supporting the notion that PKA phosphorylation promotes dimer formation. Surprisingly, however, when two engineered cysteines at the dimer interface form a disulfide bond, the channels still show slow open-close transitions, suggesting that the coupling of NBD dimerization and opening/closing of the gate may be more complicated. For example, Csanady et al. (61), by examining temperature dependence of CFTR gating, reported evidence that the NBD dimer is already formed but the gate (presumably located in MSDs) is yet to open.

F. Are the Two ATP-Binding Sites Functionally Distinct?

Although it is now well accepted that each of the two ATP binding pockets in an ABC protein consists of components from both NBDs, it should be noted, however, in all the crystal structures of monomeric NBDs (including CFTR’s NBD1), that ATP is bound to the Walker A and B motifs, but not the signature motif, suggesting that the signature sequence may not contribute to ATP binding before two NBDs are dimerized. Nevertheless, in theory, to understand the functional role of ATP binding at each site, one can manipulate the Walker motifs and/or the signature sequence. Many mutagenesis studies have been undertaken along this direction.

Before we cover these studies, a few technical issues that can affect how electrophysiological data are interpreted should be discussed. Single CFTR channel currents show characteristic long bursts of opening (Fig. 6A). Each burst, lasting for hundreds of milliseconds to seconds, is interrupted by brief flickery closings with a time constant of ~10 ms. Thus the closed-time histogram can be at least fitted with two components (113), with the longer closed-time constant being a function of [ATP] (34, 339). The short flickery closing within a burst is voltage dependent, but independent of [ATP]. These events are likely due to blockade of the CFTR pore by yet-to-be-identified cytoplasmic anion blockers (308, 357; cf. Ref. 40). Different methods have been employed to isolate the ATP-dependent events during single-channel kinetic analysis (7, 34, 60, 339). Thus kinetic parameters reported from different groups vary tremendously even for wild-type CFTR. For hydrolysis-deficient mutants such as K1250A, the bursting time estimated with different methods in different reports can differ by ~100-fold. For example, using macroscopic current relaxation analysis, Zeltwanger et al. (350) and Powe et al. (237) reported a locked open-time constant of ~2–3 min for K1250A-CFTR. However, a bursting duration of ~1 s was estimated for the same mutant with microscopic kinetic analysis (42, 251). Different expression systems used by different groups may further complicate the issue (see a summary in Ref. 358). It is thus very difficult, if not impossible, for the authors to make a comprehensive comparison among all the results in the literature. Instead, with a few exceptions, we set our priority in discussing papers that apply relatively similar kinetic analy-
A restrained version of CFTR gating model can be seen in Vergani et al. (324). The action of ATP presumably at ABP2. ATP binding at the ABP1 may also contribute to the stability of the open state (NBD dimer) (34, 237, 360). A hydrolysis-deficient mutant E1371S are short-lived events (Cho and Hwang, unpublished observations), a stable NBD dimer formation requires the spontaneous openings are observed in a CFTR mutant whose NBD2 is completely deleted (329). Since the spontaneous openings for the ATP-independent opening event is depicted as dimerized NBDs, but there is so far no evidence for this proposition. In fact, ATP-independent, unstable-time constant increases. However, even at 2.75 mM ATP, a saturating [ATP], the mean closed time for these closed events is still ~400 ms. This is a unique feature for CFTR as a ligand-gated channel. For classical ligand-gated channels, the ligand-bound closed state has an opening rate that is more than 1,000-fold higher than that of ATP-bound CFTR. Thus for CFTR channel opening, there exists a very slow rate-limiting step following ligand binding (perhaps reflecting a large conformational change associated with opening of the gate). The expanded trace shows one closed-open-closed gating cycle. Within one single opening, flickery closings (*) with a time constant ~10 ms can be seen. These events are voltage dependent but ATP independent.

