Metal Bridges Illuminate Transmembrane Domain Movements during Gating of the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel*

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**Background:** Opening and closing of the CFTR channel involve conformational changes in the channel pore.

**Results:** Different metal bridges stabilize the channel in the open or closed configuration.

**Conclusion:** Opening and closing result from relative lateral movement of different transmembrane segments.

**Significance:** This work defines the three-dimensional conformational changes underlying channel opening and closing.

Opening and closing of the cystic fibrosis transmembrane conductance regulator are controlled by ATP binding and hydrolysis by the cytoplasmic nucleotide-binding domains. Different conformational changes in the channel pore have been described during channel opening and closing; however, the relative importance of these changes to the process of gating the pore is unknown. We have used patch clamp recording to identify high affinity Cd\(^{2+}\) bridges formed between pairs of pore-lining cysteine residues introduced into different transmembrane \(\alpha\)-helices (TM). Seven Cd\(^{2+}\) bridges were identified forming between cysteines in TMs 6 and 12. Interestingly, each of these Cd\(^{2+}\) bridges apparently formed only in closed channels, and their formation stabilized the closed state. In contrast, a single Cd\(^{2+}\) bridge identified between cysteines in TMs 1 and 12 stabilized the channel open state. Analysis of the pattern of Cd\(^{2+}\) bridge formation in different channel states suggests that lateral separation and convergence of different TMs, rather than relative rotation or translation of different TMs, is the key conformational change that causes the channel pore to open and close.

Cystic fibrosis is caused by loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a phosphorylation-regulated, nucleotide-gated Cl\(^{-}\) channel found in the apical membrane of many epithelial cell types (1). CFTR is a member of the ATP-binding cassette (ABC) family of membrane transport proteins, with an archetypal architecture of two membrane-spanning domains (MSDs), each made up of six transmembrane \(\alpha\)-helices (TMs) and two cytoplasmic nucleotide-binding domains (NBDs) (see Fig. 1). CFTR also contains a unique cytoplasmic regulatory domain. Opening and closing of phosphorylated CFTR channels are controlled by ATP binding and hydrolysis by a dimer of the two NBDs; this is presumed to result in the opening and closing of a gate within the MSDs that allows or prevents the transmembrane movement of Cl\(^{-}\) and other small anions (2, 3). However, the overall structure of the channel pore formed by the MSDs, the location of the gate, and the structural rearrangements that occur within the pore region that control gate opening and closing are not known.

The channel pore is lined by a subset of the TMs, with TMs 1, 6, and 12 playing particularly important functional roles (2, 3) (see Fig. 1). At the functional level, the pore is divided into a central narrow region flanked by wider inner and outer vestibules (3). The contributions of different TMs, and specific amino acid side chains coming from different TMs, to each of these functionally distinct, two-dimensional regions of the pore has been reviewed recently (3); however, the three-dimensional structure of the pore is less well defined experimentally. Three-dimensional atomic homology models of CFTR have been generated based on related, non-ion channel ABC proteins (4–6); however, the structures of the pore suggested by these models show some important discrepancies from functional models based on direct experimental evidence (3).

The three-dimensional structure of the pore is not static but presumably changes as the channel opens and closes. Indeed, different conformational changes of the pore during channel gating have been proposed (2, 3). These include local constrictions/dilations of the pore (7–10), as well as rotational (11, 12) and translational (7) movements of individual TMs. However, although experimental evidence for these different kinds of movement has been put forward, the relative mechanistic importance of these movements for the process of channel opening and closing is not known. Evidence for conformational changes in individual TMs within the inner vestibule of the pore during channel gating is mainly two-dimensional in nature, coming from state-dependent differences in the accessibility of cytoplasmically applied cysteine reactive methanethiosulfonate reagents to cysteine side chains introduced into TMs 1 (8, 9, 13, 14), 6 (8, 9, 11, 15), 11 (10), and 12 (12, 16). However, how the relative positions of these TM changes in three-dimensional space during channel gating have not previously been investigated.

