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Current Topics

ATP Hydrolysis-Coupled Gating of CFTR Chloride Channels: Structure and Function†

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Ion channels, a major class of gateways for ion transport, are water-filled pores that extend across the cell membrane. Ions pass through ion channels at a rate that approximates diffusion following the electrochemical gradient (*1*). Thus, ion channels are passive transporters, contrary to active transporters such as Na/K pumps (ATPases) which utilize metabolic energy from ATP hydrolysis to move ions across the membrane against electrochemical gradients. The opening and closing of an ion channel is known as gating, and is regulated by specific perturbations of the membrane, such as a change in the membrane potential (voltage-gated channels), mechanical stimulation (stretch-activated channels), or the binding of a signaling molecule (ligand-gated channels). Generally speaking, ion channels do not utilize ATP hydrolysis as the energy source for ion transport. One exception is the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride channel with complex regulation (see refs *2*−*4* for extensive reviews). ATP hydrolysis is proposed to be a requisite for CFTR channel function. Nevertheless, while the CFTR may serve other functions (*5*), there is little doubt that the CFTR itself is an ion channel because, when the channel opens, chloride ions move across the CFTR pore along the electrochemical gradient. It is suggested that the opening and closing of the channel gate require an input of free energy from ATP hydrolysis. Understanding this unique gating mechanism of the CFTR is a long-time fantasy for ion channel studies.

The CFTR protein is of significant clinical importance. Abnormal function of the CFTR is associated with several disease states. Mutations that diminish the CFTR channel activity cause cystic fibrosis (CF), the most common lethal genetic disease in Caucasians (*6*). About 1 out of 2000−2500 newborns in northern Europe and the United States are affected by CF. CF is primarily a disease of the exocrine glands and is characterized by thick mucous secretions that plug airways and lead to chronic infection and destruction of the lung. Most CF patients are diagnosed with a high salt concentration in the sweat, resulting from defective Cl^- absorption by epithelial cells in sweat ducts (*7*). On the other hand, increased activity of the CFTR Cl^- channel, usually caused by bacterial toxins, results in secretory diarrhea which incapacitates millions of people each year in developing countries.

On the basis of topological analysis, the CFTR is classified as a member of the ATP-binding cassette (ABC) transporter (also known as traffic ATPase) superfamily (*8*), likely the largest family of homologous proteins. Most members in this family function as active transporters to move substrates across cell membranes, utilizing ATP hydrolysis as the energy source. Structure−function studies of the CFTR may therefore advance our understanding of other important members of the ABC transporter family, including P-glycoprotein (PGP), involved in multidrug resistance (MDR)

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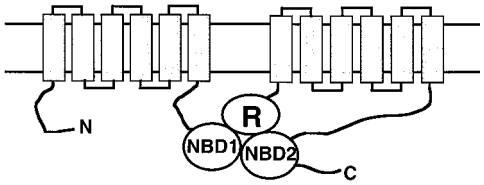

FIGURE 1: Predicted CFTR topology. N is the amino terminus, C the carboxyl terminus, NBD the nucleotide binding domain, and R the regulatory domain.

to chemotherapy for cancers.

Extensive reviews of CFTR function and pharmacology have been published recently (*2*, *3*, *9*). In the current article, we will focus on recent theories about how ATP controls CFTR gating. Specifically, we will provide a structural perspective on the ATP hydrolysis mechanism of the CFTR and compare it with ATP hydrolysis in related ABC transporters whose crystal structures have been recently determined.

Architecture of the CFTR

The human CFTR protein consists of 1480 amino acid residues with a molecular mass of ~168 kDa (*8*). It is predominantly expressed in epithelial cells of the lung, pancreas, sweat glands, salivary glands, intestine, and reproductive tract. The predicted topology of the CFTR is illustrated in Figure 1. Like other ABC transporters, the CFTR is composed of two similar halves, each consisting of a six-helical membrane-spanning domain (MSD) followed by an intracellular nucleotide binding domain (NBD). Unlike other members in the superfamily, the CFTR contains a unique cytoplasmic regulatory (R) domain.

The two MSDs are believed to form the transmembrane channel of the CFTR, and are responsible for the anion selectivity and permeation of the channel (*10*). It is believed that protein kinase A (PKA)-dependent phosphorylation of the R domain is obligatory for CFTR channel function. However, after the CFTR is activated by PKA-dependent phosphorylation, the opening and closing (i.e., gating) of the phosphorylated channel requires the presence of hydrolyzable nucleoside triphosphates (see below for details). It is also demonstrated biochemically that purified CFTR proteins can hydrolyze ATP (*11*).

