The Two ATP Binding Sites of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Play Distinct Roles in Gating Kinetics and Energetics

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Cystic fibrosis transmembrane conductance regulator (CFTR), a member of the ABC (ATP binding cassette) transporter family, is a chloride channel whose activity is controlled by protein kinase–dependent phosphorylation. Opening and closing (gating) of the phosphorylated CFTR is coupled to ATP binding and hydrolysis at CFTR's two nucleotide binding domains (NBD1 and NBD2). Recent studies present evidence that the open channel conformation reflects a head-to-tail dimerization of CFTR's two NBDs as seen in the NBDs of other ABC transporters (Vergani et al., 2005). Whether these two ATP binding sites play an equivalent role in the dynamics of NBD dimerization, and thus in gating CFTR channels, remains unsettled. Based on the crystal structures of NBDs, sequence alignment, and homology modeling, we have identified two critical aromatic amino acids (W401 in NBD1 and Y1219 in NBD2) that coordinate the adenine ring of the bound ATP. Conversion of the W401 residue to glycine (W401G) has little effect on the sensitivity of the opening rate to [ATP], but the same mutation at the Y1219 residue dramatically lowers the apparent affinity for ATP by >50-fold, suggesting distinct roles of these two ATP binding sites in channel opening. The W401G mutation, however, shortens the open time constant. Energetic analysis of our data suggests that the free energy of ATP binding at NBD1, but not at NBD2, contributes significantly to the energetics of the open state. This kinetic and energetic asymmetry of CFTR's two NBDs suggests an asymmetric motion of the NBDs during channel gating. Opening of the channel is initiated by ATP binding at the NBD2 site, whereas separation of the NBD dimer at the NBD1 site constitutes the rate-limiting step in channel closing.

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INTRODUCTION

As a member of the ABC transporter superfamily, cystic fibrosis transmembrane conductance regulator (CFTR) has two nucleotide binding domains, NBD1 and NBD2 (Riordan et al., 1989). For CFTR to function as a chloride channel, the CFTR protein needs to be phosphorylated in the regulatory (R) domain; subsequent interactions of ATP with the two NBDs control the opening and closing of the channel (i.e., gating). Several properties of CFTR's two NBDs distinguish CFTR among members of the ABC family. First, CFTR's two NBDs exhibit only moderate sequence homology. Second, biochemical studies show that NBD2, but not NBD1, hydrolyzes ATP (Szabo et al., 1999; Aleksandrov et al., 2002; Basso et al., 2003). Third, NBD1 displays a higher ATP binding affinity than NBD2 (Szabo et al., 1999; Aleksandrov et al., 2001; Aleksandrov et al., 2002; Basso et al., 2003; Zhou et al., 2005). Despite more than a decade of study, the functional significance of this structural asymmetry of NBDs in CFTR gating remains unclear. It is generally agreed that ATP hydrolysis at NBD2 precedes channel closing since mutations (e.g., K1250A and E1371S) that abolish ATP hydrolysis at the NBD2 site drastically prolong the open time (Carson et al., 1995; Gunderson and Kopito, 1995; Zeltwanger et al., 1999; Vergani et al., 2003; Bompadre et al., 2005b). However, recent studies suggest that ATP binding at NBD1 may also modulate the closing rate (Bompadre et al., 2005a,b; Zhou et al., 2005). How ATP binding catalyzes channel opening also remains unsettled. One popular hypothesis is that ATP binding to both NBD1 and NBD2 is required for channel opening (Aleksandrov et al., 2001; Vergani et al., 2003; Berger et al., 2005; Vergani et al., 2005; for review see Gadsby et al., 2006). An alternative view is that ATP binding at NBD2 plays a major role in channel opening, whereas ATP binding at NBD1 may not be absolutely required for channel opening (Powe et al., 2002; Bompadre et al., 2005b). One probable reason for these controversies is the fact that previous studies were mostly designed with limited structural information about CFTR's NBDs. Very often, manipulations that affect ATP binding also

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CFTR, cystic fibrosis transmembrane conductance regulator; P-ATP, N^6-(2-phenylethyl)-ATP; WT, wild type.

perturb ATP hydrolysis (e.g., Gunderson and Kopito, 1995; Basso et al., 2003; Vergani et al., 2003).

Ideally, to understand the roles of CFTR's two NBDs, one needs to manipulate the ligand binding affinity at each NBD by mutating amino acid residues that interact with ATP in the binding pockets and/or by chemically engineering ATP to alter its affinity. These strategies have been successfully employed in other ATP binding proteins (e.g., Shah et al., 1997; Gillespie et al., 1999). Recent success in solving the crystal structures of CFTR's NBD1 (Lewis et al., 2004; Lewis et al., 2005) and in designing high-affinity hydrolyzable ATP analogues for CFTR gating (Zhou et al., 2005) has made structure-guided functional studies possible for the first time in the CFTR field. A good example is the utilization of homology modeling of NBDs and mutant cycle analysis to demonstrate that the opening of the CFTR channels is associated with the dimerization of CFTR's two NBDs (Vergani et al., 2005).

Capitalizing on the crystal structures of CFTR's NBD1, we made mutations of the aromatic residues that interact (or are predicted to interact) with the adenine ring of ATP in CFTR's NBD1 (tryptophan 401, i.e., W401) and NBD2 (tyrosine 1219, i.e., Y1219). Macroscopic and microscopic kinetic analyses of ATP-dependent gating reveal functional asymmetry of these two ATP binding sites. Nonconserved mutations of Y1219 cause a rightward shift of the relationship between [ATP] and the opening rate, suggesting that ATP binding at the NBD2 site plays a critical role in channel opening. Although conversion of W401 to glycine (i.e., W401G) has little effect on the sensitivity of the channel opening rate to [ATP], this mutation shortens the mean open time. The shortening of the open time by the W401G mutation is also seen with the hydrolysis-deficient mutant background (i.e., E1371S), suggesting that the effect of W401G mutation is not through a perturbation of ATP hydrolysis. Kinetic and energetic asymmetry of CFTR's two ATP binding sites will be discussed.