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2 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; ABC, ATP-binding cassette; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; TM, transmembrane \(\alpha\)-helix.
Conformational Changes during CFTR Channel Opening

Direct information concerning the three-dimensional structure of the pore has come from disulfide cross-linking experiments (7, 13, 17), as well as from experiments in which anionic channel blocker binding was altered by introduction of positively charged amino acid side chains (17, 18). Previously, we have used the oxidizing agent copper(II)-α-phenanthroline to show that disulfide bonds can be formed between cysteine side chains introduced into different TMs, and we used this information to infer the relative proximity of residues in these different TMs lining the inner vestibule of the pore (13, 17). However, because these disulfide bonds are essentially irreversible under non-reducing conditions, this approach has several disadvantages, such as the potential for disulfide bonds to form during rare or unnatural channel conformations when normally distant cysteine side chains transiently approach close together. Furthermore, the irreversible nature of disulfide bonds makes it difficult to investigate state-dependent changes in the proximity of pairs of cysteine side chains. An alternative approach is to use weaker Cd\textsuperscript{2+} bridges that are more likely to catch the channel in native conformations (19–22). An additional advantage is that these weaker, reversible interactions can break and reform, allowing channel gating to proceed relatively normally (22) and so potentially providing information on the relative propensity of Cd\textsuperscript{2+} bridges to form in different gating states. In the present study, we have investigated the potential of Cd\textsuperscript{2+} to form metal bridges between nearby pore-lining cysteine side chains introduced into TMs 1, 6, and 12 lining a localized region of the inner vestibule of the channel pore close to an anionic blocker-binding site (17, 18). In particular, we have taken advantage of the dynamic nature of Cd\textsuperscript{2+} bridges to associate the formation of these bridges with different channel conformational states. Our results show that Cd\textsuperscript{2+} bridges form selectively between a limited number of cysteine pairs in TMs 6 and 12, suggesting a relative alignment of these two TMs. Interestingly, each of these bridges appears to form only in closed channels, and they stabilize the channel in the closed state. In contrast, a single identified Cd\textsuperscript{2+} bridge between TMs 1 and 12 stabilized the channel in the open state. These results support a model in which relative lateral movement of different TMs toward and away from each other in the inner vestibule, rather than helical rotation or translation of individual TMs, is required for the channel to open and close.

EXPERIMENTAL PROCEDURES

Experiments were carried out in baby hamster kidney cells transiently transfected with CFTR. In this study, we have used a human CFTR variant in which all cysteines had been removed by mutagenesis (as described in Ref. 23) and that includes a mutation in NBD1 (V510A) to increase protein expression in the cell membrane (24). This Cys-less variant, which we have used in previous studies of pore conformational change (7–9), has functional properties that are very similar to those of wild-type CFTR (25). Additional mutations were introduced into the Cys-less background using the QuikChange site-directed mutagenesis system (Agilent Technologies, Santa Clara, CA) and verified by DNA sequencing. In some cases, cysteine mutants were combined with the NBD2 mutation E1371Q, which we (7–9) and others (12, 26) have used to abolish ATP-dependent channel gating and lock CFTR channels in the open state. In our expression system, the open probability of channels bearing the E1371Q mutation is >95% (7).

To investigate potential Cd\textsuperscript{2+} bridges formed between pore-lining cysteine side chains exposed in the inner vestibule of the CFTR pore, we combined individual cysteines that we previously found to be accessible to cytoplasmically applied methanethiosulfonate reagents in three important pore-lining TMs: TM1 (K95C, Q98C) (13), TM6 (I344C, V345C, M348C, A349C) (15), and TM12 (M1140C, S1141C, T1142C, Q1144C, W1145C, V1147C, N1148C) (16), to generate a total of 50 double cysteine mutants (8 TM1:TM6; 14 TM1:TM12; 28 TM6:TM12).

Macroscopic CFTR currents were recorded using patch clamp recordings from inside-out membrane patches, as described in detail recently (17). Following patch excision and recording of background currents, CFTR channels were activated by exposure to protein kinase A catalytic subunit (PKA; 20 nm) plus MgATP (1 mM) in the intracellular solution. In some cases (see Fig. 4), patches were subsequently treated with 2 mM sodium pyrophosphate (PP), to stabilize the channel open state (27, 28), as described previously in studies of state-dependent modification of Cys-less CFTR (8, 15). Both intracellular (bath) and extracellular (pipette) solutions contained (in mM): 150 NaCl, 2 MgCl\textsubscript{2}, 10 N-tris[hydroxyethyl]methyl-2-aminoethanesulfonate, pH 7.4. Current traces were filtered at 100 Hz using an eight-pole Bessel filter, digitized at 250 Hz, and analyzed using pCLAMP software (Molecular Devices, Sunnyvale, CA). CFTR current amplitudes were monitored during brief voltage deflections (to ±50 mV) from a holding potential of 0 mV applied every 6 s (10). In some cases, the identity of CFTR currents in inside-out patches was confirmed by their sensitivity to the specific inhibitor CFTRinh-172 (5 μM). Different concentrations of CdCl\textsubscript{2} were applied directly to the cytoplasmic face of inside-out patches from stock solutions made up in normal intracellular solution. In those cases where exposure to Cd\textsuperscript{2+} caused inhibition of current amplitude, concentration-inhibition relationships were fitted by the equation.

