Conformational Changes in a Pore-lining Helix Coupled to Cystic Fibrosis Transmembrane Conductance Regulator Channel Gating

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Cystic fibrosis transmembrane conductance regulator (CFTR), the protein dysfunctional in cystic fibrosis, is unique among ATP-binding cassette transporters in that it functions as an ion channel. In CFTR, ATP binding opens the channel, and its subsequent hydrolysis causes channel closure. We studied the conformational changes in the pore-lining sixth transmembrane segment upon ATP binding by measuring state-dependent changes in accessibility of substituted cysteines to methanethiosulfonate reagents. Modification rates of three residues (residues 331, 333, and 335) near the extracellular side were 10–1000-fold slower in the open state than in the closed state. Introduction of a charged residue by chemical modification at two of these positions (residues 331 and 333) affected CFTR single-channel gating. In contrast, modifications of pore-lining residues 334 and 338 were not state-dependent. Our results suggest that ATP binding induces a modest conformational change in the sixth transmembrane segment, and this conformational change is coupled to the gating mechanism that regulates ion conduction. These results may establish a structural basis of gating involving the dynamic rearrangement of transmembrane domains necessary for vectorial transport of substrates in ATP-binding cassette transporters.

ATP-binding cassette (ABC) transporters are a large family of integral membrane proteins that actively transport a broad range of substrates across cell membranes. Despite their diverse functions, they share a common basic architecture comprised of two transmembrane domains (TMDs) that function as a pathway for the permeation of substrates and two cytoplasmic nucleotide-binding domains (NBDs). The highly conserved NBDs are the molecular motors that transform the chemical potential energy of ATP into conformational changes that drive substrate molecules through the TMDs (1). Recent biochemical, structural, and genetic studies have led to a common mechanism in which ATP binding and hydrolysis induce the formation and dissociation of an NBD dimer, respectively. This regulated switch induces conformational changes in the TMDs to mediate vectorial transport of substrates across cell membranes (2–4). However, the structural bases for the propagation of conformational changes in the NBDs to the TMDs, and the conformational changes in TMDs are not well understood.

Cystic fibrosis transmembrane conductance regulator (CFTR; ATP-binding cassette transporter subfamily C member 7), the product of the cystic fibrosis gene, is unique among ABC transporters in that its TMDs provide a conductive channel for anions. Phosphorylation of serines in the regulatory domain by cAMP-dependent protein kinase activates CFTR (see Fig. 1A). Once phosphorylated, ATP-induced dimerization of NBDs opens the channel, and their subsequent dissociation upon ATP hydrolysis closes the channel (5). Despite extensive biochemical, structural, and functional studies, the nature of conformational changes in TMDs associated with CFTR channel gating remains elusive.

The structure of the pore of CFTR is poorly understood. It is not known how many of the predicted 12 transmembrane segments contribute to formation of the pore. Nevertheless, several studies have suggested that the sixth transmembrane segment (TM6) in TMD1 plays a key role in the pore structure and determining its functional properties (6–8). The positively charged residue, Arg<sup>334</sup>, in the putative outer mouth of the pore, facilitates the entry of Cl<sup>−</sup> ions into the pore (9, 10), and the side chain of Thr<sup>338</sup>, located one helical turn away, lies in the pore (11, 12). We have investigated the structure of TM6 and probed for its conformational changes during channel gating using the substituted cysteine accessibility method (13). Each residue in and flanking TM6 (amino acids 325–353) was replaced individually with cysteines, and the rates of modification by water-soluble thiol-reactive reagents were assessed in different channel gating states. State-dependent differences in the effects of Cd<sup>2+</sup> and reactivities of sulfhydryl reagents were interpreted as reflecting changes in the local environment and accessibility of the substituted cysteines to the aqueous phase. During channel opening, there is a structural rearrangement in TM6 that results in decreased accessibility of three residues near the extracellular end to the aqueous phase. This conformational change is local, because it does not affect nearby pore-lining residues, and furthermore, it is required for the channel...
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to open. These results establish a structural basis for CFTR gating involving ATP-induced conformational changes in TMDs, which may be relevant for other members of the ABC transporter family.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—Mutations were constructed in pSP-CFTR (14) plasmid containing the cDNA of human CFTR, using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Each mutation was confirmed by sequencing. Each cDNA was linearized and transcribed using a SP6 promoter based *in vitro* transcription method (Ambion, Austin, TX). For channel expression, *Xenopus* oocytes were injected with cRNA, stored at 18 °C, and used for recordings 2–5 days after injection. All of the chemicals was purchased from Sigma-Aldrich unless otherwise stated.