B: a simplified kinetic model for CFTR gating proposed by Zhou and Hwang (358). The two NBDs dimerize upon ATP binding in a head-to-tail configuration. The head of each NBD, containing the Walker A and Walker B motifs, is represented by a lighter color, while the tail, containing the signature sequence, is represented by a darker color. In the absence of ATP, the channel can open, but this spontaneous ATP-independent opening rate is fairly small, ~0.006 s⁻¹ in Bompadre et al. (33, 35). When the ABP1 site is occupied by ATP (C₁ state), the opening rate is not increased. However, binding of ATP to ABP2 increases the opening rate irrespectively if ABP1 is occupied or not. Once the channel is opened by ATP binding to ABP2, hydrolysis of ATP rapidly closes the channel (~2-3 s⁻¹). When ATP hydrolysis is abolished (e.g., by mutations), the nonhydrolytic closing proceeds at a slow rate (<0.01 s⁻¹). It is interesting to note that the spontaneous ATP independent open state (O₁) is unstable-time constant ~400 ms in Bompadre and co-workers (33, 35), although this closing does not involve ATP hydrolysis. For simplification, the ATP-independent opening event is depicted as dimerized NBDs, but there is so far no evidence for this proposition. In fact, ATP-independent, spontaneous openings are observed in a CFTR mutant whose NBD2 is completed deleted (329). Since the spontaneous openings for the hydrolysis-deficient mutant E1371S are short-lived events (Cho and Hwang, unpublished observations), a stable NBD dimer formation requires the action of ATP presumably at ABP2. ATP binding at the ABP1 may also contribute to the stability of the open state (NBD dimer) (34, 237, 360). A restrained version of CFTR gating model can be seen in Vergani et al. (324).

sis methods as ours. In light of these complications, we apologize in advance if certain papers are not well presented. Since there are two ATP-binding pockets (ABP1 and ABP2) in CFTR, questions relating these two binding sites to CFTR gating include the following: 1) Which ATP binding site is coupled to the ATP-dependent opening? 2) Is binding of ATP to both sites necessary to open the channel? 3) Since only ABP2 contains the catalytic glutamate for ATP hydrolysis, is the role of ATP binding at ABP1 in CFTR gating? 4) What is the functional role of the signature sequence? This last question is especially important since many CF-associated mutations are found in the signature sequence of both NBDs.

As described above, several early studies suggest a role of ATP binding at ABP1 in channel opening based on studies of mutations at the Walker A lysine residues, K464 and K1250 (42, 112, 350). This idea was first challenged by Ramjeesingh et al. (251). They showed that purified K464A-CFTR exhibits a nearly identical opening rate as wild-type channels at 1 mM ATP. Powe et al. (237) not only confirmed this result, but also showed that the relationship between [ATP] and the opening rate is not affected by the K464A mutation, thus casting serious doubt on the role of ATP binding at ABP1 in channel opening.

However, two other studies provide evidence for the essential role of ATP binding at both ABPs for channel opening (29, 324). Vergani et al. (324) reached this conclusion based on the observation that mutations of Walker A lysine (K464 and K1250) or Walker B aspartate in NBD2 (D1370) decrease the apparent affinity for ATP (cf. Ref. 17). Single-channel kinetic analysis suggested that it is the relationship between the opening rate and [ATP] that is affected by these mutations (324; cf. Ref. 237). It seems safe to assume that these mutations affect ATP-binding affinity, since crystal structures of the NBD-ATP complex in many ABC proteins indicate that these charged residues interact with MgATP. It is therefore concluded that ATP binding at either site can be made rate-limiting for channel opening (97). This then leads to the suggestion that channel opening takes place after both ABPs are occupied by ATP. Berger et al. (29) tackled the same issue with a different approach. They showed that the opening rate is diminished by introducing bulky entities into either ATP binding pockets. Since photolabeling with
8-N$_3$-ATP to either ABP is also decreased by these maneuvers, they concluded that ATP binding to both ABPs is required to open the channel. However, it is unclear if the decrease of the opening rate is due to a decrease of ATP binding affinity rather than due to a steric hindrance for NBD dimerization resulting from the introduced bulky side chains. It will be interesting to test if increasing [ATP] can restore the normal opening rate since an effect on ATP binding affinity is expected to be ameliorated by increasing [ATP].