The NBDs of ABC transporters, including the CFTR, are characterized by three unique consensus sequences: (1) Walker A (also termed the phosphate-binding loop or P-loop), G-X-S/T-G-X-G-K-S/T; (2) Walker B, R-X_{6−8}-h-h-h-h-D; and (3) C motif (or signature sequence), L-S-X-G-X-R/K. Here X stands for any residue, and h stands for any hydrophobic residue. The two highly conserved Walker motifs have been shown to form part of the ATP binding fold in several proteins that hydrolyze ATP or GTP (*12*). The C motif of ABC transporters is postulated to function as a transducer that transfers the free energy from ATP hydrolysis to the conformational changes in transport function (*13*). Although NBDs in the CFTR share these motifs with other members of the ABC family, the overall degree of homology of NBD sequences between the CFTR and other members of the family is low. In addition, unlike other ABC transporters of which the sequences of the two NBDs are very similar if not identical, the sequence degree of identity between the two NBDs of the CFTR is quite low (~33%), suggesting that NBD1 and NBD2 may have very distinct functions in controlling CFTR gating.

Function of the CFTR

CFTR as an Ion Channel. Because of its homology to other ABC transporters, the CFTR was first thought, as its name implies, to function as a transporter that somehow regulates Cl^- channels. Although the CFTR may function as a channel regulator (*5*), it is undisputed that the CFTR is indeed a Cl^- channel as demonstrated by several lines of evidence.

First, expression of the CFTR in a wide variety of nonepithelial cells produces Cl^- currents with properties resembling those of a class of small conductance Cl^- channels found in native epithelia (*14*−*16*). These characteristic conductance properties include the following. (1) The whole-cell currents are time- and voltage-independent. (2) The channel is anion selective, following the halide sequence ($Br^- > Cl^- > I^-$). (3) The single-channel conductance is low, in the range of 6−10 pS. (4) The channel is activated by PKA-dependent phosphorylation.

Second, mutations in the putative membrane spanning domains of the CFTR alter the anion selectivity and conductance of the expressed channel, consistent with the idea that the CFTR serves as the channel itself rather than acting as a regulator of an endogenous channel. For instance, replacing two positively charged residues with negatively charged residues in the M1 and M2 α-helices of MSD (K95D and K335E) alters the anion selectivity sequence of CFTR (*17*). Neutralizing a positively charged residue (R334W or R347P) diminishes the channel conductance (*18*).

The most compelling evidence is the demonstration that the purified CFTR protein, when reconstituted into artificial lipid bilayers, forms a PKA-activated, ATP-dependent Cl^- channel with a single-channel conductance of ~10 pS (*19*).

Functional Role of the R Domain. In cell-free systems, such as artificial lipid bilayers or excised inside-out membrane patches, the CFTR will only function as an ion channel in the presence of PKA and ATP, suggesting that PKA-dependent phosphorylation is a necessity for channel activation. In intact cells (likely also in vivo), however, the CFTR is activated by reagents that stimulate the cAMP signaling pathway (*20*). The R domain contains multiple consensus sequences for PKA-dependent phosphorylation (nine classic dibasic consensus serine or threonine residues), and biochemical studies have demonstrated that most of these dibasic consensus serine residues can be phosphorylated by PKA in vitro or in vivo. Therefore, the R domain has been the target of studies tackling the molecular mechanism of PKA-dependent activation of the CFTR. For example, partial or complete removal of the R domain renders the channel constitutively active without the need for PKA phosphorylation (*21*). Thus, the R domain appears to exert an inhibitory effect on the CFTR, and phosphorylation may release this inhibition. On the other hand, an increased degree of phosphorylation appears to be accompanied by an increase in CFTR channel activity (*22*−*24*). Interestingly, however, converting all dibasic consensus sites to alanine (including one serine at NBD1, i.e., 10SA) generates channels that still can be activated by PKA phosphorylation, suggesting that some other sites may be involved. Seibert et al. (*24*) further identified five serines that are phosphorylated but have only

one preceding basic amino acid (monobasic consensus sites). Converting those five sites to alanine under the 10SA background completely abolished PKA-dependent CFTR activity. Surprisingly, however, unlike the wild-type channel, this 15SA mutant CFTR possesses some constitutive PKA-independent channel activity. One caveat of the mutagenesis approach needs to be kept in mind. Mutations can potentially give rise to more drastic structural changes that complicate the data interpretation. Because of the complexity of the phosphorylation-dependent regulation of the CFTR, the readers are referred to recent extensive reviews for details (*3*, *24*).

Gating of CFTR by ATP Hydrolysis. Even though PKA phosphorylation of the CFTR is a prerequisite for channel activation, the phosphorylation itself is not sufficient for channel opening. This is first demonstrated by Anderson et al. (*25*) in that phosphorylated CFTR channels in membrane patches excised from transfected 3T3 and HeLa cells require the continued presence of MgATP to function. Hydrolyzable nucleoside triphosphates other than ATP, such as GTP, UTP, CTP, or AMP-CPP, can sustain the activity of the phosphorylated channel, but poorly hydrolyzable analogues such as AMP-PNP, AMP-PCP, or ATPγS, at a concentration of 1 mM, cannot open the phosphorylated CFTR. As in other enzyme-catalyzed ATP hydrolysis reactions, Mg^{2+} is a common cofactor that ATP needs to open the channel. These results were subsequently confirmed in membrane patches excised from guinea pig cardiac myocytes (*26*, *27*). Thus, it appears that ATP opens the CFTR channel not simply by binding; opening of the phosphorylated CFTR may occur after the bound ATP is hydrolyzed.