MATERIALS AND METHODS

Transient Expression System

Wild-type (WT) or mutant CFTR channels were expressed transiently in CHO cells. The cDNA constructs were cotransfected with pEGFP-C3 (CLONTECH Laboratories, Inc.), encoding the green fluorescent protein, using SuperFect transfection reagent (QIAGEN) according to manufacturer's instruction. Electrophysiological recordings were performed 2–7 d after the transfection.

Electrophysiological Recordings

Inside-out patch-clamp recordings were performed according to methods described previously (e.g., Zhou et al., 2002). In brief, the pipette solution (i.e., extracellular solution) contained (in mM) 140 NMDG-Cl, 2 $MgCl_2$, 5 $CaCl_2$, and 10 HEPES (pH 7.4 with NMDG). Cells were perfused with a bath solution containing (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 5 HEPES, and 20 sucrose (pH 7.4 with NaOH). After the establishment of an inside-out configuration, the patch was perfused with a standard perfusion solution (i.e., intracellular solution) containing (in mM) 150 NMDG-Cl, 2 $MgCl_2$, 10 EGTA, and 8 Tris (pH 7.4 with NMDG). Patches were held at −50 mV in all experiments. Downward deflections of the current trace represent channel opening. Currents were recorded at room temperature using an EPC10 patch-clamp amplifier (Heka Electronic). Current traces were filtered at 100 Hz with a built-in 4-pole Bessel filter and digitized online at 500 Hz.

Data Analysis

Steady-state macroscopic current amplitude, used for construction of the ATP dose–response relationship, was measured using Igor Pro program (version 4.07; Wavemetrics). Specifically, the mean current amplitude was measured as an average of all data points from ~1 min of continuous recording. The mean baseline current was measured in the same way and then subtracted. Only recordings from patches containing no more than eight channels (estimated from maximal number of simultaneous openings during at least 20 min of recording) were selected for single-channel kinetic analysis. These data were further filtered at 50 Hz and analyzed using software written by Dr. Csanády (2000). A three-state kinetic model, C↔O↔B, was adopted to extract kinetic parameters as described previously (Csanády et al., 2000; Vergani et al., 2003; Bompadre et al., 2005a). To estimate the time constant of current relaxation, a single exponential function was fitted to the data using Igor Pro program. The first 5 s of the current relaxation were excluded from curve fitting due to the limited speed of solution exchange.

All averaged data are presented as means ± SEM.; *n* represents the number of experiments. Student *t* tests were performed using SigmaPlot (version 8.0), and results were considered significant when $P < 0.05$.

Homology Modeling of Human CFTR NBD2

The modeled structure was generated by using the program MODELLER. The crystal structure of human F508A NBD1-ATP complexes (pdb code: 1xmi, chain A) was used as a template. Y1219 was aligned to W401 (also see Callebaut et al., 2004; Moran et al., 2005). The ATP molecule was included in the modeled structure.

Online Supplemental Material

The online supplemental material (text and Fig. S1) is available at http://www.jgp.org/cgi/content/full/jgp.200609622/DC1. The online supplement shows our energetic analysis of the closing rate for hydrolysis-deficient mutants.

RESULTS

The crystal structure of the NBD1 of human CFTR (Fig. 1 A) reveals that W401 forms a ring–ring stacking interaction with the adenine ring of the bound ATP (Lewis et al., 2005). Sequence analyses suggest that Y1219 at NBD2 is the equivalent amino acid residue of W401 (Fig. 1 B). The physiological importance of Y1219 in coordinating ATP is implicated by the fact that Y1219 is perfectly conserved in CFTR from 36 different species. In fact, we collected 299 sequences of the ABC transporter proteins from the gene bank; a tyrosine residue is found at the equivalent position in 86% of these proteins (the rest are phenylalanine [12%], tryptophan [1%], and histidine [1%]). This is perhaps not surprising since the importance of aromatic residues in ligand