A major disadvantage with mutating conserved Walker A and Walker B residues, particularly at the NBD2 site, is that these mutations likely affect ATP hydrolysis. For example, the hydrolysis-deficient mutant K1250A-CFTR, once opened by ATP, stays open for minutes (but cf. Refs. 42, 251). This prolonged open time exerts tremendous effects on the ATP dose-response based on macroscopic current measurement, an assay that is usually used as the first step to assess mutational effects on ATP sensitivity. To circumvent this problem, Zhou et al. (359) initiated a new approach. Instead of examining the residues that interact with the phosphate group of ATP, they focused on aromatic amino acid residues that coordinate the adenine ring of ATP (12). In the modified human NBD1 structure, an aromatic residue, W401, interacts directly with the adenine ring of ATP by a ring-ring stacking mechanism (168). A homology model of CFTR’s NBD2 based on the human CFTR’s NBD1 structure was made. Together with sequence analysis, a conserved residue Y1219 in NBD2 (thus in ABP2) was identified as the counterpart of W401 in NBD1 (or ABP1).

When Y1219 is mutated to a glycine (Y1219G), the ATP dose-response relationship shows a dramatic rightward shift with a $K_{0.5} >$ 50-fold higher than that of wild-type channels. A more conservative mutation (Y1219W), however, did not change the $K_{0.5}$ value significantly. The ATP dose-response relationships of Y1219F and Y1219I mutants lie between those of wild type and Y1219G, suggesting a correlation between changes of the ATP sensitivity and the chemical natures of the side chain at this position. Single-channel kinetic analysis indicates that the shifts of the ATP dose-response relationships in Y1219G and Y1219I mutants are mainly due to changes of the opening rate (360). These results are consistent with the idea that Y1219 mutations change the ATP binding affinity at the ABP2 and confirm the idea that ATP binding at the ABP2 plays a critical role in catalyzing channel opening (112, 237, 324). Unlike the mutation at the Walker A lysine residue, the Y1219G mutation does not affect the open-time constant significantly, suggesting that the mutation does not alter ATP hydrolysis at ABP2. This latest study also provides the first piece of evidence that ATP binding at ABP1 alone (ABP2 is empty) does not increase the opening rate. This is perhaps not surprising since the conserved pair of amino acids (R555 and T1246) important for NBD dimerization (323) is only present in ABP2, but not in ABP1. In addition, whether ATP binding at ABP1 is essential for channel opening by ATP binding at ABP2 is questioned since, unlike the Y1219G mutation, the W401G mutation in the ABP1 has little effect on the apparent affinity for ATP in both macroscopic and microscopic measurements (360).

Although the mutations that presumably decrease ATP affinity at the ABP1 (K464A and W401G) have questionable effects on the ability of ATP to increase the opening rate of CFTR, both mutants show a shortened open time, suggesting a destabilization of the open state by the mutations (237, 360). The effect of K464A on the open time was seen even in early studies (42, 251). The shortened opening bursts of K464A-CFTR, compared with wild-type channels, are visually discernable in Ramjesi-ngh et al. (251) and Powe et al. (237). In contrast, Vergani et al. (324) reported that the open time for K464A-CFTR is not significantly different from that of wild-type channels. Although it is unclear why the same mutation behaves differently in different laboratories, differences in the expression system may be partly responsible. It is, however, important to note that the K464A mutation does shorten the locked open time of hydrolysis-deficient mutants in two different studies (237, 324), suggesting that this effect on the open time is not due to a potential allosteric action of the mutations at the ABP1 on the ATP hydrolysis rate at the ABP2, which normally determines the rate of channel closing.

Based on the idea that CFTR’s open channel conformation is associated with an NBD dimer embedded with two ATP molecules (323), these results can be explained by an energetic mechanism since the ligand-binding energy will be part of the overall energetics of the open-channel conformation. Thus a reduction of the free energy of ATP binding could be reported as a decreased open-time constant as seen with the K464A and W401G mutants. If we consider ATP as a “glue” that bonds two NBDs together, mutations that loosen the strength of the interaction between the glue and NBDs could destabilize the NBD dimer. This mechanism also predicts that an ATP analog with a higher binding affinity (i.e., a “superglue”) should stabilize the open state. Zhou et al. (359) demonstrated that $N^6$-(2-phenylethyl)-ATP (or P-ATP), an $N^6$-modified ATP analog that has been shown to bind more tightly to other ATP-binding proteins than ATP (e.g., Ref. 103), activates CFTR with a 50-fold higher apparent affinity than ATP. P-ATP indeed prolongs the locked open time of WT-CFTR. P-ATP also increases the locked open time of the hydrolysis-deficient mutant E1371S-CFTR, suggesting that this effect of P-ATP is through a tighter binding. The observation that P-ATP also prolongs the locked open time of WT-CFTR with AMP-PNP is particularly interesting (359). If the conventional idea is correct that when CFTR is locked opened by the combined action of ATP
and AMP-PNP, ABP1 and ABP2 are occupied by ATP and AMP-PNP, respectively, this finding indicates that P-ATP stabilizes the open state by binding to ABP1.