Fractional unblocked current = 1/(1 + ([Cd\textsuperscript{2+}]/K\textsubscript{i}))

(Eq. 1)

Experiments were carried out at room temperature (21–24°C). The values are presented as the means ± S.E. Tests of significance were carried out using an unpaired t test, with p < 0.05 being considered statistically significant. All chemicals were from Sigma-Aldrich, except for PKA (Promega, Madison, WI).

RESULTS

Metal Bridge Formation between TMs 5 and 12—Pore-lining TMs 6 and 12 make important functional contributions to the inner vestibule of the pore (2, 3) and occupy symmetrical positions within the MSDs (Fig. 1). To investigate the potential for Cd\textsuperscript{2+} bridge formation between pore-forming side chains coming from TMs 6 and 12, we introduced cysteines at each of four sites in TM6 (Ile-344, Val-345, Met-348, Ala-349) and seven sites in TM12 (Met-1140, Ser-1141, Thr-1142, Gln-1144, Trp-1145, Val-1147, Asn-1148) that we have previously found to be accessible to cysteine-reactive methanethiosulfonate reagents.

28150 JOURNAL OF BIOLOGICAL CHEMISTRY ASMBMB VOLUME 289 • NUMBER 41 • OCTOBER 10, 2014
applied to the cytoplasmic side of the membrane (15, 16). Combination of each single cysteine mutant from each TM then gave a total of 28 double-cysteine mutants containing one cysteine in TM6 and one cysteine in TM12 within the putative inner vestibule of the pore.

Cys-less CFTR itself was weakly inhibited by the addition of millimolar concentrations of Cd\(^{2+}\) to the cytoplasmic side of the membrane (Fig. 2, A and B), presumably acting in a cysteine-independent manner. All 11 single cysteine mutants tested were more strongly inhibited by Cd\(^{2+}\) (Fig. 2, A and B), with estimated \(K_i\) values in the range of 70–250 \(\mu\)M (Fig. 3). This suggests that Cd\(^{2+}\) is able to bind to each of these introduced cysteine side chains (29), which is consistent with the proposed pore-lining location of the cysteine residues introduced. The effects of Cd\(^{2+}\) appeared independent of voltage (Fig. 2, A and B).

Of the 28 double-cysteine mutants tested, most (twenty-one) did not result in an increase in sensitivity to Cd\(^{2+}\) as compared with the corresponding single-cysteine mutants (Fig. 4). In contrast, the remaining seven double cysteine mutants, namely I344C/S1141C (Fig. 2, C and D), V345C/S1141C, M348C/S1141C (Fig. 2, C and E), M348C/V1147C, M348C/W1145C, M348C/V1145C, and M348C/N1148C, all showed increased sensitivity to Cd\(^{2+}\), leading to a significant decrease in \(K_i\), as compared with either of the single cysteine mutants from which they were derived (estimated \(K_i\) values < 50 \(\mu\)M, Fig. 3). In theory, this apparent increase in Cd\(^{2+}\) potency could reflect additive effects of two Cd\(^{2+}\) ions bound simultaneously to different cysteine side chains. However, if the inner vestibule of the pore were able to accommodate two Cd\(^{2+}\) ions simultaneously, we would expect most double cysteine mutants to show strong Cd\(^{2+}\) binding, especially where the two cysteine side chains are expected to be relatively far apart (see “Discussion”). Instead, we propose that the relatively small number of double cysteine mutant channels that show strengthened Cd\(^{2+}\) binding results from specific coordination of a single Cd\(^{2+}\) ion interacting simultaneously with both cysteine side chains. This proposal is consistent with recent studies showing that cysteine side chains introduced into ion channel pores bind a single Cd\(^{2+}\) ion and that nearby cysteine side chains then increase the strength of binding of this single Cd\(^{2+}\) ion (29). In effect, these combinations of cysteine pairs that led specifically to a strengthened binding of Cd\(^{2+}\) within the pore (but no other combination of cysteines tested) are proposed to support the formation of Cd\(^{2+}\) bridges between TMs 6 and 12 that impair channel function. In particular, M348C/S1141C was associated with a dramatic increase in sensitivity to Cd\(^{2+}\) (Fig. 2, C and E, and Fig. 3) with an estimated \(K_i\) of 0.14 ± 0.02 \(\mu\)M (\(n = 5\)).