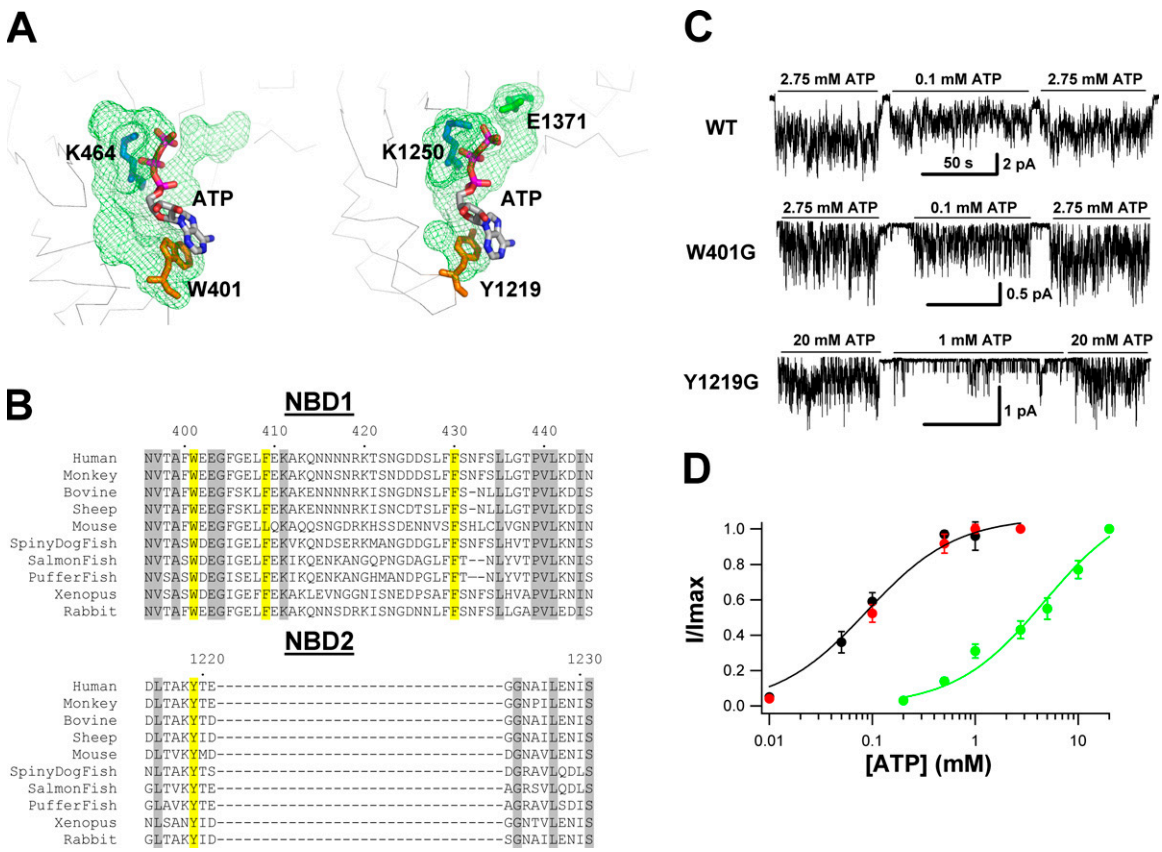

Figure 1. Tryptophan 401 and tyrosine 1219 residues interact with the adenine ring of ATP in the human CFTR NBD1 and NBD2, respectively. (A) Interactions between ATP and key amino acids in the NBD1 binding pocket, adopted from the monomeric crystal structure of the human F508A NBD1-ATP complexes (pdb code: 1xmi, chain A) (left). A similar picture of modeled NBD2 binding pocket is shown on the right. Residues of interest are represented by sticks, including those that interact with adenine ring (W401 in NBD1 and Y1219 in NBD2), the Walker A lysines (K464 in NBD1 and K1250 in NBD2), and the catalytic Walker B glutamate residue, E1371. The protein backbone atoms are plotted in thin lines and colored in gray. The figures were prepared by PYMOL. In other members of the ABC family, there is either an E or a D at the position equivalent to E1371 of CFTR NBD2, however, it is a serine in CFTR's NBD1 that is believed to be the structural basis for CFTR NBD1's inability to hydrolyze ATP (Lewis et al., 2004; Lewis et al., 2005). Since ATP is found to be associated with the Walker A and B motifs in all crystal structures of NBDs resolved so far (e.g., Hung et al., 1998; Karpowich et al., 2001; Yuan et al., 2001; Lewis et al., 2004), for the sake of clarity, we define the NBD1 ATP-binding site (or NBD1 site) as the binding pocket containing Walker A and Walker B motifs in the NBD1 sequence. An equivalent definition is applied to the NBD2 site. (B) Sequence alignment of the N-terminal part of the NBD1 and NBD2 of CFTR from 10 species (chosen randomly out of 36). Aromatic residues studied in this paper are highlighted in yellow. Conserved residues are highlighted in gray. Note that a stretch of ~30 amino acids (from 404 to 435) is present in NBD1 (i.e., regulatory insertion) but absent in NBD2 (dashed line). (C) Representative bracketed current traces at different [ATP] for constructing ATP dose–response relationships. Current induced by various [ATP] was normalized to 2.75 mM ATP in the case of WT and W401G and to 20 mM ATP in the case of Y1219G. Horizontal bars represent 50 s. (D) ATP dose–response relationships of WT (black), W401G (red), and Y1219G (green). Solid lines are Michaelis-Menten fits to the data. The $K_{1/2}$ values are 0.09 ± 0.02 mM, 0.11 ± 0.02 mM, and 4.72 ± 1.12 mM for WT, W401G, and Y1219G, respectively. Each data point represents the average from three to eight experiments.

binding has been well established in other nucleotide binding proteins (e.g., Hung et al., 1998; Gillespie et al., 1999; Hopfner et al., 2000; Huai et al., 2003, 2004; Zhang et al., 2004). Based on this sequence information, we made a homology model of CFTR's NBD2 using the coordinates of NBD1 as a template (Fig. 1 A).

To examine the role of ATP binding at CFTR's two NBDs, we converted W401 or Y1219 to glycine and examined the effect of these mutations on the apparent affinity for ATP ($K_{1/2}$). Wild-type (WT) and mutant CFTR channels in excised inside-out membrane patches were activated by the catalytic subunit of PKA plus ATP. Different concentrations of ATP were then applied to obtain a macroscopic ATP dose–response relationship. Fig. 1 C shows three representative traces. For WT channels, 0.1 mM ATP generates a CFTR activity about half of that with 2.75 mM ATP, a saturating concentration for WT CFTR gating (e.g., Zeltwanger et al., 1999). For the W401G mutant, 0.1 mM ATP elicits a similar level of activity. However, for the Y1219G mutant, 0.1 mM hardly induces any current different from the basal activity. Unlike WT and W401G, 2.75 mM ATP does not saturate