Nonhydrolyzable ATP analogues such as AMP-PNP indeed bind to CFTR, but cannot open the CFTR. Recently, two independent studies (*28*, *29*) demonstrated elegantly that in the presence of AMP-PNP, the rate of opening of the CFTR by ATP is slowed, suggesting that AMP-PNP is able to compete with ATP for a nucleotide binding site and that ATP cannot open the channel as long as this site is occupied by AMP-PNP. A second AMP-PNP binding site was elucidated by Hwang and colleagues (*30*). In the whole-cell recording mode, introducing AMP-PNP together with ATP into cells whose CFTR channels are activated by cAMP agonists increases the whole-cell macroscopic current. In this case, a simple competition between ATP and AMP-PNP for one nucleotide binding site cannot explain this result since a decrease in the CFTR current would be expected from a simple competitive inhibition. In excised inside-out patches, Hwang et al. (*30*) showed that the PKA-phosphorylated CFTR assumes a prolonged open state (also termed a "locked open" state in the literature) in the presence of a mixture of ATP and AMP-PNP. This prolonged open state can last for minutes even when all the nucleotides are removed from the bath solution. To explain this result, Hwang et al. (*30*) proposed that once the channel is opened by ATP at the first nucleotide binding site, AMP-PNP keeps the channel in the open state by binding to a second site. The slow closing of the channel after AMP-PNP occupies the second site suggests that the dissociation of AMP-PNP from the second nucleotide binding site is extremely slow. Since ATP and AMP-PNP are highly structurally similar, it is further suggested that ATP can also occupy this second site.

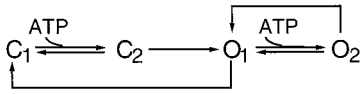

FIGURE 2: CFTR gating scheme with irreversible kinetic steps. C_1 and C_2 are closed states and O_1 and O_2 open states.

In this case, why does the channel open only briefly when ATP is bound to the second site? The hypothesis that has been advanced is that channel closing occurs after hydrolysis of ATP at the second site. Since AMP-PNP cannot be hydrolyzed, it thereby stabilizes the open state conformation. The time the channel remained locked open is then determined by the slow dissociation of the bound AMP-PNP. This interpretation is analogous to the function of the G-protein, where GTPase activity is used as a biological clock to time the duration of the active G-protein conformation. Thus, when GMP-PNP binds to the G-protein, the lifetime of the activated state is greatly prolonged.

One observation made by Hwang et al. (*30*) is an interesting functional coupling between the two nucleotide binding sites. If the interpretation that AMP-PNP dissociates slowly from the second binding site is correct, it is then expected that ATP applied shortly after exposure of the channel to a pulse of AMP-PNP should lock the channel in the prolonged open state. This is not observed. The simplest interpretation for this latter result is that the second binding site is not available to AMP-PNP when the channel is closed. Thus, AMP-PNP can only occupy the second site after ATP has opened the channel at the first site.

Thus, ATP hydrolysis at one nucleotide binding site controls the opening, whereas ATP hydrolysis at the second nucleotide binding site closes the channel. It seems logical to assume that the two nucleotide binding sites inferred from gating studies correspond to the two NBDs of the CFTR discovered through sequence analysis (Figure 1). A basic prediction is that perturbation of ATP hydrolysis at two NBDs of the CFTR should yield different effects on CFTR gating. Initial studies by Gunderson and Kopito (*31*) and Carson et al. (*32*) support the notion that NBD1 opens the channel while NBD2 closes the channel. Both groups show that mutations of the Walker A lysine residues (essential for ATP hydrolysis) in NBDs affect either the rate of opening or the rate of closing of CFTR.

Other gating models, based on a pure ligand-gated scheme, have also been proposed to explain ATP-dependent CFTR gating (*33*−*35*). These models propose that following ATP binding, the channel undergoes a conformational change to the open state. Recent demonstrations of channel opening by AMP-PNP (*36*) or ATP in the absence of Mg^{2+} (*37*) seem to support this idea (see below for details). However, it is puzzling why a high concentration of AMP-PNP (5 mM) is required to sustain CFTR activity (*36*) whereas the $K_{1/2}$ for ATP is in the micromolar range (*38*). There are also conflicting accounts about the dependence of CFTR gating on Mg^{2+} (*25*, *27*, *36*, *37*). Furthermore, Zeltwanger et al. (*38*) provided kinetic evidence for the presence of irreversible steps in the gating scheme, which can be best explained by an incorporation of energy-driven conformational changes in the gating transitions (Figure 2).