**Electrophysiology**—Conventional two-electrode voltage-clamp methods were used to measure membrane currents in CFTR expressing oocytes, using an OC-725C oocyte clamp amplifier (Warner Instruments) connected to a computer via an ITC-18 interface (Instrutech Corp, Elmont, NY). Single oocytes were placed in a chamber (25 μl of volume) containing LCa96 (96 mM NaCl, 1 mM KCl, 0.2 mM CaCl2, 5.8 mM MgCl2, 10 mM HEPES, pH 7.5, by NaOH) and continuously perfused at the rate of 1.5 ml/min. Pulse software (HEKA Electronics, Inc.) was used to ramp the applied transmembrane potential (∆Vm) at regular intervals. Vm was clamped at −20 mV in between the voltage ramps. Transmembrane current (Im) and Vm were digitized at 50 Hz during the voltage ramps and written directly onto hard disk. In data analysis with Igor Pro (WaveMetrics, Lake Oswego, OR) software, a fifth order polynomial was fitted to the raw, monotonically increasing signal with a resolution of 10−9 amperes and 5−10 mV data from each voltage ramp. The whole oocyte membrane conductance and the reversal potential (Vrev) were evaluated simultaneously as the slope of data from each experiment condition by TAC software (Bruxton, Seattle, WA) for ∆Pevaluation. The total number of channels in a patch was assumed to be the maximum number of open channel current levels observed over the full duration of the experiment (5–15 min). The data were fitted and modeled using Igor Pro (Wave Metrics, Lake Oswego, OR).

For kinetic analysis, the current records were filtered digitally at 50Hz and idealized using half-amplified threshold crossing, with imposition of fixed dead time of 6.5 ms. Events lists were fitted with a simple model in which all principal gating transitions were pooled into a closed-open scheme, with fickler closure were modeled as pore blockage events resulting in the three state closed-open-blocked scheme (C-O-B). Rate constants rCO, rOC, rOB, and rBO were extracted by a simultaneous fit to the dwell time histograms of all conductance levels, as described (15). The mean interburst and burst durations were then calculated as τib = 1/rCO and τb = (1/rOC)(1 + rOB/rBO), respectively. The data are presented as the means ± S.E. Student’s unpaired (2-tailed) t test was used to determine the significance (p < 0.05).

**Cysteine Modification**—Stock solutions of 100 mM MTS reagents (Toronto Research Chemicals, North York, Canada) were made, and aliquots were frozen at −80 °C. For every experiment, single aliquots were thawed and diluted in LCa96, or in LCa96 containing forskolin and IBMX to the indicated concentration and used immediately. The effects of externally applied MTSEA, MTSET, MTSES, and Cd2+ on whole oocyte conductance, and the percentage of change in conductance was calculated for each oocyte. MTS reagents were applied for 3–8 min until modification reached a steady state. The whole cell conductance was plotted as a function of cumulative time and fitted with a simple model in which all principal gating transitions were modeled as pore blockage events resulting in the three state closed-open-blocked scheme (C-O-B).