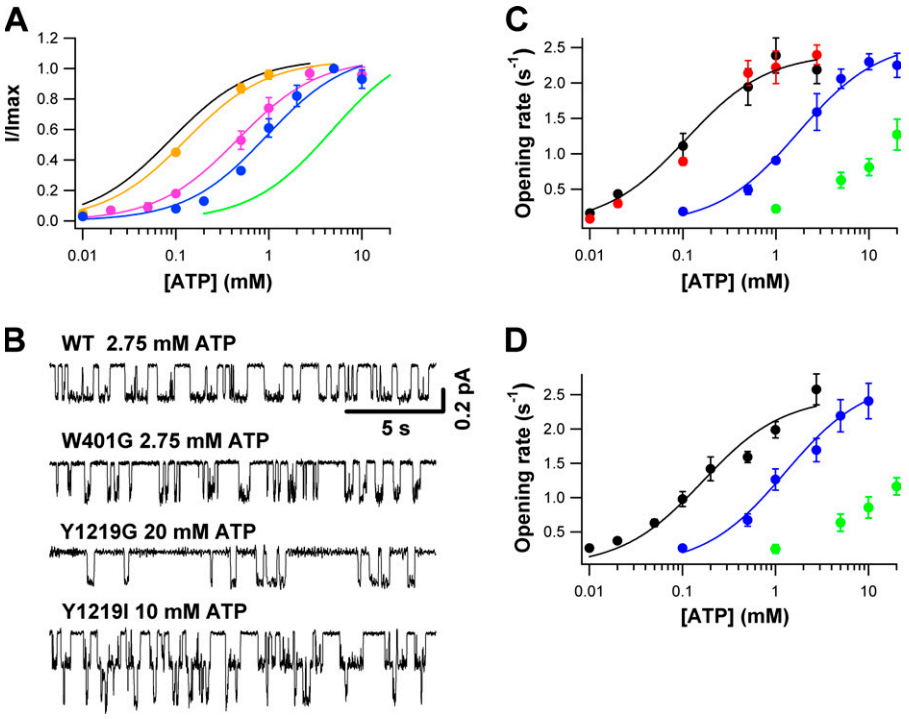

Figure 2. Effects of mutations at W401 and Y1219 on the opening rate. (A) Normalized ATP dose–response relationships of WT (black line, Michaelis-Menten fit from Fig. 1 D), Y1219W (brown), Y1219F (pink), Y1219I (blue), and Y1219G (green line, Michaelis-Menten fit from Fig. 1 D). Solid lines are the Michaelis-Menten fits to the data. $K_{1/2}$ values are 0.13 ± 0.02 mM (Y1219W), 0.46 ± 0.06 mM (Y1219F), and 0.94 ± 0.20 mM (Y1219I), respectively. (B) Representative single-channel current traces of WT, W401G, Y1219G, and Y1219I in response to [ATP] as marked. (C) Relationships between channel opening rates and [ATP] for WT (black), W401G (red), Y1219I (blue), and Y1219G (green). Solid lines are Michaelis-Menten fits to the data of WT (black) and Y1219I (blue). The maximal opening rate and $K_{1/2}$ values are 2.42 ± 0.11 s^{-1} and 0.11 ± 0.02 mM for WT, and 2.60 ± 0.11 s^{-1} and 1.73 ± 0.26 mM for Y1219I, respectively. (D) Relationships between channel opening rates and [ATP] for ΔR-CFTR (black), ΔR-Y1219I (blue), and ΔR-Y1219G (green). $K_{1/2}$ from Michaelis-Menten fits (solid lines) are 0.16 ± 0.04 mM and 1.27 ± 0.16 mM for ΔR-CFTR and ΔR-Y1219I, respectively. (Data for ΔR-CFTR were obtained from Bompadre et al., 2005a.)

the current for Y1219G (unpublished data). As seen in Fig. 1 C, 1 mM ATP, a concentration that induces ∼80% of maximal current for WT, only elicits a small fraction of current compared with that by 20 mM ATP, the highest [ATP] tested. Fig. 1 D summarizes normalized macroscopic ATP dose–response relationships of WT, W401G, and Y1219G. Although the W401G mutation does not affect the apparent affinity, converting Y1219 to glycine causes a dramatic rightward shift of the ATP dose–response curve with a $K_{1/2}$ of 4.72 ± 1.12 mM, >50-fold higher than that of WT (0.09 ± 0.02 mM).

We further characterized several mutations at the Y1219 residue (Fig. 2 A). A more conserved mutation (Y1219W) does not change the $K_{1/2}$ value significantly. The ATP dose–response relationships of Y1219F and Y1219I mutants lie between those of WT and Y1219G. These results suggest that observed changes of the ATP sensitivity are not due to a nonspecific mutational effect. Since the macroscopic ATP dose–response mostly reflects the potency of ATP in opening the channel, we hypothesize that ATP binding at the NBD2 site plays a critical role in channel opening.

To test this hypothesis, we examined single-channel kinetics of WT, W401G, and Y1219G. Fig. 2 B shows representative single-channel traces. At 2.75 mM ATP, WT and W401G channels close for hundreds of milliseconds between opening bursts. However, even at 20 mM ATP, most of the closed events for Y1219G last for several seconds. Since the opening rate of Y1219G is not saturated at 20 mM ATP, we also studied single-channel kinetics of Y1219I, which shows a smaller shift in the ATP dose–response relationship (Fig. 2 A). The relationship between the opening rate and [ATP] reaches a saturation at ∼10 mM ATP. The opening rate of Y1219I at 10 or 20 mM ATP is very similar to that of WT at 2.75 mM ATP (Fig. 2, B and C), indicating that mutations at the Y1219 residue likely affect the ATP binding step with minimal effect on the post-binding events. Fig. 2 C shows the relationship between the opening rate and [ATP] for WT, W401G, Y1219I, and Y1219G. It should be noted that these relationships virtually mirror the macroscopic ATP dose–response relationship (Fig. 2 A), consistent with the notion that the opening rate is the major determinant for the macroscopic ATP dose–response curve (Zeltwanger et al., 1999; Powe et al., 2002; Vergani et al., 2003).