The finding that Cd\(^{2+}\) bridges between TMs 6 and 12 inhibited channel function suggested to us that these bridges may stabilize the channel closed state. To test this idea, we applied Cd\(^{2+}\) following channel treatment with 2 mM PPi to stabilize the channel open state (see “Experimental Procedures”). In each case, PPi treatment resulted in a weakening of Cd\(^{2+}\) inhibition (Fig. 4A) and a significant increase in \(K_i\) (Fig. 4B) of between 2.3-fold (in I344C/S1141C) and 97-fold (in M348C/S1141C). This weakened interaction is consistent with Cd\(^{2+}\) normally interacting with the closed state of these mutant channels to impair channel opening. To confirm this apparent state dependence of Cd\(^{2+}\) bridge formation, we combined selected single and double cysteine mutants with the E1371Q mutation in NBD2 that results in constitutively open channels in our expression system (see “Experimental Procedures”). As shown in Fig. 5, all E1371Q-containing channels tested were only weakly sensitive to inhibition by Cd\(^{2+}\), resulting in a significant increase in \(K_i\) both in single cysteine (I344C, M348C, S1141C) and in double cysteine (I344C/S1141C, Fig. 5, A–C; M348C/S1141C, Fig. 5, A, D, and E) mutant channels. However, the effect of the E1371Q mutation was greater in the double cysteine mutants; this gating mutation increased \(K_i\) 30-fold in I344C/S1141C (Fig. 5C) and 2500-fold in M348C/S1141C (Fig. 5E). In fact, in striking contrast to normally gating CFTR channels in which these double cysteine mutants showed greatly increased sensitivity to Cd\(^{2+}\) as compared with the correspond-
ing single cysteine mutants (Figs. 3 and Fig. 5, C and E), in E1371Q mutant channels, the $K_i$ for the double cysteine mutants was not significantly different from the single cysteine mutants (Fig. 5, B–E). The finding that these cysteine pairs fail to increase $\text{Cd}^{2+}$ sensitivity in constitutively open channels (relative to single cysteines) suggests that $\text{Cd}^{2+}$ bridge formation effectively does not occur in the open state and supports the idea that the inhibitory effect of $\text{Cd}^{2+}$ bridge formation between TMs 6 and 12 results from a specific interaction with, and resulting stabilization of, the channel closed state.

**Metal Bridge Formation between TMs 1 and 6 and 12**—In addition to TMs 6 and 12, TM1 also plays an important func-
Conformational Changes during CFTR Channel Opening

**A** Single cysteine mutants

| TM6   | TM12 |
|-------|------|
| D348C |      |
| M348C |      |
| A349C |      |

**B** Double cysteine mutants

| I344C | V345C | M348C | A349C |
|-------|-------|-------|-------|
|       |       |       |       |

FIGURE 3. Mean inhibitory effect of Cd^{2+} on single and double cysteine mutant channels in TMs 6 and 12. Mean K_i values were calculated from fits to data from individual patches as those shown in Fig. 2, D and E, A. single cysteine mutants in TM6 (left) and TM12 (right) as indicated. B, double cysteine mutants that combine one cysteine substituted in TM6 (listed above the panel) with one in TM12 (listed below the panel). Those double mutants that showed a significantly different K_i from that of either of the corresponding single cysteine mutants (p < 0.05) are filled black, whereas those that with no significant difference are filled gray. Note that the K_i for M348C/S1141C (0.14 ± 0.02 μM, n = 5) is too small to be visible on this scale, but was significantly different from either M348C or S1141C alone (p < 0.0005; see also Fig. 2E). Also note that the estimated K_i values for some double mutants were ≈300 μM (V345C/M1140C, 316 ± 38 μM, n = 3; A349C/M1140C, 345 ± 58 μM, n = 3; A349C/T1142C, 231 ± 68 μM, n = 3). Error bars indicate the means ± S.E. from 3–7 patches.