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                                                  Walker-A
S.t.HisP      1 MMSENKLHVI DLHKRYGGHE VLKGVSLQAR AGDVISIIGS SGSGKSTFLR CINFLEKPSE   60
T.l.MalK      1 ---MAGVRLV DVWKVFGEVT AVRELSLEVK DGEFMILLGP SGCGKTTTLR MIAGLEEPSR   57
CFTRNBD1    421 -TSNGDDSLF FSNFSLLGTP VLKDINFKIE RGQLLAVAGS TGAGKTSLLM MIMGELEPSE  479
CFTRNBD2   1210 ----MTVKDL TAKYTEGGNA ILENISFSIS PGQRVGLLGR TGSGKSTLLS AFLRLLN-TE 1264
                                                  #   ^^ ^ ^#

                                                Lid (Q-loop)
S.t.HisP     61 GAIIVNGQNI NLVRDKDGQL KVADKNQLRL LRTRLTMVFQ HFNLWSHMTV LENVMEAPIQ  120
T.l.MalK     58 GQIYIGDK-- -LVADPEKGI FVPPKDRD-- ----IAMVFQ SYALYPHMTV YDNIAFPLKL  108
CFTRNBD1    480 GKIKHSG--- ---------- ---------- ---RISFCSQ FSWIMPG-TI KENIIFGVSY  512
CFTRNBD2   1265 GEIQIDG--- -VSWDSITLQ Q-WRKA---- ----FGVIPQ KVFIFSG-TF RKNLDPYEQW 1310
                                                  **#      *

                                              Signature           Walker-B
S.t.HisP    121 --VLGLSKHD ARERALKYLA KVGIDERAQG KYPVHLSGGQ QQRVSIARAL AMEPDVLLFD  178
T.l.MalK    109 ---RKVPRQE IDQRVREVAE LLGLTELLN- RKPRELSGGQ RQRVALGRAI VRKPQVFLMD  164
CFTRNBD1    513 DEYRYRSVIK ACQLEEDISK FAEKDNIVLG EGGITLSGGQ RARISLARAV YKDADLYLLD  572
CFTRNBD2   1311 SDQEIWKVAD EVGLRSVIEQ FPGKLDFVLV DGGCVLSHGH KQLMCLARSV LSKAKILLLD 1370
                                                  **#* #^** ***** *  ^ ** **

S.t.HisP    179 EPTSALDPEL VGEVLRIMQQ LAE-EGKTMV VVTHEMGFAR HVSSHVIFLH QGKIEEEGDP  237
T.l.MalK    165 EPLSNLDAKL RVRMRAELKK LQRQLGVTTI YVTHDQVEAM TMGDRIAVMN RGVLQQVGSP  224
CFTRNBD1    573 SPFGYLDVLT EKEIFESCVC KLM-ANKTRI LVTSKMEHLK KA-DKILILH ------EGSS  624
CFTRNBD2   1371 EPSAHLDPVT YQIIRRTLKQ AFAD--CTVI LCEHRI---- ---EAMLECQ QFLVIEENKV 1421

S.t.HisP    238 EQVFGNPQSP RLQQFLK                                               254
T.l.MalK    225 DEVYDKPANT FVAGFIG                                              241
CFTRNBD1    625 YFYGTFSELQ NLQPDFS                                            641
CFTRNBD2   1422 RQYDSIQKLL NERSLFR                                           1438
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FIGURE 3: Sequence alignment of MalK, HisP, and the NBDs of the human CFTR. The threading alignment is slightly different from the sequence alignments in refs *43* and *45*. CF-associated mutations in the Walker A motif, the Walker B motif, the signature motif, and the Lid region are marked: *, ∧, and # stand for these mutations in NBD1 only, in NBD2 only, and in both NBDs, respectively.

Structures of the NBDs of the CFTR Based on the Crystal Structures of MalK and HisP

To better understand the mechanism of gating of the CFTR by ATP hydrolysis at the two NBDs, it would be ideal to know the atomic structures of the NBDs in the CFTR. Unfortunately, no such structural information is available for the CFTR protein. Early homologous modeling studies were carried out on the basis of adenylate kinase (*39*) or F_1-ATPase/recA (*40*, *41*), the few nucleotide binding proteins with known three-dimensional structures. Because the overall topology of adenylate kinase and F_1-ATPase is significantly different from the topology of the NBDs of the ABC transporter, the insight provided by these modeled structures is limited.

As a member of the ABC transporter superfamily, the CFTR is believed to share similar structural features in nucleotide binding domains with other members in this protein superfamily. This speculation is supported by the experimental findings of Teem et al. on CFTR−STE6 chimeras. When part of NBD1 of STE6, another member of the ABC transporter superfamily, is replaced with the analogous portion of NBD1 of the CFTR, the modified STE6 was still capable of transporting the substrate, a-factor (*42*). Therefore, before the crystal structure of CFTR can be determined, our quest for a structural mechanism of CFTR channel gating may be facilitated by the knowledge of the high-resolution structure of ABC transporters.