It is known that the opening rate of CFTR is very sensitive to the influence of PKA-dependent phosphorylation (Mathews et al., 1998; Wang et al., 1998). Before we draw the conclusion that these mutational effects at the Y1219 residue result from a perturbation of ATP binding at the NBD2 site, we need to rule out the possibility that mutations at the Y1219 residue somehow hinder phosphorylation of the R domain, and thus the observed decrease of the opening rate is secondary to a potential mutational effect on phosphorylation. To exclude this possibility, we introduced the mutations of interest into a CFTR construct with the entire R domain

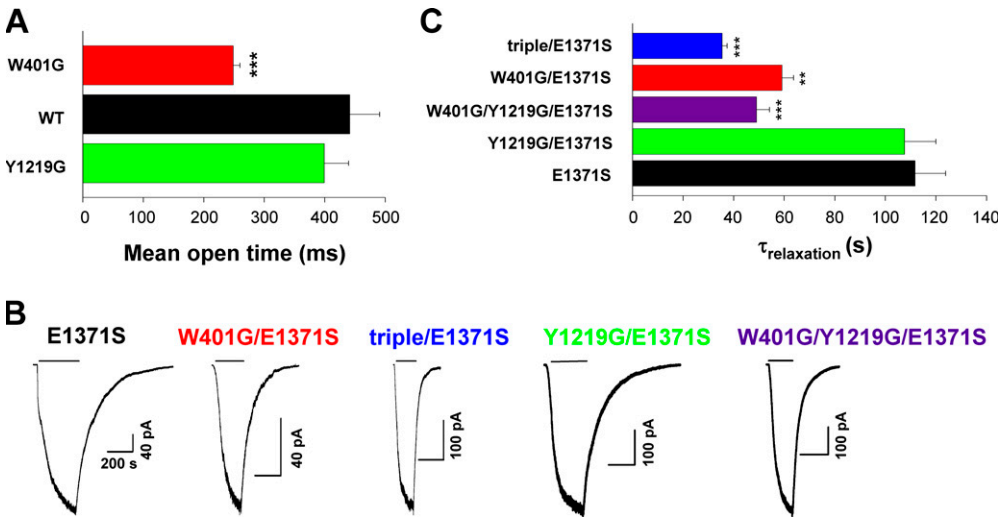

Figure 3. Effect of mutations at W401 and Y1219 residues on channel open time. (A) Mean open times of WT and W401G in the presence of 2.75 mM ATP are 441.3 ± 49.4 ms (n = 13) and 248.7 ± 11.3 ms (n = 22), respectively. The mean open time of Y1219G in the presence of 20 mM ATP is 399.1 ± 40.4 ms (n = 5). *** indicates $P < 0.001$ between WT and W401G. (B) Representative current relaxation traces upon withdrawal of 1 mM ATP plus PKA for E1371S, W401G/E1371S, triple/E1371S, Y1219G/E1371S, and W401G/Y1219G/E1371S. Solid lines above the traces indicate the duration of application of 1 mM ATP plus PKA. Horizontal scale bars represent 200 s. (C) Mean relaxation time constants upon withdrawal of 1 mM ATP plus PKA. ** indicates $P < 0.01$ and *** indicates $P < 0.001$ (compared with E1371S).

deleted (ΔR-CFTR) (Csanády et al., 2000). We have extensively characterized this phosphorylation-independent CFTR channel and shown that its ATP sensitivity and single-channel kinetic parameters are very similar to those of WT CFTR (Bompadre et al., 2005a). Fig. 2 D shows a similar rightward shift of the ATP dose–response relationships for Y1219I and Y1219G mutants under the ΔR-CFTR background. These results strongly support the hypothesis that ATP binding at the NBD2 site catalyzes channel opening.

Although mutations of the W401 residue at the NBD1 site had minimal effect on the relationship between [ATP] and the opening rate (Fig. 2, B and C), close inspection of the single-channel current trace reveals that the channel open time is shorter for the W401G mutant (Fig. 2 B). Fig. 3 A summarizes the mean open time for WT, W401G, and Y1219G. Although the Y1219G mutation causes a dramatic change of the relationship between [ATP] and the opening rate, it has negligible effect on the mean open time. In contrast, W401G exhibits ∼40% decrease of the mean open time, suggesting that mutations that decrease the ATP binding affinity at the NBD1 site destabilize the open channel conformation (i.e., increase the closing rate). It should be noted that this shortening of the mean open time by W401G mutant is not readily reflected in the ATP dose–response relationship (Fig. 1 D). Indeed, according to our simulation, if the relationship between the opening rate and [ATP] remains unchanged, a 40% shortening of the mean open time only results in ∼15% change of the apparent K_d, which is within the experimental error range (Fig. 1 D, legend).

We considered three possibilities for the shortened open time seen with the W401G mutation at NBD1. First, since it is established that ATP hydrolysis at NBD2 leads to channel closing during the ATP hydrolysis–driven gating cycle, it is possible that the W401G mutation accelerates the ATP hydrolysis rate of NBD2. Second, it has been demonstrated that the open state of the channel corresponds to a dimer conformation of NBDs (Vergani et al., 2005), and the two ATP molecules are sandwiched at the dimer interface as shown in other ABC family members (Smith et al., 2002; Chen et al., 2003). If this were the case, the binding energy of ATP could contribute to the overall stability of the dimer (or open state). It is probable then that mutating W401 in NBD1 decreases the binding energy of ATP so that the resulting dimer, thus the open state, becomes less stable. Third, the shortened open time of the W401G could be due to an allosteric effect.