In striking contrast to cysteine substitution in TMs 6 and 12, we found that K95C channel currents were weakly, but significantly, increased in amplitude by Cd^{2+} at concentrations of 30–100 μM (Fig. 6). Because K95C is the only single mutant we have studied that involves replacement of a positively charged amino acid side chain by cysteine, and because removal of this positive charge by mutagenesis is known to reduce single channel conductance by ~85% (17, 18, 30), we considered whether this apparent stimulatory effect of Cd^{2+} could result from a bound Cd^{2+} ion acting as a surrogate fixed positive charge in the pore and thereby increasing Cl^- conductance. If this were the case, Cd^{2+} should have a consistent stimulatory effect irrespective of channel gating. However, we found that constitu- tively open K95C/E1371Q channels were insensitive to Cd^{2+} at concentrations up to 100 μM (Fig. 6B), as were K95C channels following treatment with 2 mM PPi (data not shown), inconsis- tent with an effect of Cd^{2+} on Cl^- conductance. Instead, these results suggest that Cd^{2+} binding to K95C results in a small but significant stabilization of the channel open state, an effect that is not observed in E1371Q channels that are already open almost 100% of the time (7). In contrast to these results with K95C, but in common with TMs 6 and 12 single cysteine mutants, Q98C channels were inhibited by Cd^{2+} (Fig. 7).

Most (18 out of 19) double cysteine mutants including K95C or Q98C were weakly inhibited by Cd^{2+}, consistent with Cd^{2+} effects on single cysteine mutants in TMs 6 or 12 (or indeed in Q98C) (Fig. 7). Thus, evidence for Cd^{2+} bridge formation involving TM1 is scant. However, one mutant, K95C/S1141C, was apparently stimulated by Cd^{2+} (Fig. 6A), and this stimulation was of a greater magnitude than that seen in the K95C single cysteine mutant (Figs. 6C and 7). This suggests that a Cd^{2+} bridge may form between K95C in TM1 and S1141C in TM12. As with K95C itself, the stimulatory effects of Cd^{2+} on K95C/S1141C were abolished by the E1371Q mutation (Fig. 6C) or by treatment with PPi (data not shown), suggesting that the observed effects of Cd^{2+} are due to an increase in channel open probability and a stabilization of the channel open state. Thus, although Cd^{2+} bridges between TMs 6 and 12 consistently stabilize the closed state, this single Cd^{2+} bridge between TMs 1 and 12 appears to stabilize the channel in the open state.

**DISCUSSION**

The inner vestibule of the CFTR channel pore is lined by TMs 1, 6, and 12 (3); however, their relative arrangement in three-dimensional space is not known. Consistent with their known pore-forming roles, cysteine residues introduced into each of 13 putative pore-lining positions in these three TMs resulted in macroscopic current sensitivity to submillimolar concentrations of cytoplasmically applied Cd^{2+} ions (Figs. 3A and 7) that was not observed in Cys-less background channels (Fig. 2). Similar Cd^{2+} sensitivity has previously been described for cysteine mutants in other parts of the pore (7, 26) and presumably reflects Cd^{2+} ion binding to these 13 different pore-exposed cysteine side chains (29). Channels bearing two cysteine residues, one in each of two different TMs, were also sensitive to cytoplasmic Cd^{2+} (Figs. 3B and 7). In most cases, combining two cysteines in different TMs did not lead to a significant change in apparent Cd^{2+} binding (Figs. 3B and 7), suggesting that the two cysteine side chains in question were unable to act together to coordinate Cd^{2+} binding and also that simultaneous binding of two Cd^{2+} ions to both cysteines in this localized region of the pore is unlikely. However, in a limited
number of cases, double cysteine mutants exhibited tighter 
Cd$^{2+}$ binding than either of the single cysteine mutants from 
which they were derived (Fig. 3). In highly localized regions 
of ion channel pores, this is generally considered consistent with 
the formation of a metal bridge between the two cysteine side 
chains (29). Indeed, the ability of a restricted subset of cysteine 
pairs specifically to coordinate binding of a single Cd$^{2+}$ ion in 
the inner vestibule is consistent with the proposed alignment of 
TM1, 6, and 12 around this region of the pore (Fig. 8A). In 
contrast, binding of a second Cd$^{2+}$ ion would be expected to be 
favored when two cysteine side chains were further apart, when 
simultaneously bound Cd$^{2+}$ ions would be expected to experi-
ence less steric and electrostatic interference.