The idea that ATP binding at the ABP1 stabilizes the open state probably also explains the decrease of the open time by ADP, first discovered by Weinreich et al. (333) from macroscopic relaxation analysis, and later confirmed at a single-channel level (33). Also consistent with this idea, Csanady et al. (59) reported that the removal of part of the “regulatory insertion” in NBD1 does not affect apparent $K_d$ for ATP, but significantly shortens the open time. Perhaps the function of this regulatory insertion is to provide a chemical mechanism for a tighter binding of ATP at ABP1. It should be noted that the “regulatory insertion” contains two additional aromatic amino acids (F409 and F430 in human NBD1) that interact with the adenine ring of ATP in the crystal structure of mouse NBD1 (167). Since multiple aromatic amino acids have been shown to be important in forming a high-affinity binding pocket for nucleotides (103, 133, 134) and biochemical studies demonstrated that NBD1 assumes a higher affinity for ATP than NBD2 (10), Zhou et al. (360) examined the effect of mutations and a combination of mutations at W401, F409, and F430 residues on the open state (or NBD dimer) stability under the E1371S background. The locked open time of E1371S-CFTR was shortened in a graded manner as the number of altered aromatic amino acids was increased. Interestingly, this effect of mutation on the open state can be partly compensated by using P-ATP, supporting an energetic mechanism instead of nonspecific effects on channel structures. By applying mutant cycle analyses, Zhou et al. (360) were able to show that P-ATP binds to ABP1 to prolong the open time of E1371S-CFTR. It should be noted that this latest proposition suggests a ready association/dissociation of ATP from ABP1 at room temperature where the experiments were carried out. This contradicts biochemical results suggesting that ATP is occluded for minutes in ABP1 (24). More studies are needed to elucidate the functional significance of this ATP-occluded state.

Irrespective of the duration ATP stays bound in ABP1, the consequential stabilization of the open state could provide a distinct advantage for CFTR to function as an ion channel rather than a transporter since only the open state conducts ions. On the flip side of the coin, the substrate transport rate for a transporter is decided by the turnover rate of a full hydrolysis cycle. If the aforementioned energetic mechanism is applicable to a transporter, decreasing the ATP binding affinity at the catalysis-incompetent site (i.e., the NH2-terminal NBD) is expected to increase the substrate transport rate for the transporter in the ABCC subfamily. A recent report by Yang et al. (344) demonstrated that the solute transporter rate of MRP1, a member of the ABCC subfamily, is indeed increased in mutants whose ATP affinity at NBD1 is decreased. One important functional implication of this latest biochemical result is that a complete transport cycle involves not only ATP hydrolysis at the catalysis-competent site but also ATP dissociation from the catalysis-incompetent site. If we consider the ABP2 as a catalytic site for CFTR or other members of the ABCC family, ABP1 may serve as an allosteric site that modulates the whole reaction cycle.

Altogether, these results suggest a gating model that incorporates CFTR’s two ABPs. In the absence of ATP, the opening rate of CFTR channels is extremely small (33, 35). ATP binds to the low-affinity ABP2 to increase the rate of channel opening through NBD dimerization (97, 323). For wild-type CFTR, it is the hydrolysis of the bound ATP at ABP2 that closes the channel. When ATP hydrolysis is abolished by mutations, the channel is locked in a stable open state; without the free energy from ATP hydrolysis, channel closing through thermal agitation takes a long time (Fig. 6B). Although it remains controversial whether ATP binding to ABP1 is required for channel opening by ATP, data in Zhou et al. (360) suggest that ATP binding to ABP1 alone fails to increase the opening rate. Thus the functional asymmetry of the two ATP binding sites echoes abundant evidence for a biochemical/structural asymmetry of CFTR’s two NBDs described above.