**RESULTS**

**Identification of Substituted Cysteines Accessible to Cd2+ and MTSEA**—In this study, each of 29 consecutive residues between amino acids 325 and 353 comprising extracellular loop 3 and TM6 (Fig. 1A) was substituted one at a time with cysteine (except for native Cys435), and the mutant channels were expressed in *Xenopus* oocytes. Twenty-six of the 28 mutants produced CFTR Cl− currents following activation with a mixture of forskolin (10 μM) and IBMX (1 mM). We examined the effects of two thiol-reactive agents on channel function: Cd2+, a cation capable of binding to SH groups, and MTSEA, a partially ionized primary amine. We assumed that Cd2+ only binds to

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**Note:** The above text is a natural representation of the content of the image, formatted for clarity and readability. The document is a scientific paper from the Journal of Biological Chemistry, discussing CFTR conformation changes during gating, molecular biology, and electrophysiology experiments. It includes details on experimental procedures, results, and statistical methods, along with a discussion on the implications of these findings for understanding CFTR function. The text is a comprehensive overview of the study, covering the methods used, the outcomes, and the conclusions drawn. The document is intended for readers with a background in biochemical and biophysical sciences, focusing on the molecular mechanisms of CFTR protein function and modulation.
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FIGURE 1. Membrane topology of CFTR and accessibility of residues in and flanking the sixth transmembrane helix to thiol-reactive reagents. A, schematic model of CFTR domain architecture. This model shows 12 transmembrane helices organized into two TMDs, two NBDs, and a regulatory domain (RD). The region highlighted in red indicates the putative location of the residues analyzed. B, and C, typical recording of whole cell conductance of oocytes expressing WT (8) or R334C-CFTR (C). CFTR channels were stimulated by application of IBMX/forskolin mixture (horizontal black bar). After the conductance has reached a plateau, dithiothreitol (blue bar), CdCl$_2$ (green bar), and MTSEA (red bar) were added during the indicated times. Vertical lines with double arrowheads indicate the measured changes in conductance caused by cAMP (black), Cd$^{2+}$ (green), and MTSEA (red). All of the reagents were used at 1 mM. D, summary of percent change in cAMP-activated whole cell conductance of various cysteine-substituted CFTR mutants. Both Cd$^{2+}$ and MTSEA had significant effects on the conductances of only five (I331C, L333C, R334C, K335C, and T338C) of the 26 Cys-substituted channels examined. Cd$^{2+}$ had a small but significant potentiating effect on K329C and T338C, whereas MTSEA might also modify residues partially buried in those cysteines with side chains in a water-accessible surface, whereas MTSEA might also modify residues partially buried in the membrane (16). Applications of dithiothreitol, Cd$^{2+}$, or MTSEA (all reagents at 1 mM) were without significant effect on the whole cell Cl$^{-}$ conductance of oocytes expressing wild type CFTR (Fig. 1B). The positively charged residue Arg$^{334}$ influences Cl$^{-}$ permeation properties and is therefore expected to be near the aqueous pore (9, 10). Application of Cd$^{2+}$ to activated R334C CFTR reduced whole cell Cl$^{-}$ conductance by >80%, with the inhibition completely reversible upon Cd$^{2+}$ removal (Fig. 1C). A brief application of dithiothreitol accelerated reversal associated with the removal of Cd$^{2+}$. Subsequent application of MTSEA sharply and irreversibly increased the Cl$^{-}$ conductance, confirming previous studies (10). Subsequent application of Cd$^{2+}$ was without further effect, suggesting that both MTSEA and Cd$^{2+}$ reacted with the same cysteine, viz. Cys$^{334}$, and its complete modification by MTSEA abolished the binding of Cd$^{2+}$. Representative experiments of other Cys-substituted CFTR mutants can be found in supplemental Fig. S1.