Of eight putative Cd$^{2+}$/H11001 bridges identified (out of a total of 50 
double cysteine mutants tested), all involved a cysteine substit-
tuted either for Met-348 (TM6) or for Ser-1141 (TM12) (Fig. 
8B). Furthermore, when cysteine was present at both of these 
positions, an unusually high affinity Cd$^{2+}$/H11001 bridge was formed 
(Figs. 2–5). These results suggest that cysteine side chains sub-
stituted for Met-348 or Ser-1141 are particularly well posi-
tioned to form inter-TM Cd$^{2+}$/H11001 bridges.

Consideration of the different Cd$^{2+}$/H11001 bridges that can form 
between different TMs may provide some information on the 
relative alignment and orientation of these TMs. Thus, M348C 
is able to form Cd$^{2+}$/H11001 bridges with cysteines at multiple positions 
in TM12 (S1141C, Q1144C, W1145C, V1147C, N1148C) (Fig. 
8B), and S1141C is able to form Cd$^{2+}$/H11001 bridges with cysteines 
both in TM1 (K95C) and in TM6 (I344C, V345C, M348C) (Fig. 
8C). The variety of bridges that can be formed from these two 
positions suggests that some degree of conformational flexibil-
ity must exist within these TMs. For example, M348C is able to 
form Cd$^{2+}$/H11001 bridges with TM12 sites on a broad face of the 
TM12 helical face (Fig. 8B, top panel), suggesting some degree 
of relative rotational flexibility or movement, and also across a 
distance spanning two helical turns (from Ser-1141 to Asn- 
1148) (Fig. 8B, bottom panel), suggesting also some transla-
tional flexibility or movement. Similarly, S1141C is able to form 
Cd$^{2+}$/H11001 bridges with different sites in TM6 (Fig. 8C). Thus, our 
results suggest that different TMs surrounding the inner vesti-
bule do not occupy rigid positions relative to one another. Cad-
imium bridges between cysteine side chains are thought to 
require sulfur distances $\leq$ 6.5 Å (31).

Seven different Cd$^{2+}$/H11001 bridges were identified as forming 
between cysteine side chains in TMs 6 and 12 (Figs. 3B and 8A). Interestingly, all of these Cd$^{2+}$/H11001 bridges appear to form prefer-
entially in the closed state and to stabilize the channel in this 
state. Thus, if channels are stabilized in the open state, either 
using PPi to manipulate NBD-driven gating (Fig. 4) or using 
E1371Q channels that cannot hydrolyze ATP (Fig. 5), the inhib-
itory effects of Cd$^{2+}$ are greatly reduced, suggesting that Cd$^{2+}$ 
binds more strongly to the closed state than to the open state. Most striking in this respect was the M348C/S1141C mutant 

**FIGURE 4. Effect of increasing channel open probability with PPi on Cd$^{2+}$/H11001 sensitivity of TM6:TM12 double cysteine mutant channels.** A, mean fractional current remaining following the addition of different concentrations of 
Cd$^{2+}$ in M348C/S1141C channels in the presence of PKA and ATP (●) or follow-
ing activation by PKA and ATP followed by treatment with 2 mM PPi, to 
maximize channel open probability (○). B, mean $K_i$ values estimated for the 
double cysteine mutant channels listed, either in the presence of PKA and 
ATP (black bars) or following treatment with PPi, (gray bars). Asterisks indicate 
a significant difference in the presence of PPi, (*, $p < 0.05$; **, $p < 0.00005$). Error bars indicate the means ± S.E. from 3–7 patches.
that appears to bind \( \text{Cd}^{2+} \) ~2500 times more strongly in channels that are gating normally as compared with constitutively open E1371Q channels (Fig. 5). The unusually high apparent \( \text{Cd}^{2+} \) binding affinity of this double cysteine mutant (Figs. 3B and 4B), more than 700-fold lower \( K_i \) than the corresponding single cysteine mutants (Figs. 2E and 3), suggests that M348C and S1141C are uniquely well positioned to coordinate tight \( \text{Cd}^{2+} \) binding in closed channels. However, when the channel is open, these two cysteines do not appear to coordinate \( \text{Cd}^{2+} \) at all because the apparent \( \text{Cd}^{2+} \) affinity in M348C/S1141C/E1371Q channels appears the same as in M348C/E1371Q or S1141C/E1371Q (Fig. 5, D and E).

In contrast, a single identified \( \text{Cd}^{2+} \) bridge between TMs 1 and 12 (K95C/S1141C) appears to stabilize the channel open state, leading to a significant increase in channel current (Fig. 6). Because this effect was abolished in E1371Q channels (Fig. 6), it appears to reflect a \( \text{Cd}^{2+} \) bridge-induced change in channel gating rather than channel conductance. No evidence for \( \text{Cd}^{2+} \) bridge formation was observed in the limited number of TM1:TM6 double cysteine mutants that could be studied.