This molecular picture of how ATP binding to two functionally distinct ABPs induces NBD dimerization predicts different functional anomalies caused by disease-associated mutations at each ABP. More than 1,000 different mutations have been identified to cause CF. Relevant to the current article is the gating dysfunction caused by mutations in ABPs. Most notable are G551D and G1349D, two mutations located in the signature sequence of NBD1 (ABP2) and NBD2 (ABP1), respectively. G551D is the third most common CF mutation, with a worldwide frequency of ~3% (www.genet.sickkids.on.ca/cftr). This mutation is associated with a severe phenotype characterized by pulmonary dysfunction and pancreatic insufficiency (63). The G551D mutation results in a significantly decreased chloride current due to a drastic reduction of the channel activity (41, 73, 334, 337). On the contrary, the G1349D mutation causes a mild form of CF (271). Although some functional studies have shown that these two mutant channels exhibit low $P_o$ with altered pharmacological properties (e.g., Ref. 41), it is unclear how these two mutants respond differently to ATP to account for the phenotypical difference in patients carrying the mutated genes. Recent studies by Bompadre et al. (35) indicate that the G551D mutation completely abolishes ATP-dependent gating of CFTR (also see Ref. 329), whereas G1349D-CFTR retains some ATP dependence albeit with a $P_o$ ~10-fold lower than that of WT-CFTR. These distinct gating defects manifested in mutations at CFTR’s two signature sequences again resonate the functional asymmetry of the two ABPs in controlling ATP-dependent gating of CFTR.
G. Outstanding Issues and Future Directions

In the last few years we have witnessed rapid progress in our structural and functional understanding of CFTR and other ABC proteins. A dynamic picture of CFTR channel gating has begun to emerge. This picture addresses some critical issues in the CFTR field and lays a new foundation for future studies. At the same time, it also raises new questions. One obvious question is how ATP binding catalyzes dimer formation. For instance, after ATP binds to the Walker A and Walker B of one NBD, how is the signature sequence of the partner NBD recruited? While most of the studies of CFTR gating discussed above focus on residues in the Walker A and Walker B motifs, very few have tackled the role of signature sequences, which constitute the complementary partner to form each ABP. The physiological importance of CFTR's signature sequences is demonstrated by the fact that many disease-associated mutations are found in these regions. As discussed in section VIII, mutations at the conserved glycine residues in the two signature sequences result in distinct gating dysfunction. More thorough studies of these mutants are needed to elucidate the functional role of the signature sequences. This line of research is perhaps even more critical given that mutating the Walker A and B motifs has resulted in much conflicting data. It is interesting to note that, although the activity of G551D-CFTR is ATP independent, 2'-deoxy-ATP increases the $P_o$ of this mutant mainly by prolonging the open time (41). How and where this ATP analog works to enhance the channel activity are yet to be elucidated. Nevertheless, understanding the fundamental mechanisms responsible for the defective gating function could pave the way for development of tailored interventions in CF treatment.

Another important area of research is how the R domain interacts with NBDs to affect the dimer formation. As described above, removal of the R domain renders the channel activity phosphorylation independent, implying an inhibitory function of an unphosphorylated R domain. However, the open-time constant of the $\Delta R$-CFTR is slightly smaller than that of wild-type channels (60). Thus the interaction between NBDs and the R domain may somehow stabilize NBD dimer formation. Recent biochemical studies also demonstrated that phosphorylation promotes not only NBD dimerization (207), but also the association of the R domain with other domains of CFTR (44). In addition to the well-established activation role for PKA-dependent phosphorylation, the demonstration that some of the phosphorylation sites may inhibit CFTR activity (43, 62, 338) further complicates this already complex issue. Although early studies suggest that the more than 10 PKA consensus serines in the R domain may be functionally redundant in activating the channel (260, 284), it seems important, but quite challenging, to ask how individual phosphorylation sites modulate these interactions. A new approach to address R-domain function emerges recently. By comparing NMR spectra of the R domain in the presence and absence of NBD1, Baker et al. (22) provided evidence for interactions between NBD1 and multiple sites in the R domain with measured fractional helical propensity. This approach together with careful functional studies should propel us toward a molecular understanding of how the R domain modulates CFTR function.