Recently, three crystal structures have been determined for NBDs (or ATPase domain) of the ABC transporter family. Among them, HisP is the ATP binding subunit of the histidine permease, a bacterial transporter protein responsible for the import of histidine (*43*). A preliminary description of the RbsA structure, the ATPase domain of the *Escherichia coli* ribose transporter, is published in an abstract (*44*). MalK is the ATP-hydrolyzing subunit of the maltose transport complex (*45*). Although these transporters transport different substrates, HisP and MalK appear to have almost identical backbone structures (*45*), suggesting that the fundamental construction of the ATP hydrolysis machinery is conserved.

Figure 3 shows the sequence alignment of MalK, HisP, and the NBDs of the human CFTR. It is perhaps not surprising that most of the critical regions show a high degree of sequence homology between these bacterial NBDs and those in the CFTR. The ribbon diagram of the NBD1−NBD2 complex, modeled on the basis of the crystal structure of MalK and HisP (manuscript in preparation), is shown in Figure 4.

The overall dimensions of the modeled NBD1−NBD2 complex of the CFTR are ~70 Å × 60 Å × 50 Å. As seen in Figure 4, the dimer has significantly asymmetric structures, similar to MalK dimers. The overall fold of each modeled monomer is very similar to that of MalK. Each monomer is composed of three layers (*45*). The top layer (Figure 4) is formed by an antiparallel β-sheet. The middle layer is a mixture of α- and β-type structures. The bottom layer contains mostly α-helices, facing the transmembrane domains. The top layer and the middle layer are roughly perpendicular to each other, forming the so-called L-arm (*43*) (Figure 4A, rotating NBD1 180° counterclockwise). The well-conserved Walker A and B motifs (shown in magenta and red, respectively) are located in the middle layer, surrounding the bound ATP. The signature motif (cyan) is at the bottom of the helical layer, suggesting that this conserved region may be involved in coupling the ATP hydrolysis in the NBDs to the conformational changes of the transmembrane domains. The less conserved Q-loops or "lids" (blue) are near the nucleotide binding sites and are facing each other. The two glutamine residues (Q493 in NBD1 and Q1291 in NBD2, corresponding to Q88 in MalK, not shown in Figure 4) are 2.8 Å apart, implying that they may help stabilize dimer formation. The structure of the

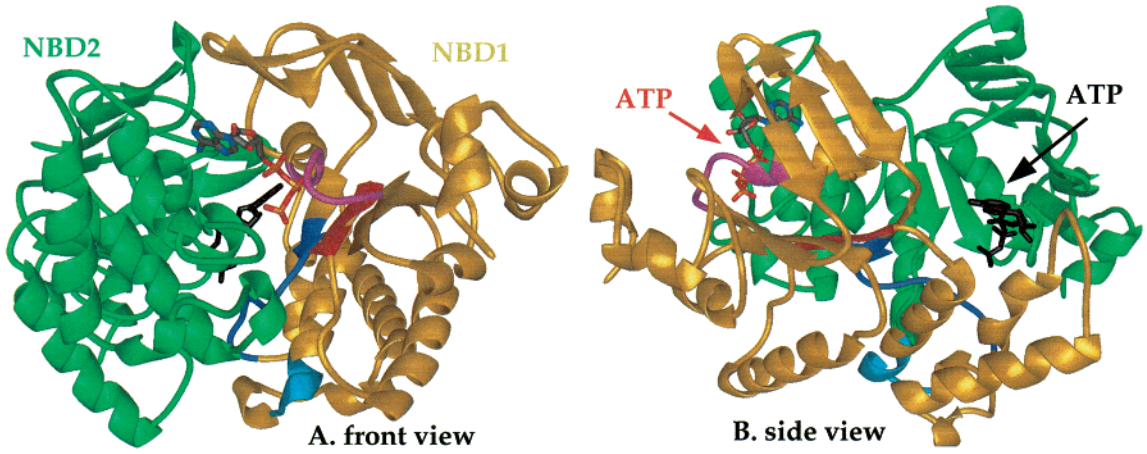

FIGURE 4: Ribbon diagram of the modeled NBDs of the CFTR with bound ATP molecules. The three-dimensional structure of the complex was built using the loop search features of SYBYL (Tripos, Inc.). (A) View of the NBD1−NBD2 dimer along an axis perpendicular to its 2-fold axis. (B) View by a 90° rotation along the 2-fold axis. NBD1 and NBD2 are shown in gold and green, respectively: magenta, Walker A motif; red, Walker B motif; cyan, signature motif; and blue, lid region. The bound ATP molecules are in stick representation. The ATP bound to NBD1 is colored by atom type, and the ATP bound to NBD2 is in black. This figure was prepared with MidasPlus.

Walker A region of CFTR NBD1 is modeled on the basis of the same region in HisP. More detailed comparisons between the structure of the modeled NBD1−NBD2 complex of the CFTR and the X-ray structure of MalK will be given in a future paper. The model will be used to understand the functional roles of critical residues in the NBDs of the CFTR at a molecular level.