To examine the potential effect of mutation in NBD1 on ATP hydrolysis at NBD2, we introduced the W401G mutation into the E1371S background, a mutant CFTR whose ATP hydrolysis is abolished (Moody et al., 2002; Tombline et al., 2004; Vergani et al., 2005). If the shortened open time seen with the W401G mutant is indeed due to an increased ATP hydrolysis rate, the effect on the open time should be at least reduced once the ATP hydrolysis is eliminated. Since the gating cycle of channels in the E1371S background is extremely long, it is technically difficult to do microscopic single-channel kinetic analysis. We, therefore, resorted to macroscopic current relaxation experiments, which has been used previously to assess the mean open time of hydrolysis-deficient CFTR channels (Vergani et al., 2003, 2005; Bompadre et al., 2005a). It should be noted that the relaxation time constant upon withdrawal of ATP is determined by the channel closing rate, opening rate, and the ATP dissociation rate if a simply ligand-gated kinetic scheme is assumed. However, since the ATP dissociation rate is much faster than the channel opening rate and closing rate, this relaxation time constant reflects

mainly the closing rate of the channel (see online supplemental material for detail, available at http://www.jgp.org/cgi/content/full/jgp.200609622/DC1). Fig. 3 B shows experiments using current relaxation analysis to estimate the open time constants for E1371S and W401G/E1371S. Our results show that the relaxation time constant for W401G/E1371S (59.1 ± 4.6 s, $n = 8$) is shortened by ~50%, compared with that of E1371S (111.7 ± 12.1 s, $n = 15$) (Fig. 3 C), suggesting that the shorter open time of W401G is not secondary to an altered ATP hydrolysis rate. In contrast, although the Y1219G mutation greatly decreases the apparent affinity of ATP (Fig. 2 C), introducing this mutation into the E1371S background has little effect on the relaxation time constant (107.6 ± 12.4 s, $n = 7$) (Fig. 3, B and C). In addition, W401G/Y1219G/E1371S has a relaxation time constant of 49.0 ± 5.3 s (Fig. 3, B and C), which is similar to that of W401G/E1371S, indicating that W401, but not Y1219, plays a dominant role in modulating the open time.

Although we cannot rule out the possibility of an allosteric effect of mutations on the stability of the open state, the following results are more in line with the idea that it is the binding energy of ATP that determines the stability of the open state. First, it has been shown that mutations at the K464 residue (Fig. 1 A) decrease ATP binding affinity at the NBD1 site (Basso et al., 2003) and shorten the open time (Carson et al., 1995; Gunderson and Kopito, 1995; Sugita et al., 1998; Powe et al., 2002; compare with Vergani et al., 2003). Second, we have recently characterized the effects of an ATP analogue, N^6-(2-phenylethyl)-ATP (denoted as P-ATP), on CFTR gating. Not only is P-ATP >50-fold more potent than ATP in opening CFTR, it also increases the open time by ~30% (Fig. 4 A; also see Zhou et al., 2005). Third, the shortened relaxation time constant due to the W401G mutation can be prolonged by P-ATP (see below).

The idea that ATP binding at the NBD1 site stabilizes the open channel conformation is also supported by the recent report by Csanády et al. (2005) An interesting feature revealed by the comparison of NBD1 and NBD2 sequences (Fig. 1 B) is that NBD1 has a "regulatory insertion" consisting of amino acids 404–435 (Lewis et al., 2004, 2005). Deletion of part of this regulatory insertion destabilizes the open state (Csanády et al., 2005). Interestingly, two additional aromatic amino acids (F409 and F430) are located in this regulatory insertion. Furthermore, the crystal structure of NBD1 from mouse CFTR shows interactions between ATP and all three amino acids including W401, F409 (leucine in the mouse NBD1), and F430. Since multiple aromatic amino acids can be used to form a high affinity nucleotide binding pocket (e.g., Gillespie et al., 1999; Huai et al., 2003, 2004), we further examine the contribution of F409 and F430 in stabilizing the open state.

We converted all three aromatic amino acids, including W401, F409, and F430 to glycine in the E1371S background and examined current relaxations of the W401G/F409G/F430G/E1371S (or triple/E1371S). Compared with the current relaxation of W401G/E1371S (Fig. 3 B), the triple/E1371S mutation further shortens the time course of current decay. Thus the relaxation time constants show a graded change as additional mutations in NBD1's ATP binding pocket were introduced (Fig. 3 C). The simplest interpretation of these results is that these three aromatic amino acids contribute to the stability of the open state by stabilizing ATP binding at the NBD1 site.

To further examine the role of ligand binding in stabilizing the open state, we considered nucleoside triphosphates that have been characterized previously, such as GTP, CTP, UTP (Zeltwanger, 1998), and P-ATP (Zhou et al., 2005). Unlike GTP, CTP, and UTP, which only change the apparent affinity modestly, P-ATP has an apparent affinity >50-fold of that for ATP. We thus consider P-ATP an ideal ATP analogue for this type of energetic analysis. If the hypothesis that ligand binding energy can contribute to the stability of the open state is correct, one would expect that P-ATP should prolong the open time constant (Fig. 4 A). Fig. 4 B shows experiments examining current relaxations upon removal of ATP or P-ATP for E1371S, W401G/E1371S, Y1219G/E1371S, and triple/E1371S. As demonstrated previously (Zhou et al., 2005), the relaxation time course upon washout of P-ATP for E1371S is approximately twofold longer than that with ATP. P-ATP also increases the relaxation time constant of Y1219G/E1371S by approximately twofold. However, this prolongation effect of P-ATP is significantly larger for W401G/E1371S (2.7-fold) and triple/E1371S (greater than fourfold; Fig. 4 C). These data suggest an energetic coupling between mutations at the NBD1 site and alterations of the bound ligand. On the other hand, mutating the NBD2 residue and changing of the binding energy of the ligand are two independent events (see online supplemental material for detailed analysis). We thus propose that ligand binding at the NBD1 site stabilizes the open state.