A major challenge in understanding CFTR gating is to relate biochemical events in CFTR's two ABPs to gating transitions. Our discussion has been focused on relating ATP binding/hydrolysis to gating since the gating cycle ($-1$ s$^{-1}$) is compatible with the measured ATP hydrolysis rate for purified CFTR (170). However, biochemical studies with isolated NBD2 showed adenylate kinase activity (107, 253). Using a variety of pharmacological maneuvers, Randak and Welsh (254) demonstrated a correlation between adenylate kinase activity and CFTR gating function. Furthermore, contrary to previous studies showing that ADP inhibits CFTR by a competitive inhibition mechanism (17, 33, 281, 333), recent data suggest that ADP modulates CFTR function by perturbing adenylate kinase activity (255). Since most of these conclusions were made based on macroscopic measurements, microscopic kinetic studies that may directly relate adenylate kinase reactions to gating transitions are needed. Moreover, a direct demonstration of adenylate kinase activity with the whole CFTR protein seems critical at this juncture. It might be worth noting that in most of the studies mentioned here, CFTR gating was observed in excised patches with no ADP or AMP present. Under such conditions, adenylate kinase activity is hardly conceivable, suggesting that adenylate kinase activity is at least not required for channel gating.

The difficulty of relating biochemical data to functional results is amplified by the fact that these two lines of studies very often are carried out in different experimental conditions. For example, solutions with a low ionic strength, commonly used in biochemical studies for cell lysis (10), may affect gating dramatically (341). Since CFTR gating is very sensitive to temperature changes (8, 61, 204), low-temperature incubation, a common practice in biochemical experiments (9, 24, 305), could affect data interpretations. In addition, the phosphorylation status of CFTR, a known modulating factor for CFTR gating (96) and NBD dimerization (207), may not be the same in biochemical experiments as in functional studies. Nevertheless, the field will benefit tremendously from development of a readily accessible method for large-scale production of CFTR proteins suitable for both biochemical and functional investigations.

In spite of a sweeping pace toward a better understanding of the action of NBDs, CFTR's gating machinery, numerous puzzling questions remain unanswered. For ex-
ample, how is CFTR function modulated by scores of proteins shown to interact with CFTR (110, 169)? How does extracellular [Cl\textsuperscript{−}] affect CFTR gating (340)? Is gating control in native tissues different from what has been unveiled in heterologous expression systems (256, 257)? It also has been known for years that CFTR gating in intact cells is very different from that in cell-free systems such as inside-out membrane patches (119, 139, 350). Although a clear transition of CFTR gating patterns can be discerned from cell-attached to excised patches, the fundamental mechanism for this difference in gating is still unknown. Answering these questions may pose significant impacts on development of therapeutical interventions for CF patients carrying mutations that affect CFTR gating.

For conformational changes associated with CFTR gating, immediate questions include the following: Where is the channel gate? How is the signal transduced from the NBDs to the channel gate? Is this process similar to those for classical ligand-gated ion channels such as acetylcholine-gated cation channels (reviewed in Ref. 294), where many subtle conformational changes spreading from the ligand binding site to the gate? Recent studies of CFTR gating energetics indeed suggest a spreading conformational change initiated by NBD dimerization (61). By carefully examining temperature dependence of CFTR gating through hydrolytic and nonhydrolytic paths, Csanady et al. (61) discovered large enthalpy and entropy changes through hydrolytic and nonhydrolytic paths, Csanady et al. (61) discovered large enthalpy and entropy changes initiated by NBD dimerization (61). By carefully examining temperature dependence of CFTR gating through hydrolytic and nonhydrolytic paths, Csanady et al. 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By carefully examining temperature dependence of CFTR gating through hydrolytic and nonhydrolytic paths, Csanady et al. (61) discovered large enthalpy and entropy changes initiated by NBD dimerization (61). By carefully examining temperature dependence of CFTR gating through hydrolytic and nonhydrolytic paths, Csanady et al. (61) discovered large enthalpy and entropy changes initiated by NBD dimerization (61). By carefully examining temperature dependence of CFTR gating through hydrolytic and nonhydrolytic paths, Csanady et al. (61) discovered large enthalpy and entropy changes initiated by NBD dimerization (61).