If we assume that the effects of \( \text{Cd}^{2+} \) bridges on the stability of the channel open and closed states reflects changes in the relative positions of cysteine side chains introduced into different TMs in these states, then our results would suggest that a number of side chains in TMs 6 and 12 are in close proximity in closed channels, and K95C (TM1) and S1141C (TM12) are in close proximity in open channels. Loss of \( \text{Cd}^{2+} \) coordination in these TM6:TM12 mutants in the open state would then reflect reduced physical proximity of these specific pairs of cysteine side chains concomitant with channel opening. What kinds of physical rearrangements of the TMs during channel gating might result in these changes in side chain relative positions?

Changes in accessibility of different side chains in TMs 6 and 12 from the cytoplasm during channel opening and closing have been interpreted as reflecting rotational movements of these TMs within the inner vestibule (11, 12). Such rotational movements could also lead to state-dependent changes in the relative positions of different side chains such as those we infer from our results. However, helical rotation alone does not appear to be able to explain the effects of \( \text{Cd}^{2+} \) bridges on chan-

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**FIGURE 5.** Effect of the E1371Q mutation on the effects of \( \text{Cd}^{2+} \) on single and double cysteine mutant channels in TMs 6 and 12. **A**, sample time courses and \( I-V \) curves illustrating the low \( \text{Cd}^{2+} \) sensitivity of constitutively active I344C/S1141C/E1371Q (left panels) and M348C/S1141C/E1371Q (right panels) channels in inside-out patches. Experiments were performed as described in the legend for Fig. 2. Note that although currents were relatively insensitive to \( \text{Cd}^{2+} \), they were inhibited by CFTRinh-172 (5 \( \mu \text{M} \); indicated by hatched bars marked inh-172). **B–E**, mean fractional current remaining following the addition of different concentrations of \( \text{Cd}^{2+} \) (B and D) and mean \( K_i \) values for the single and double cysteine mutants listed (C and E). In C and E, black bars indicate channels gating normally, and gray bars indicate the corresponding cysteine mutants with the E1371Q mutation; asterisks indicate a significant difference between the two for the same cysteine constructs \((p < 0.02)\). Error bars indicate the means \( \pm \) S.E. from 3–7 patches.
nel gating. If the only movement of TMs 6 and 12 during channel opening and closing was a relative rotation, then we would expect some Cd\textsuperscript{2+}/H11001 bridges to stabilize the closed state, but others (following rotation of the helices) to stabilize the open state. In fact, we found that Cd\textsuperscript{2+}/H11001 bridges only stabilize the closed state and that these bridges appear incapable of forming in the open state. A similar argument suggests that relative translational movement of TMs 6 and 12 does not underlie channel opening and closing; if this were the case, some Cd\textsuperscript{2+} bridges should again be expected to stabilize the open state. Instead, we propose that the most likely physical explanation for this state-dependent Cd\textsuperscript{2+}/H11001 bridge formation is that one face of each of TMs 6 and 12 is physically close together when the channel is closed and that these two TMs then move apart when the channel is open, such that the distance between the two TMs in the open state is too great for Cd\textsuperscript{2+}/H11001 bridges to form between any pairs of cysteine residues exposed at this level of the pore (Fig. 8, D and E). This does not imply that these helices do not rotate and/or translate during channel opening and closing, only that the rotation and/or translation (at this level) appears coincidental to opening and closing, with physical separation and convergence of these two TMs being the mechanistically necessary conformational change that results in opening and closing. Thus, forcing TMs 6 and 12 to remain in close physical proximity (by formation of Cd\textsuperscript{2+} bridges between the two) stabilizes the channel closed state (Fig. 8E). Furthermore, this stabilization of the closed state appears relatively independent of the exact location of the Cd\textsuperscript{2+} bridge (Fig. 8, A–C). As described above, several different Cd\textsuperscript{2+} bridges can form between M348C (TM6) and TM12 (Fig. 8, A and B), as well as between S1141C (TM12) and TM6; all appear to stabilize the closed state (Figs. 3 and 4). Thus, for the reasons described earlier, there may be

![Graphical representation of Cd\textsuperscript{2+} concentration-dependent increases in macroscopic current amplitude in K95C and K95C/S1141C channels.](image)