Fig. 1D summarizes the effects of external Cd$^{2+}$ and MTSEA on the whole cell conductances of various cysteine-substituted CFTR mutants. Both Cd$^{2+}$ and MTSEA had significant effects on the conductances of only five (I331C, L333C, R334C, K335C, and T338C) of the 26 Cys-substituted channels examined. Cd$^{2+}$ had a small but significant potentiating effect on K329C channels, but MTSEA, which by itself is without any significant effect, was able to abolish the potentiating effect of Cd$^{2+}$ (supplemental Fig. S2). Surprisingly, MTSEA did not have a functional effect on any residues that were not identified by Cd$^{2+}$ reactivity, even though it can permeate the membrane to reach residues on the other side of the ion permeation gate, as evidenced by its modification of K95C, a residue that is on the cytoplasmic side of the channel (17, 18). These experiments were performed on activated channels that undergo rapid transitions between the closed and open states. Thus, the observed reactivities represent composite of reactivities with closed and open channel conformations. The thiol side chains of these residues are presumably exposed to the aqueous phase during part or all of the gating cycle, and their modification by Cd$^{2+}$ and MTSEA affected either the single-channel conductance or channel gating.

Accessibility of Substituted Cysteines to MTSEA in the Inactive Closed State—To determine whether there was a state dependence to the observed reactivities, we examined the reactivity of these five residues (residues 331, 333, 334, 335, and 338) to MTSEA applied to the external side of the channel in the closed state. Before activation of CFTR by the cAMP mixture, the conductance of CFTR-expressing oocytes was very low and comparable with control water-injected oocytes. The oocytes and MTSEA (red bar) were added during the indicated times. Vertical lines with double arrowheads indicate the measured changes in conductance caused by cAMP (black), Cd$^{2+}$ (green), and MTSEA (red). All of the reagents were used at 1 mM. D, summary of percent change in cAMP-activated whole cell conductance for WT CFTR (Cys$^{334}$) and 26 Cys-substituted mutants. Negative change indicates inhibition and positive change indicates potentiation. The mean and S.D. are shown (n = 6). An asterisk indicates mutants for which the change was significantly different for both Cd$^{2+}$ (green bar) and MTSEA (red bar) from WT by one-way analysis of variance (p < 0.01).
were exposed to MTSEA (1 mM) for 3 min before cAMP stimulation, when the channels were in the inactive closed state. The MTSEA was then washed away, and the channels were subsequently activated by perfusion of the cAMP mixture. The effect of Cd$^{2+}$ on whole cell Cl$^{-}$ conductance was then assayed. We reasoned that if a cysteine side chain was not accessible to MTSEA in the inactive closed state, the activated channel would retain its sensitivity to Cd$^{2+}$, whereas if the cysteine was modified by MTSEA in the closed state, then subsequent application of Cd$^{2+}$ to activated channels would be without effect on whole cell conductance. An example of this protocol applied to R334C-CFTR expressing oocytes is shown in Fig. 2A. The normal inhibitory effect of Cd$^{2+}$ was nearly absent for channels that had been pre-exposed to MTSEA in the closed state. The magnitude of the loss of Cd$^{2+}$ sensitivity was similar to that observed for activated R334C-CFTR that had been modified by MTSEA (e.g. Fig. 1C). The results for all five Cys-substituted channels are summarized in Fig. 2B. Strikingly, for all the five Cys-substituted channels that were pre-exposed to MTSEA in the closed state, the normal inhibitory effect of Cd$^{2+}$ was either absent or substantially reduced. These results suggest that the thiol side chains of cysteines in these five positions are accessible to MTSEA in the inactive closed state.