DISCUSSION

Gating of the CFTR chloride channel is unique in that the ligand ATP, used for opening the channel, is hydrolyzed. Studies using different mutations that perturb ATP hydrolysis (e.g., K1250A, E1371S) indicate that ATP hydrolysis drives channel closure. This input of the free energy of ATP hydrolysis into gating transitions poses great challenges in understanding CFTR's gating mechanism since it is often difficult to separate the functional roles of ATP binding and ATP hydrolysis in CFTR gating. Previous mutagenesis approaches to studying ATP-dependent gating focus on amino acid

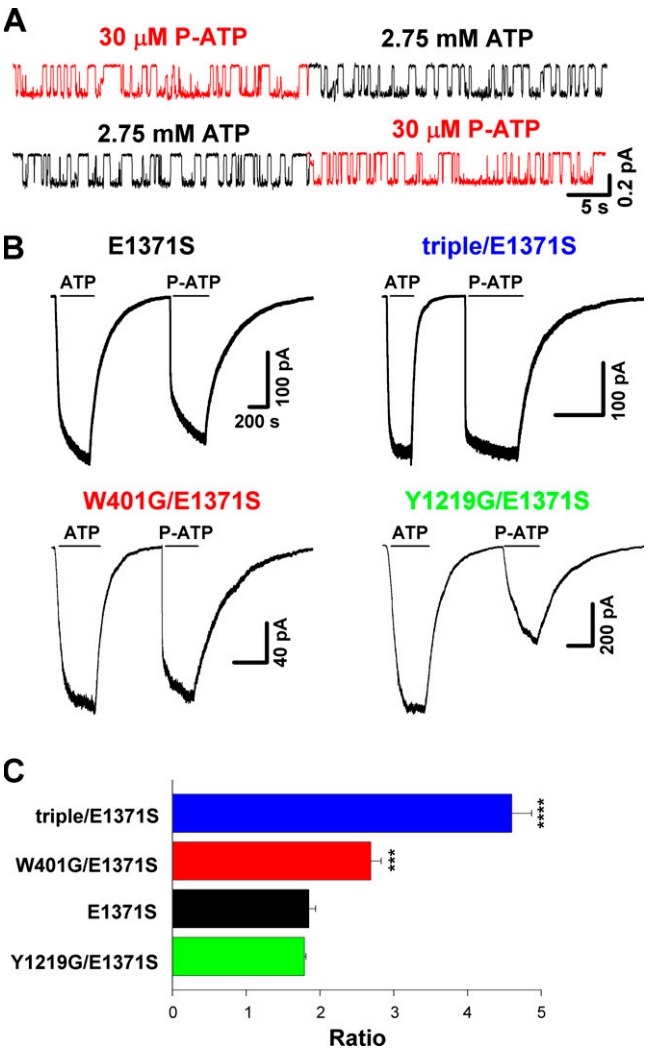

Figure 4. Effects of P-ATP on the relaxation time constant. (A) Two representative single-channel current traces of WT-CFTR in the presence of P-ATP (red) or ATP (black). The mean open time for P-ATP or ATP opened channels are 583.9 ± 60.6 ms ($n = 6$) and 441.3 ± 49.4 ms ($n = 13$), respectively. (B) Representative current relaxation traces of E1371S, W401G/E1371S, triple/E1371S, and Y1219G/E1371S after withdrawal of 1 mM ATP plus PKA or 50 μM P-ATP plus PKA. Horizontal scale bars represent 200 s. (C) The ratio of the relaxation time constant upon withdrawal of 50 μM P-ATP plus PKA to that upon withdrawal of 1 mM ATP plus PKA from the same patch was calculated for E1371S and various mutants in the E1371S background. The mean values are taken from 3–11 experiments. *** indicates $P < 0.001$ and **** indicates $P < 0.0001$ (compared with E1371S).

residues located in the well-conserved motifs (e.g., K464, K1250 in Walker A and D572, D1370, E1371 in Walker B motifs). These residues are conserved because they interact with the phosphate groups and Mg ions in the ATP binding pockets. Although these studies have provided significant insight into the role of ATP hydrolysis in CFTR gating, this kind of approach does not provide a distinct advantage in understanding the role of ATP binding since altering the ligand binding affinity with these mutations is often complicated by the mutational effect on ATP hydrolysis (e.g., K1250A in Vergani et al., 2003).

Recent successes in solving the crystal structures of CFTR's NBD1 (Lewis et al., 2004, 2005) have laid the foundation for a new era of gating studies using structure-guided mutagenesis approaches. The crystal structure of human CFTR's NBD1 reveals that the side chain of the W401 residue forms ring–ring stacking interaction with the adenine ring of the bound ATP molecule. Previous studies of other ATP binding proteins (MacLeod et al., 1998; Zhao and Chang, 2004; compare with Shyamala et al., 1991) suggest that aromatic amino acids that interact with the adenine ring of ATP may be the ideal candidates for investigating the role of ATP binding. Although the crystal structure of CFTR's NBD2 has yet to be solved, using sequence analysis and homology modeling, we identified the Y1219 residue as the equivalent amino acid at the NBD2 site that interacts with the adenine ring of ATP (Fig. 1). A model by H. Senderowitz and collaborators from Predix Pharmaceuticals also reveals the same role of Y1219 in ATP binding (personal communication). Functional studies of the Y1219 mutations indeed suggest a critical role of this residue in channel opening (Figs. 1 and 2). The Y1219I mutation lowers the sensitivity of the opening rate to [ATP] without altering the maximal opening rate (Fig. 2), indicating that this mutation indeed decreases the binding affinity for ATP at the NBD2 site. Interestingly, although the Y1219G mutation causes a drastic shift of the ATP dose–response relationship (Figs. 1 and 2), it does not affect the mean open time. If we accept the idea that the open time is determined by the rate of ATP hydrolysis (Gadsby et al., 2006), this latter result suggests that the mutations at the Y1219 residue preferentially decrease ATP binding affinity without a significant effect on ATP hydrolysis. Thus, these novel mutations investigated in this paper could be useful tools to discern the roles of ATP binding for future CFTR gating studies.