Structure and Function of the NBDs of the CFTR

The importance of the NBDs in CFTR gating is reflected by numerous studies that characterize the functional perturbations generated by mutating critical amino acid residues in the consensus regions of NBDs.

Walker A Lysines (K42 in MalK, K45 in HisP, K464 in NBD1, and K1250 in NBD2). In the crystal structure of MalK and the modeled structure of NBD2 of the CFTR, both the main chain and side chain nitrogens of the conserved Walker A lysine interact with the β-phosphate of the bound ATP via hydrogen bonding. Converting lysine 1250 to alanine (K1250A) almost completely abolishes the ATPase activity (*46*). A critical role of this NBD2 lysine in CFTR gating was demonstrated by the result that the open time of the K1250A CFTR mutant is significantly prolonged from hundreds of milliseconds to minutes (*38*), an effect similar to the effect of AMP-PNP on wild-type channels (see the previous section). These observations thus support the assignment of NBD2 as the engine that closes the channel as described above.

Whether NBD1 is the site that opens the channel remains controversial. Although earlier studies showed that converting lysine 464 to alanine (K464A) slowed opening (*31*, *32*), a more recent report found that single-channel gating kinetics of the K464A mutant was almost indistinguishable from that of the wild-type CFTR (*46*−*48*). The apparent affinity for ATP is little changed for the K464A channel (*48*). Nevertheless, the ATP hydrolysis rate of the K464A mutant is decreased (*46*). Ramjeesingh and his colleagues (*46*) thus propose a loose coupling between ATP hydrolysis and CFTR gating transitions. It is worth noting that in the crystal structure of HisP, it is the main chain nitrogen rather than the side chain nitrogen of the Walker A lysine that forms a hydrogen bond with the β-phosphate of the bound ATP. Perhaps a more drastic mutation at this position is required to disrupt the interaction between ATP and the backbone nitrogen. Interestingly, the K45P, K45V, and K45N mutants of HisP are defective in nucleotide binding, ATP hydrolysis, and ligand translocation (*49*).

Kinetic studies of the K1250A CFTR channels have refined the function of NBD2. It was first suggested that hydrolysis of ATP at NBD2 plays an obligatory role in closing of the channel since the K1250A mutant CFTR exhibits a much longer open time (*31*, *32*). This model predicts that the open time of the K1250A mutant CFTR will be independent of the concentration of ATP. In other words, no matter how much ATP is used to open the channel, the channel can only close after another ATP molecule binds and is subsequently hydrolyzed at NBD2. Thus, when the hydrolysis rate is slowed by a lysine-to-alanine mutation, the open time for the mutant channel is expected to be long irrespective of the concentration of ATP. However, while the K1250A CFTR channel, once opened by a millimolar level of ATP, can remain open for minutes, the mean open time is only ~250 ms when the channel is opened by a low micromolar ATP concentration. Furthermore, this mean open time of 250 ms is very similar to the value for wild-type CFTR at a micromolar ATP concentration (*38*). Assuming that lowering the concentration of ATP only affects the probability of the occupancy of NBDs, Zeltwanger et al. (*38*) hypothesize that at low micromolar ATP concentrations, the K1250A CFTR channels close before ATP binds at NBD2. Thus, NBD1 alone is sufficient not only for opening the channel but also for closing the channel. The function of NBD2 is to prolong the lifetime of the open state (Figure 2).

Walker B Aspartates (D164 in MalK, D178 in HisP, D572 in NBD1, and D1370 in NBD2). In the crystal structures of MalK and HisP and the modeled structure of the NBDs of the CFTR, the side chain of the conserved Walker B aspartate coordinates the Mg^{2+} ion or interacts with the γ-phosphate of ATP via intermediate water molecules, similar to the

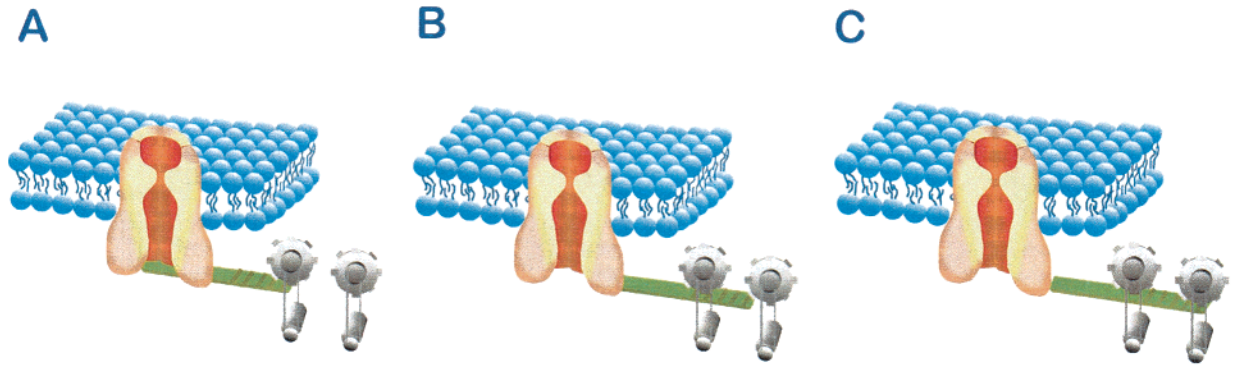

FIGURE 5: Schematic representation of CFTR channel gating. The two engines that drive individual gears represent the two NBDs of the CFTR. The gate can be opened by the action of the first engine (from A to B). Once the gate is latched on the second gear, the channel can stay open until the second engine completes its cycle (C).