**Functional Effects of Cd$^{2+}$ and MTSEA on Substituted Cysteines in E1371Q-CFTR**—To study the accessibility of substituted cysteines in the open state, the effects of Cd$^{2+}$ and MTSEA were examined for CFTR channels bearing the E1371Q mutation. This Glu to Gln mutation in NBD2 prevents ATP hydrolysis without affecting ATP binding and stabilizes the open state of the channel by almost 1000-fold compared with WT-CFTR (19, 20). Because the average burst duration of this mutant is 7–8 min (19), most of the channels bearing this mutation are likely to be locked in the open state for the entire duration of the experiment (3–5 min). Hence, the observed effects of Cd$^{2+}$ and MTS reagents, on E1371Q-CFTR represent effects on open channel state. As shown in Fig. 3A, the open probability ($P_o$) of wild type CFTR channels was about 0.2, whereas it was nearly 1 for E1371Q-CFTR. The open probability of various mutant channels in 1371E (WT), and in Gln$^{1371}$ background can be found in supplemental Fig. S3. Depending on the position of the introduced cysteine, dramatic differences in the magnitude of the functional effects between Glu$^{1371}$ (WT) and Gln$^{1371}$ channels were observed. For example, whereas L333C in the Glu$^{1371}$ (WT) channel was inhibited by either Cd$^{2+}$ or MTSEA, neither reagent was particularly effective when this mutation was present in the Gln$^{1371}$ background.
MTSEA had a small potentiating effect on K335C in the wild type channel background, whereas it was inhibitory in the "locked open" 1371Q channels (Fig. 3). In contrast, the pore-lining residues R334C and T338C exhibited very small or no differences in their functional effects in the Glu1371 and Gln1371 channels, respectively. The differences between Glu1371 and Gln1371 backgrounds in the effects of Cd^{2+} and MTSEA on I331C, L333C, R334C, K335C, and T338C channels are summarized in Fig. 3 (C and E), respectively. The differences in the magnitude of MTS modification suggest that in E1371Q background there is a significant change in TM6 structure. This structural change, which manifests as a change in the efficacy of MTS modification, affects some residues but has no effect on nearby pore-lining residues.

**Effect of E1371Q Mutation on Thiol Modification Rates**—To obtain quantitative information about changes in reactivity, the rates of substituted cysteine modification were calculated by fitting the time course of Cl− conductance modification with a single exponential, which was then used to calculate second order reaction rate constants for various MTS reagents. This analysis was carried out in both Glu1371 (WT) and Gln1371 backgrounds (Fig. 4A; summarized in Fig. 4 (B and C); MTSET data can be found in supplemental Fig. S4). The cysteine residues R334C and T338C, postulated to be pore-lining residues, showed no changes in their rates of modification by either MTSEA or MTSES. In contrast, I331C, L333C, and K335C reacted faster in the Glu1371 background (Fig. 4, B and C). These results reveal clearly that modification of I331C, L333C, and K335C by both these reagents was much slower in the Gln1371 mutational background than in the WT Glu1371 channels. The modification rates of both positively and negatively charged reagents were reduced, indicating that the slower reactivities were due to steric effects rather than electrostatic ones. The difference in reaction rates between WT and Gln1371 channels was greatest for K335C, which reacted nearly 800 times more slowly in E1371Q background. L333C showed a relatively smaller 10-fold

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**FIGURE 4.** MTSEA, and MTSES modification rates of residues in TM6 in WT and E1371Q backgrounds. A, representative experiments showing time-dependent changes in whole cell conductance caused by MTSEA (left panels) and MTSES (right panels) modification of various Cys-substituted CFTR channels (top to bottom) in Glu1371 (blue) and Gln1371 (red) backgrounds. For each experiment, the MTS reagents were used at the indicated concentrations. B and C, comparison of MTSEA (B) and MTSES (C) modification rate constants in 1371E (blue) and 1371Q (red) backgrounds for various Cys-substituted CFTR channels. The mean (±S.E.; n ≥ 6) rate constants for both closed (square) and open (circle) states are plotted on log scale for each mutant. The length of the black bar connecting the symbol shows fold change in the rates between the open and closed conformation states.

(Fig. 3B). MTSEA had a small potentiating effect on K335C in the wild type channel background, whereas it was inhibitory in the “locked open” 1371Q channels (Fig. 3D). In contrast, the...
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FIGURE 5. Effects of MTSEA, and MTSES depend on CFTR activation levels. A, typical recording of whole cell conductance of oocytes expressing K335C-CFTR. CFTR channels were stimulated by application of forskolin/IBMX (0.02 mM; black bar) followed by forskolin/IBMX (1 mM; gray bar). B, change in the whole cell conductance of oocytes stimulated by 0.02 mM IBMX expressed as a fraction of conductance change elicited by 1 mM IBMX. C, the percentage of change in whole cell conductance affected by MTSEA (left panel) and MTSES (right panel) in oocytes stimulated by 0.02 mM IBMX (black) and 1 mM IBMX (gray) are plotted for each Cys-substituted CFTR (mean ± S.E.; n ≥ 4). For each residue, an asterisk indicates a significant difference.