Although the crystal structure of human CFTR's NBD1 reveals the importance of W401 in ATP binding at the NBD1 site, the W401 mutation does not affect the ATP dose–response relationship, an inexact but direct way to estimate changes of ATP binding affinity. This negative result presents challenges for one to quantitatively gauge ATP–NBD1 interactions. Our energetic analysis shown in the supplemental material, albeit imperfect and with its own assumptions, provides a potential quantitative assay to assess the functional consequence of mutations that decrease ATP binding affinity at the NBD1 site. Using this analysis, we show that two additional aromatic amino acids (F409 and F430) also play a role in determining the closing rate. It should be noted that F409 and F430 are found to interact with ATP in the crystal structure of mouse NBD1, but not

human NBD1. The reason for this discrepancy is unclear. But X-ray crystallography provides only static snapshots of protein structures. Moreover, the numerous artificial mutations introduced into human CFTR's NBD1 for optimal crystallization may cause distortion of the structure. A more interesting possibility is that these minor differences in the binding pocket for the adenine ring of ATP may reflect different NBD1 structures in different functional states. Future studies of CFTR gating using these mutations may provide definitive answers.

The current studies of mutations in CFTR's ATP binding pockets suggest that the two ATP binding sites differ in their gating functions both kinetically and energetically. Since the closed state of the channel represents NBDs in the monomeric configuration (Vergani et al., 2005), the relationship between [ATP] and the opening rate reflects the ATP binding affinity at the site that controls channel opening. Our results with the Y1219 and W401 mutations (Fig. 2 C) suggest that ATP binding at the NBD2 site, but not the NBD1 site, is critical for channel opening. However, whether ATP binding at the NBD1 site is essential for channel opening (e.g., Vergani et al., 2003) remains unknown. On the other hand, although both ATP binding sites play a role in channel closing, each site utilizes ATP differently. During normal hydrolysis-driven gating, it appears that it is the ATP hydrolysis at the NBD2 site that catalyzes channel closing (Carson et al., 1995; Zeltwanger et al., 1999; Vergani et al., 2003; Bompadre et al., 2005b), whereas ATP binding at the NBD1 site modulates the closing rate by stabilizing the open channel conformation.

The kinetic and energetic asymmetry in CFTR's NBDs described above imparts several structural and biochemical implications. First, we propose an asymmetrical molecular motion of NBDs during gating. Our results suggest that channel opening is initiated by ATP binding at the NBD2 site. Indeed, the critical pair of amino acids (R555 and T1246 in Vergani et al., 2005) identified for lowering the transitional state energy for NBD dimerization is absent in the NBD1 site (T460 and L1353). Thus, it is possible that the molecular motion of NBD dimerization that leads to opening of the gate may not proceed as a simple symmetrical movement of two ATP-bound NBDs. We propose that NBD dimerization is triggered by ATP binding at the NBD2 site and subsequently progresses to close the gap at the NBD1 site. Since the open state represents a head-to-tail dimer of CFTR's two NBDs with two ATP molecules sandwiched in between, the ligand ATP now becomes part of the whole protein molecule in the open channel configuration. Thus, the ligand binding energy is expected to be part of the overall energetics of the open channel conformation. This idea predicts that manipulating the ligand binding energy with mutations or alterations of ligands can affect the stability of the open state. It seems puzzling that mutations at the NBD1 site, but not the NBD2 site, affect the open time. However, these results are consistent with the idea that separation of the NBD dimer at the NBD1 site is coupled to channel closing (compare Vergani et al., 2003). Taking one step further, we speculate that the molecular motion that couples NBD dimerization and opening of the gate resides in the NBD1 site.

Second, it is known that NBD1 does not hydrolyze ATP (Szabo et al., 1999; Aleksandrov et al., 2002; Basso et al., 2003). Unlike the NBD2 site, the ATP binding pocket at NBD1 lacks the crucial glutamate residue that serves as a catalytic base for ATP hydrolysis (Lewis et al., 2004, 2005). In addition, the Walker A lysine (K464 in human CFTR) is not perfectly conserved across species. If the hypothesis that the free energy of ATP binding at the NBD1 site is used to stabilize the open channel configuration is correct, it is perhaps not surprising that the NBD1 site is not designed to hydrolyze ATP.

Third, to harvest a high binding energy at NBD1, it is necessary to construct a binding pocket that binds ATP tightly. It is interesting to note that the two aromatic residues, F409 and F430, in the ATP binding pocket of the NBD1 site, are located in the regulatory insertion that is unique to CFTR's NBD1. The existence of multiple aromatic residues in ATP binding pockets have been reported previously in many ATP binding proteins with high affinity to nucleotides, such as phosphodiesterases (Huai et al., 2003, 2004) and myosin isozymes (Gillespie et al., 1999). It is tempting to speculate that the higher affinity for nucleotides in NBD1 compared with NBD2 (Szabo et al., 1999; Aleksandrov et al., 2001; Aleksandrov et al., 2002; Basso et al., 2003; Zhou et al., 2005) is at least partly due to the existence of multiple aromatic residues in the ATP binding pocket of NBD1.

Regardless of how NBD1 assumes a high affinity for ATP, given the fact that the intracellular [ATP] is nearly always maintained in the millimolar range, it seems puzzling why one of CFTR's two NBDs (i.e., NBD1) exhibits a higher affinity for ATP, while most of the other ABC family members have two very similar NBDs. If we accept that CFTR is evolved from a primordial ABC transporter that functions as an ATP hydrolysis–driven pump, this structural modification of CFTR's NBD1 may serve a teleological purpose. For a pump molecule to transport its substrate efficiently, it is preferable for most of the intermediate states during a transport cycle to be short-lived. This basic design principle for efficient pumps may not be ideal for a channel because only the open state of the channel conducts ions. It seems possible that the purpose of rendering NBD1 with a high affinity for ATP is to allow the channel to stay in the open state longer so that more Cl^- ions can go through the channel during each gating cycle.

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