function of the conserved aspartate in G-proteins. Contrary to results with mutations of the Walker A lysines in NBDs, converting Walker B aspartates to asparagines seems to yield outcomes that support the notion that NBD1 and NBD2 play distinct roles in opening and closing. Gunderson and Kopito (*31*) demonstrated that the mean open time for the D1370N mutant is longer than that of the wild-type CFTR. In a preliminary report, Vergani et al. (*47*) showed that the opening rate of the D572N mutant is reduced.

Lid Region Glutamines (Q88 in MalK, Q100 in HisP, Q493 in NBD1, and Q1291 in NBD2). There is a minor discrepancy between the crystal structures of HisP and MalK at a conserved glutamine residue (Q100 for HisP and Q88 for MalK). In HisP, this glutamine likely interacts with the γ-phosphate via a water molecule and thus was postulated to play an essential role in catalyzing ATP hydrolysis. Converting this glutamine to leucine (Q100L) eliminates transport activity of HisP while the ATP binding property is normal. However, the role of the corresponding glutamine residue in MalK is unknown. The determined structure of MalK contains pyrophosphate instead of ATP, but Q88 is close enough to interact with the putative γ-phosphate position via a water molecule. Interestingly, even relatively conserved mutations of these two glutamine residues (e.g., Q493R in NBD1 and Q1291R or Q1291H in NBD2) in the CFTR cause CF (Figure 3). Although how mutations at these positions affect CFTR function has not yet been deciphered, it is worth noting that these mutations represent very rare examples of disease-associated mutations that affect either one of the corresponding residues in NBDs.

Residues in the Signature Motif. The signature motif is unique in the ABC transporter family. This motif seems to place itself at a strategic location between the ATP binding pocket and the membrane, supporting the hypothesis that this region serves as a transducer that couples ATP hydrolysis to the transport function of membrane-spanning domains (*13*). Several disease-associated mutations have been identified in this motif (Figure 3). Among them, the glycine-to-aspartate mutation (G551D) has a worldwide frequency of ~2%. It has been shown that the G551D CFTR has a diminished ATPase activity, and the open probability of this mutant CFTR is less than 10% of that of wild-type channels (*11*). By comparing ATP hydrolysis rates and gating parameters of wild-type and G551D CFTR channels, Bear et al. (*50*) were able to confirm the proposal that ATP hydrolysis is directly coupled to the individual gating transition (*30*, *38*, *51*; cf. ref *46*). It is interesting to note that mutations of numerous residues in the signature motif of either NBD are associated with cystic fibrosis (Figure 3).

Other Residues in Walker A Motifs. The crystal structures of HisP and MalK proteins also reveal the potential role of amino acid residues other than the Walker A lysine. For example, several conserved Ser/Thr residues in the Walker A motif (S41, S46, and S47 in HisP or T43 and T44 in MalK**)** form hydrogen bonds with bound ATP (*43*, *45*). Mutations of some of the corresponding amino acids in CFTR (S466L in NBD1 or T1246I, S1251N, and T1252P in NBD2) are associated with CF (Figure 3). The main chain of G39 in HisP or G36 in MalK also forms a hydrogen bond with the side chain of the conserved lysine in the Walker A motif (*43*, *45*). Mutations of G39 in HisP cause defective nucleotide binding, ATP hydrolysis, and ligand translocation (*49*). The other two conserved glycines in the same domain (corresponding to G42 and G44 in HisP) have main chain nitrogens that form hydrogen bonds with the β-phosphate of ATP. Although most of these residues in the CFTR have been identified as disease-associated (Figure 3), the mechanism awaits future investigations. Biological assays and electrophysiological recordings are needed to determine whether the mutations cause CF by impairing ATP binding and hydrolysis or by paralyzing the traffic of the CFTR to the cell surface.

Different Models of CFTR Gating

Figure 5 illustrates schematically how two NBDs operate as two engines to control the channel gate. Unlike MDR proteins where the two NBDs appear to be structurally and functionally symmetrical (*52*), the two NBDs of the CFTR likely play distinct roles in channel gating. While it is hypothesized that the two NBDs in MDR work in parallel and alternate in controlling the transport of substrate across the membrane, the two NBDs in the CFTR may operate in series. ATP hydrolysis at NBD1 drives the conformational change that opens the gate. Although NBD1 alone can also close the gate perhaps after the hydrolytic products are released, NBD2 helps CFTR function by maintaining the gate in an open position. Hydrolysis of ATP at NBD2 terminates its function much like how GTPase activity controls the lifetime of functional G-proteins. This oversimplified model (cf. a slightly modified model in ref *53*) represents our view

of how the two NBDs control CFTR gating, but the following controversial issues await resolution.