**FIGURE 6.** Cadmium increases macroscopic current amplitude in K95C and K95C/S1141C channels in a gating-dependent manner. A, sample time courses and I–V curves showing Cd\textsuperscript{2+} concentration-dependent increases in macroscopic current amplitude in K95C (left panels) and K95C/S1141C (right panels) channels in inside-out patches. Experiments were performed as described in the legend for Fig. 2. Note that although currents were increased by Cd\textsuperscript{2+}, they were inhibited by CFTRinh-172 (5 \textmu M). B, concentration-dependent increases in current amplitude in K95C were not observed in K95C/E1371Q. Asterisks indicate a significant difference from K95C (p < 0.05). C, Cd\textsuperscript{2+} causes a significantly greater increase in K95C/S1141C current amplitude as compared with K95C (p < 0.0001); this increase is not observed in K95C/S1141C/E1371Q channels. Error bars indicate the means ± S.E. from 3–6 patches.

![Graphical representation showing mean inhibitory effect of Cd\textsuperscript{2+} on channels bearing cysteine side chains in TM1.](image)

**FIGURE 7.** Mean inhibitory effect of Cd\textsuperscript{2+} on channels bearing cysteine side chains in TM1. Shown is the mean effect of 100 \textmu M Cd\textsuperscript{2+} on macroscopic current amplitude for two single cysteine mutants (K95C, Q98C; black bars) and 19 double cysteine mutants that combine the TM1 cysteine listed above the panel with the TMs 6 or 12 cysteine listed below. Daggers indicate three mutants that did not express measurable macroscopic current in inside-out patches. Asterisk indicates a significant difference from the corresponding single cysteine mutant in TMs 6 or 12 (p < 0.000005). Error bars indicate the means ± S.E. from 3–5 patches.
some degree of rotational and/or translational flexibility between TMs 6 and 12 in closed channels. On the other hand, maintaining the channel in the open state prevents Cd\(^{2+}\)/H11001 bridge formation between cysteines in TMs 6 and 12 (Figs. 4 and 5), suggesting that these two TMs have separated during channel opening (Fig. 8E).

In contrast to this consistent pattern of Cd\(^{2+}\) bridge formation between TMs 6 and 12 stabilizing the closed state, a single Cd\(^{2+}\) bridge between K95C (TM1) and S1141C (TM12) appears to stabilize the open state (Fig. 6). Because only a single Cd\(^{2+}\) bridge between these TMs was identified functionally, it is difficult to draw any conclusion regarding conformational rearrangements between these TMs during channel gating. Thus, relative rotation or translational movement of these two TMs could bring these two cysteines into close proximity in the open state and separate them when the channel closes.
natively, it has been suggested that Lys-95 is not even exposed to the pore lumen when the channel is closed (14), although this suggestion is at odds with functional evidence that the native positive charge at this position can interact with cytoplasmically applied pore-blocking ions not only in the open state but also in the closed state (32). Finally, this state-dependent Cd$^{2+}$ bridge formation could result from the same kinds of changes in relative proximity of TMs 1 and 12 as we propose for TMs 6 and 12 (Fig. 8E). This kind of model would suggest that TMs 1 and 12 are closer together in open channels and move further apart when the channel closes (Fig. 8E). No evidence was obtained for Cd$^{2+}$ bridge formation between TMs 1 and 6, and so we cannot draw any inference on proximity of these TMs in either the open or the closed state. Functional evidence suggests that Lys-95, Ile-344, Val-345, and Ser-1141 are all close together in the inner vestibule of the pore (17, 18, 32).

Our results suggest that different TMs converge and separate during channel gating, implying that ATP-dependent channel opening and closing may involve lateral movements of different TMs surrounding the inner vestibule (Fig. 8E). Furthermore, the physical requirement for these movements in order for channel opening and closing to proceed is underscored by the fact that constraining these movements (by the formation of Cd$^{2+}$ bridges between different TMs) is capable of either stabilizing the closed state (preventing channel opening) or stabilizing the open state (preventing channel closing). Thus, although the conformational changes of the MSDs that underlie channel opening and closing are ultimately controlled by ATP interactions with the NBs, these results offer encouragement that altering interactions within the MSDs themselves can, in theory, directly influence the overall activity of the CFTR channel. A substance that could influence TM:TM interactions in this way to stabilize the channel open state could theoretically represent a novel mechanism to increase CFTR function in cystic fibrosis patients.

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