**IX. EVOLUTION AND SPECIATION**

By operational definition, both CLC-0 and CFTR, two structurally unrelated proteins, are ligand-gated chloride channels. Several biophysical properties, however, distinguish these two proteins from classical ligand-gated ion channels such as acetylcholine- or GABA-gated ion channels. First, both CLC-0 and CFTR are considered “slow” channels. The maximal opening rate for CFTR (i.e., at a saturating [ATP]) is \( \sim 3 \text{s}^{-1} \), in contrast to \( \sim 2000 \text{s}^{-1} \) for liganded GABA receptor channels (106) and \( \sim 10000 \text{s}^{-1} \) for acetylcholine receptor channels (182). Even with the so-called fast-gating of CLC-0, the opening rate is on the order of \( \sim 100 \text{s}^{-1} \), whereas the time constant for the slow-gating is in tens to hundreds of seconds (50, 243). Second, even in the absence of ligand, CFTR channels have a finite significant basal activity for CLC-0. Ligand increases the gating equilibrium constant for a doubly-liganded acetylcholine receptor channel can be \( 10^7 \)-fold of that for an unliganded channel (294). Third, while traditional ligand-gated ion channels follow an allosteric mechanism with kinetic steps that are in thermodynamic equilibrium, this microscopic reversibility is violated in the gating process of CLC-0 and CFTR channels because of an input of free energy. For CLC-0, the gating cycle is coupled to the flow of Cl\textsuperscript{−} (and/or H\textsuperscript{+}). In the case of CFTR, the gating cycle is driven by ATP hydrolysis. This last biophysical property not only accounts for a strict stoichiometry of one (or two) ligand/one gating cycle, but also adequately explains why these two channels evolved not from the ion channel families, but from transporter proteins. Capitalizing on recent breakthroughs in high-resolution structure determination of transporter proteins as well as from sequence data mining in genomic research and functional insights from decades of single-channel studies, we intend to speculate daringly here on the potential evolutionary trajectory of transporter-to-channel metamorphosis.

Based on the idea that gene duplication is the major force driving evolution (228), we reason that the first primordial ABC transporter is composed of two identical NBDs and MSDs, encoded by two separate genes. Assembling two MSDs en face in the membrane and two NBDs in the juxtamembrane cytosol can generate a functional transporter with a three-dimensional structure similar to those in Figure 4. Although this ABC protein ancestor possesses two identical half-molecules (each is equipped with a transport engine), hydrolysis of one ATP is probably sufficient to complete the transport cycle, since eliminating one ATP hydrolysis site of HisP, an ABC protein with this primitive structural design, can still sustain the transport function (226).

If one hydrolysis cycle can effectively fuel the transport cycle, it is not surprising that transporters in the now ABCC subfamily emerge through “selection-directed mutagenesis” by the invisible force of nature. Members in this subfamily, including CFTR, contain only one NBD with the canonical sequences for ATP hydrolysis. Interestingly, many members of the ABCC subfamily serve as transporters that export organic anions (69). To perform
this function, the ABC proteins in this subfamily should be equipped with high-affinity anion binding site(s) facing the cytoplasmic side of the transport pathway. In addition, the “rocking banana” model (Fig. 1C) dictates that the anion-binding site needs to be sandwiched by two physical gates. Suppose one of these two gates is degraded in the process of speciation; NBD dimerization is now coupled to movement of only one gate that guards the negatively charged substrate bound in the transport pathway (which now can be called a “pore”). As described above, using organic anionic blockers as probes, numerous studies suggest the presence of an internal vestibule in the CFTR pore (reviewed by Linsdell, Ref. 178). Some results also point to a physical gate, located at the cytoplasmic end of the CFTR pore that controls the accessibility of the blocker binding site (e.g., Ref. 289). Among the numerous blockers, glibenclamide provides unique insights because both the on- and off-rates of this blocker can be measured at the single-channel level (356). The extraordinarily high voltage-dependent binding and unbinding of the gigantic glibenclamide (21.4 Å × 13.6 Å × 11.6 Å) in the CFTR pore suggest the presence of a wide and deep vestibule accessible from the cytoplasmic side of the channel (289, 356). From a teleological point of view, it seems difficult to rationalize the raison d’etre for this vestibule where many intracellular organic anions can potentially plug the pore and prevent Cl\(^{-}\) diffusion. Perhaps this functionally defined vestibule represents an evolutionary remnant.