The state-dependent reactivity of the MTS reagents with I331C, L333C, and K335C channels could indicate a change in the water accessibility of these residues caused by a conformational change in TM6 or by an alteration in the local environment surrounding these residues. We reasoned that if movement of TM6 residues from a hydrophilic to a less hydrophilic environment was coupled to channel opening, then introduction of a charged residue might interfere with such movements and therefore affect channel opening rate. We therefore investigated the effects of MTS modification on CFTR channel gating. We used the bulky, positively charged MTSET to maximize possible functional effects of modification on gating and to limit the possibility that the reagent might cross the membrane. For two of the three mutants, I331C and L333C, modification with MTSET profoundly affected channel gating (Fig. 7A). The open probabilities of MTSET-modified I331C and L333C channels were significantly smaller than those of unmodified channels. However, the single channel conductance was not affected in either channel (data not shown). In contrast, MTSET was without significant effects in both WT and K335C channels on either P_o or single channel conductance. These results indicate that MTSET reduces the whole cell conductance of I331C- and L333C-expressing oocytes by inhibiting channel gating and not by affecting channel permeation properties. Kinetic analyses of channel gating revealed that the decrease in open probability of MTSET-modified I331C and L333C channels was primarily because of an increase in the mean interburst duration of the
channels (Fig. 7B) with comparatively minimal influence on the open time. The deposition of a charge by MTSET modification at position 331 or 333 decreased the channel opening rate, consistent with the possibility that channel opening involves movement of these residues from an aqueous to a hydrophobic environment. The slowing of channel opening rate could be explained if the positive charge at position 331 or 333 helps stabilize the channel-closed state (aqueous environment) and destabilize the transition state (hydrophobic environment) for the channel opening reaction leading to an increase in activation energy for channel opening, hence decreasing the opening rate.

**DISCUSSION**

In this study, we investigated the architecture and rearrangement of the TM6 helix of CFTR. We examined the state-dependent reactivity of each residue with MTS reagents. We also studied how gating and permeation properties of CFTR were affected by site-specific modifications. Finally, we interpret our results in terms of the known structures of ABC transporters.

The substituted cysteine-accessibility method assumes that only cysteine residues at a water-accessible surface of the protein will react with hydrophilic MTS reagents and that the modification produces an irreversible change in channel function. However, it is possible that water-accessible cysteine residues will not react with MTS reagents because of an unfavorable electrostatic environment rather than a lack of accessibility (22). Moreover, modification of cysteine residues that fail to alter channel function will be undetected. We believe that K329C, whose whole cell conductance was stimulated by Cd^{2+}, is an example of one such residue that reacts with MTSEA, but the modification is without effect on channel function (supplemental Fig. S2). In our study, we identified a cluster of five residues near the extracellular end of TM6 that were accessible to MTS reagents. Our results have similarities and differences with a previous substituted cysteine accessibility study of CFTR (17). Although both studies identified I331C, L333C, R334C, and K335C as accessible to MTS reagents, we find that MTSEA increased the conductance of R334C- and K335C-expressing oocytes, whereas it was reported in the previous study to decrease channel currents. That study did not observe any effects of MTSEA on T338C channels, whereas we did here. Finally, the MTSEA reactivity was restricted to only five of twenty-six residues in and flanking TM6 in our study, whereas in the earlier study, residues F337C, S341C, I344C, R347C, T351C, R352C, and Q353C were also shown to be accessible to MTS reagents. The reasons for these discrepancies are unclear. However, our observations on the accessibility of R334C, K335C, and T338C and the inaccessibility of R347C are consistent with other studies (10, 11). Furthermore, we observed similar reactivities of these substituted cysteines in a Cys-less CFTR background, suggesting that the introduced cysteine is the site of modification (supplemental Fig. S5).