It was hypothesized 10 years ago that ATP hydrolysis is required to open the channel because Mg is a required cofactor for CFTR activity (*25*, *27*) and because AMP-PNP fails to open the channel (*30*, *32*). Two recent reports, however, dispute this view. Ikuma and Welsh (*37*) demonstrated significant CFTR activity in the absence of free Mg in excised inside-out patches. Alexandrov et al. (*36*) showed that 5 mM AMP-PNP can yield CFTR activity that is $\sim^1/_3$ of ATP-induced activity in lipid bilayers. It was also suggested that the binding energy, rather than the free energy from ATP hydrolysis, is sufficient to overcome the activation energy for CFTR opening (*54*). Although it is difficult at this moment to reconcile these new observations with previous results, several technical factors need to be taken into account. First, Mg may not be an absolutely required cofactor for ATP hydrolysis as is usually believed (e.g., ref *36*). Other divalent cations (even monovalent cations) can substitute for Mg in catalyzing ATP hydrolysis (e.g., ref *55*). Second, a high activation energy for channel opening [~25 kcal/mol (*54*)], estimated from elegant temperature jump experiments, in fact, supports the hydrolysis hypothesis since the largest known ligand binding energy is ~18 kcal/mol [biotin-streptavidin (*56*)]. Thus, although it is conceivable that the CFTR works as a classical ligand-gated channel, more studies are needed to verify this interesting idea.

Extending the idea that ATP binding opens the CFTR channel, Ikuma and Welsh (*37*) recently proposed a model in which two NBDs are somewhat equivalent and work in parallel. Their model is based mainly on the studies of the K464A and K1250A mutants, assuming mutations of the Walker A lysines diminish the level of ATP hydrolysis at respective NBDs. Noticing that both mutants support ionic currents, Ikuma and Welsh proposed that ATP binding to either NBD rather than hydrolysis can open the channel and that either hydrolysis or dissociation of bound ATP closes the channel. On the basis of the effect of ATP on gating of the Walker A lysine mutants, it was further proposed that NBD1 has a higher affinity for ATP than NBD2. Thus, at micromolar ATP concentrations, gating is controlled mostly by NBD1, whereas NBD2 only works at a higher ATP concentration. This model explains the results showing that the K464A mutant shows a longer open time at micromolar ATP concentrations than that at millimolar ATP concentrations, whereas the K1250A mutant shows an opposite pattern of gating in response to changes in the ATP concentration (cf. ref *38*). More quantitative analysis is necessary to advance this interesting idea. For example, on the basis of the assumption of two open states in the NBD1 cycle, it is desirable to observe two distinct populations of open time for the K464A mutant especially at millimolar ATP concentrations. Like any binding hypothesis, this model needs detailed kinetic studies that have been undertaken for many ligand-gated channels (e.g., ref *57*).

All the models discussed above assume explicitly that binding or hydrolysis of one or two ATP molecules is coupled to one gating cycle. The demonstration that the K1250A mutant CFTR shows a drastically reduced ATP hydrolysis rate (*48*) is consistent with this idea of tight coupling since the duration of the gating cycle of this mutant is in the range of minutes (*38*). However, it was also demonstrated that mutations of the Walker A lysine at NBD1 (i.e., K464) have only a minimal effect on CFTR gating (*46*, *48*), yet significantly decrease the rate of ATP hydrolysis (*48*). These results prompt Ramjeesingh et al. to propose that CFTR gating transitions and ATP hydrolysis are not tightly coupled. To explain this result for the K464A mutant, one needs to speculate that hydrolysis of one ATP molecule may trigger several open−close events. It is then puzzling why the ATP hydrolysis rate and the CFTR gating cycle length are compatible for the wild-type CFTR (*11*, *50*). It is unclear what accounts for this inconsistency. Could it be that hydrolysis of ATP is coupled to CFTR gating physiologically, but gating becomes uncoupled from ATP hydrolysis under some conditions? Apparently, more studies are required to address this fundamental issue.

Concluding Marks

Since the gene was cloned in 1989, a whole decade of studies on CFTR function has advanced our understanding of how channel gating is controlled. Apparently, we are still far away from a complete understanding of how NBDs function. Additional work is needed to unveil the molecular mechanisms for how NBDs interact with each other, how the R domain controls NBDs function, and how hydrolysis or binding of ATP at NBDs affects the conformational changes of the gate. As we wait for the breakthrough in CFTR structure and function that a complete three-dimensional structure will afford us, we have to be content for the moment with hints gleaned from NBDs of sister proteins in the ABC transporter family.

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