Admittedly, this naive conjecture has a few holes. First, there is only limited evidence that CFTR can conduct bulky organic anions (180). In fact, those organic blockers are so named because they are impermeant. However, this stain can be removed by imagining a selectivity filter built at the distal end of the blocker binding site as proposed by Zhou et al. (356). Second, based on the model proposed by Locher and colleagues (128), ATP binding-induced NBD dimerization actually closes the cytoplasmic access to the transport pathway. This is in direct conflict with the fact that ATP-induced dimerization of CFTR’s NBDs opens the gate of the channel. Third, if the degraded-gate idea is correct, CFTR can no longer serve as a transporter. However, recent studies by Kogan et al. (158) suggest a possible transporter function of CFTR. There is still much to learn about the fascinating and puzzling relationship between ion channels and transporters. Solving the high-resolution crystal structure of the whole CFTR protein, though a mounting challenge at this juncture, will definitely illuminate our quest.

In the CLC family, structural data have argued that the structure of the transporter CLC-ec1 does not undergo a large conformational change when the molecule carries out its function, a situation very different from the general antiporter model. As described above, a transporter is thought to have two “gates.” The side chain of Glu\(_{\text{ex}}\) is thought to be the external gate. Where is the internal gate? The constriction of the Cl\(^{-}\) transport pathway by the side chain of S107 may serve as the internal one, but MTS modification data of Y445C suggest that this constriction does not completely obstruct the transport pathway. The concept of “gate” for ion transport is not necessarily a physical one. A “virtual” gate with an insurmountable energy barrier for ion movement may also serve the purpose. The observation that Cl\(^{-}\) occupancy at Scen is critical for H\(^{+}\) transport is suggestive. If Cl\(^{-}\) occupancy at Scen is absolutely required for the H\(^{+}\) transport, the Cl\(^{-}\)-Scen complex can serve as a dynamic internal gate. When Cl\(^{-}\) occupies at Scen, the gate is open for H\(^{+}\) transport. Without Cl\(^{-}\) occupancy at this site, H\(^{+}\) cannot travel to this site as if the gate is closed for H\(^{+}\). When Cl\(^{-}\), H\(^{+}\), and Scen form a complex, the external gate is closed. Once H\(^{+}\) travels to Glu\(_{\text{ex}}\) to open this external gate, the internal virtual gate is closed because Cl\(^{-}\) may leave Scen if Cl\(^{-}\) and H\(^{+}\) bindings are synergistic. Thus there does not exist a moment when both gates are open, an essential feature for the action of transporters.

The speculated change during evolution from primordial ABC transporter molecules to CFTR Cl\(^{-}\) channels involves significant structural alterations of the protein molecule to remove one of the two gates. However, for CLC family members, the evolution from CLC antiporter to CLC Cl\(^{-}\) channels may involve more subtle changes if the speculated CLC antiporter mechanism described above is correct. To remove one gate, uncoupling the Cl\(^{-}\) and H\(^{+}\) transport can serve the purpose. Blocking the H\(^{+}\) transport, as seen in the mutational studies of Glu\(_{\text{ex}}\) and Glu\(_{\text{in}}\), has converted the bacterial antiporter into a Cl\(^{-}\)uniporter (or Cl\(^{-}\) channel). For CLC members, the residue that corresponds to Glu-203 of CLC-ec1 (the Glu\(_{\text{in}}\)) is no longer a protonatable residue in channel members, but a glutamate residue is conserved in the members that are thought to be transporters. It seems difficult to imagine that evolution converts a transporter into an ion channel by a single amino acid mutation, but such a relation between sequence analysis and functional classification is striking. Perhaps more extensive structural alterations are involved, and more theoretical and experimental work is needed to deepen our understanding of the structural/functional relationship between ion channels and transporters in this family. The residue corresponding to Glu-203 of CLC-ec1 and its surrounding region may serve as a launch platform for exploration. Clearly, most of the speculations made above will not stand the scrutiny of future work. We believe, however, that defining what makes a channel a channel and a transporter a transporter will continue to be an interesting puzzle. As more crystal structures of both CLC and ABC proteins are solved and more functional data are gathered, a clearer picture of transporter-channel ties is expected to come to light.
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