**State-dependent Reactivity Reflects a Local Rearrangement and Exposure of the Side Chains**—We observed large differences in the rates of cysteine modification at three (residues 331, 333, and 335) of the five residues in the E1371Q background. It is possible that this mutation rather than the open
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FIGURE 7. Effects of MTSET modification on CFTR channel gating. A, representative single-channel current traces of WT and various Cys-substituted CFTR channels that were either not treated (−MTSET) or treated (+MTSET) with 1 mM MTSET. The arrows indicate baseline current level, and the numbers indicate the number of open levels. B, summary of effects of MTSET on gating of WT and various Cys-substituted CFTR channels. The open probability and mean burst and interburst times (means ± S.E.) for I331C, L333C, K335C, and WT channels in untreated (−MTSET) and MTSET-treated (+MTSET) conditions are shown. The number of patches analyzed is given next to the bar. Significant differences are indicated by an asterisk.

state has altered the conformation of the thiol side chains to affect their reactivity. Furthermore, when channel $P_o$ was modulated independently by varying IBMX concentrations, only two residues, 331 and 333, were affected in their reactivity. Therefore, we cannot exclude the possibility that the combination of these two mutations, K335C and E1371Q is responsible for the observed changes in reactivity of K335C.

We propose that the large differences in reaction rates observed between the closed and open channel states result from conformation-dependent changes in the position of the thiol side chains of the substituted cysteines. In the closed channel state, the side chains of residues 331 and 333 are exposed to the aqueous environment that enables a fast reaction with modifying reagents; upon channel opening, these side chains are hidden from the aqueous phase and hence react poorly with the thiol specific reagents. Alternatively, the change in reaction rates associated with channel opening might be caused by a change in the $pK_a$ of the thiol side chains, which decreases the concentration of the reactive thiolate anion (23). If this were the case, then the reactivities with positively charged (SEA and SET) and negatively charged (MTSES) MTS reagents would be affected quite differently (11). However, we observed that all of these reagents reacted faster in the closed state. Thus, state-dependent changes in the $pK_a$ of these residues are unlikely to account for the observed changes in reactivities during gating. Furthermore, the pore-lining residues R334C and T338C showed no state-dependent changes in reactivity, which also suggests that there are no significant changes in the local electrostatic potential during channel gating. It must be pointed out that under low IBMX concentrations, a 5-fold decrease in CFTR $P_o$ cannot account for the entire differences in reactivity of I331C and L333C. High concentrations of IBMX ($K_i = 10^{-10}$ mM) are known to have an inhibitory effect on CFTR (24), most probably by decreasing its single-channel conductance (25). Hence, a small fraction of the increased reactivity of I331C, and L333C at low IBMX concentrations could be due to a relief from this block, although such an increase in reactivity is not observed for R334C and T338C. In addition, under low IBMX concentrations, CFTR is probably partially phosphorylated, resulting in decreased channel activity. Phosphorylation-dependent changes in CFTR channel structure could also account for some of the changes in reactivity.

Zhang et al. (26) reported that the rate of MTSET modification of R334C-CFTR expressed in oocytes was monotonic in the WT background but followed a bi-exponential additional slower (nearly 20 times slower) component in the K1250A background. The authors suggested that the slower reactivity in the open state that is stabilized in the K1250A mutant channel was due to changes in the local electrostatic environment (see above). In contrast, our results show that the reactivity of R334C does not exhibit state dependence either under varying activation levels or in the E1371Q channel. The reasons for this disparity are not due to differences in recording and perfusion techniques (patch clamp versus two-electrode voltage clamp; fast-perfusion of patches versus perfusion of whole oocytes), because similar rate constants for MTSET modification ($10^{-10}$ M$^{-1}$ s$^{-1}$) were observed in both studies. A notable difference between the two mutations is that the Walker A mutation K1250A, unlike E1371Q, decreases the ATP binding affinity of NBD2 (27), which profoundly reduces the channel opening rate (28, 29) in addition to decreasing the closing rate (30) of CFTR. Hence, the slower modification rate observed in the earlier study (26) may be specific to K1250A and not a general characteristic of the open state.

The Conformational Change at the Outer Mouth Is Local and Is Coupled to the Gating Mechanism—Channel opening appears to be associated with a conformational change near the extracellular end of TM6 that moves the exposed side chains of three amino acids away from the aqueous phase. However, these conformational changes are local, because residues 331, 333, and 335 undergo changes in MTS accessibility, whereas residues 334 and 338 do not. Therefore, we propose that the conformational change in TM6 associated with channel open-
ing is localized to the stretch of amino acids between Lys\textsuperscript{329} and Lys\textsuperscript{335}.

What is the significance of the apparent change in conformation during channel gating? Does it represent the movement of the actual gate that opens the channel, or does it represent a local conformational change that is coupled to channel gating but not causally involved in channel opening? Because all of these five residues are accessible from the extracellular side in the closed state, these residues may be more peripheral to the gate that physically restricts access to the intracellular side. The precise identity and motion of the intracellular gate is unknown, but MTS reagents and Cd\textsuperscript{2+} binding to introduced cysteines either at residues Ile\textsuperscript{331} or Leu\textsuperscript{333} interfered with its motion, as evidenced by reduced channel opening rate. We suspect that restricting the movement of these two residues prevents the gate from opening, either by interfering with its motion directly or by stabilizing the closed state allosterically. Residues Ile\textsuperscript{331} and Leu\textsuperscript{333} may lie on the moving part of the gate or alternatively be contained in a region involved in a multi-step gating reaction that occurs before the movement of the gate. In contrast, the observed conformational change at residue Lys\textsuperscript{335} does not appear to be tightly coupled to the gate, because deposition of positive charge there had minimal effects on gating. Nevertheless, Lys\textsuperscript{335} is near the conduction pathway and can influence Cl\textsuperscript{−} permeation via electrostatic effects, because the addition of negative charge (via MTSES\textsuperscript{−}) to this position inhibited channel conductance. The results from anion substitution experiments also support a similar conclusion (12, 31).

**Structural and Functional Implications**—Thermodynamic analysis of CFTR channel opening suggests that the transition state for channel opening is represented by a strained molecule in which the NBD dimers have already formed but the pore remains closed (32). The movement of hydrophobic side chains of Ile\textsuperscript{331} and Leu\textsuperscript{333} in to a hydrophobic environment might provide the required relief to open the channel. The observed differences in reactivities between the closed and open state conformations were restricted to a small stretch of amino acids near the extracellular end of TM6. Importantly, we observed no pattern of reactivity in the channel open state that was complementary to the pattern seen in the closed state. A complementary pattern might be expected if the closed to open state transition involved a rigid body rotation of TM6 around its long axis, thereby exposing the face of the helix buried in the closed state to the aqueous phase in the open conformation. In P-glycoprotein, large helical rotations were suggested to account for the extensive rearrangements of transmembrane helices observed upon ATP binding (33, 34). Instead, our results imply that relatively modest changes in the overall architecture of the channel may be associated with the transition between closed and open states of CFTR. Structural dynamics studies of MsbA during ATP hydrolysis have also ruled out large rigid helix rotations; instead, large conformational changes were restricted to extracellular and intracellular loops (35). A comparison of the recent crystal structures of ABC transporters suggest that differences between outward and inward facing conformations of ABC transporters may be limited and can be accommodated with little changes in the overall architecture (36–38). Although further analysis of other transmembrane segments is required to completely characterize the conformation changes in CFTR, these results may provide insights into the conformational changes and transport cycle of other ABC transporters.

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