Identification of Cystic Fibrosis Transmembrane Conductance Regulator Channel-Lining Residues in and Flanking the M6 Membrane-Spanning Segment

Min Cheung* and Myles H. Akabas*‡
*Center for Molecular Recognition and ‡Departments of Physiology and Cellular Biophysics and Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032 USA

ABSTRACT The cystic fibrosis transmembrane conductance regulator (CFTR) forms a chloride channel that is regulated by phosphorylation and ATP binding. Work by others suggested that some residues in the sixth transmembrane segment (M6) might be exposed in the channel and play a role in ion conduction and selectivity. To identify the residues in M6 that are exposed in the channel and the secondary structure of M6, we used the substituted cysteine accessibility method. We mutated to cysteine, one at a time, 24 consecutive residues in and flanking the M6 segment and expressed these mutants in *Xenopus* oocytes. We determined the accessibility of the engineered cysteines to charged, lipophobic, sulfhydryl-specific methanethiosulfonate (MTS) reagents applied extracellularly. The cysteines substituted for Ile331, Leu333, Arg334, Lys335, Phe337, Ser341, Ile344, Arg347, Thr351, Arg352, and Gln353 reacted with the MTS reagents, and we infer that they are exposed on the water-accessible surface of the protein. From the pattern of the exposed residues we infer that the secondary structure of the M6 segment includes both α-helical and extended regions. The diameter of the channel from the extracellular end to the level of Gln353 must be at least 6 Å to allow the MTS reagents to reach these residues.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette (ABC) membrane transporter gene superfamily (Riordan et al., 1989). Unlike most members of this superfamily, CFTR forms an ion channel. This channel has a small conductance and is anion-selective, with a linear current-voltage relationship (Anderson et al., 1991b; Bear et al., 1991). Channel gating is regulated by two reactions. The first is phosphorylation by cAMP-dependent protein kinase (Cheng et al., 1991; Tabcharani et al., 1991; Berger et al., 1993; Chang et al., 1993; Hwang et al., 1993; Seibert et al., 1995). The second is ATP binding and hydrolysis at the nucleotide-binding folds, which induces channel opening and closing (Anderson et al., 1991a; Smit et al., 1993; Baukrowitz et al., 1994; Hwang et al., 1994; Carson et al., 1995; Schultz et al., 1995; Gunderson and Kopito, 1995).

Examination of the amino acid sequence of CFTR suggests that it is a tandem repeat of six membrane-spanning segments and a nucleotide-binding fold; the two repeats are connected by a cytoplasmic regulatory domain (R-domain) (Fig. 1 *A*) (Riordan et al., 1989). There is considerable evidence to support the transmembrane topology predicted from hydropathy analysis. The glycosylation consensus sites in the putative extracellular loop between M7 and M8 are glycosylated in vivo (Gregory et al., 1990; Chang et al., 1994). The insertion of glycosylation sites into each of the putative extracellular loops in a mutant lacking the wild-type glycosylation sites yields functional channels that are glycosylated (Chang et al., 1994). Residues in the R-domain are phosphorylated in vivo (Cheng et al., 1991; Chang et al., 1993; Seibert et al., 1995). The binding and hydrolysis of cytoplasmic ATP, presumably at the nucleotide-binding folds, are necessary for activation of the channel (Anderson et al., 1991a; Travis et al., 1993; Baukrowitz et al., 1994; Winter et al., 1994; Gunderson and Kopito, 1995).

The structure and functions of the cytoplasmic domains have been extensively studied, but less is known about the structure of the 12 putative membrane-spanning segments and their role in forming the chloride channel. There is evidence that the sixth transmembrane segment (M6) forms part of the channel lining. A mutation, K335E, in M6 altered the permeability and conductance ratios for halides (Anderson et al., 1991b). In the mutant R347H, multiple ion occupancy was dependent on the pH of the intracellular solution, and presumably titration of the histidine altered a nearby anion-binding site (Tabcharani et al., 1993). The ability to titrate the histidine in the R347H mutant also suggests that Arg347 is on the water-exposed surface of the channel lining. Mutation of the residues Ser341 and Lys335 altered the binding of a voltage-dependent, open channel blocker, diphenylamine-2-carboxylate (McDonough et al., 1994). Taken together, these results suggest that the M6 membrane-spanning segment may form a portion of the channel lining.

To investigate further the structure of the M6 segment and its role in ion conduction and selectivity, we used the substituted-cysteine accessibility method to identify the residues in the M6 segment that form the channel lining (Akabas et al., 1992). In this method we mutate consecutive

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Address reprint requests to Dr. Myles Akabas, Center for Molecular Recognition, Columbia University, 630 West 168th Street, New York, NY 10032. Tel.: 212-305-3974; Fax: 212-305-5594; E-mail: ma14@columbia.edu.

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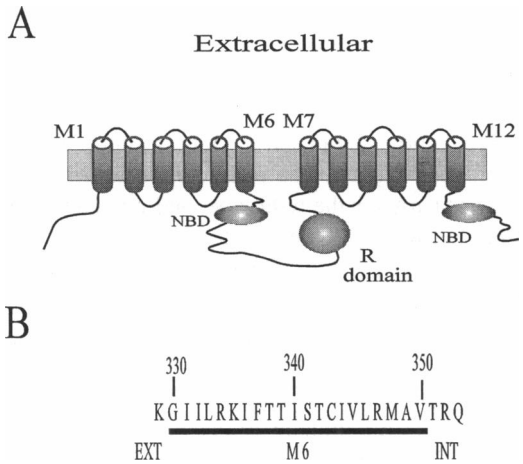

FIGURE 1 Predicted transmembrane topology of CFTR and the amino acid sequence in and flanking the M6 membrane-spanning segment. (*A*) The predicted transmembrane topology of CFTR. NBD, nucleotide binding domain. (*B*) The amino acid sequence (single letter code) in and flanking the M6 membrane-spanning segment. Each of these residues was mutated to cysteine, one at a time. The membrane-spanning segment as defined by Riordan et al. (1989) is underlined. EXT, extracellular end; INT, intracellular end.

residues in putative channel-lining segments to cysteine, one at a time. We express each cysteine substitution mutant in *Xenopus* oocytes. We test whether the engineered cysteine is on the water-accessible surface of the protein by determining the susceptibility of the cysteine to covalent modification by water-soluble, charged, sulfhydryl-specific reagents. We assume that among the residues in membrane-spanning segments only those residues exposed in the channel will be accessible to reaction with these hydrophilic reagents. We assume further that if these reagents react with a channel-lining cysteine, conduction through the channel will be irreversibly altered. We infer that if an engineered cysteine reacts with these reagents, then the corresponding wild-type residue is exposed to the inside of the pore. The reagents that we have used are derivatives of methanethiosulfonate (MTS) (Akabas et al., 1992; Stauffer and Karlin, 1994). These MTS derivatives include the negatively charged MTS-ethylsulfonate ($MTSES^-$, $CH_3SO_2SCH_2CH_2SO_3^-$) and the positively charged MTS-ethylammonium ($MTSEA^+$, $CH_3SO_2SCH_2CH_2NH_3^+$) and MTS-ethyltrimethylammonium ($MTSET^+$, $CH_3SO_2SCH_2CH_2N(CH_3)_3^+$). These reagents covalently link the charged portion $-SCH_2CH_2X$ to free sulfhydryls to form mixed disulfides. We have previously used this approach to identify three channel-lining residues in the region from Gly91 to Pro99 in the extracellular half of the M1 membrane-spanning segment; our results suggested that the secondary structure of this region is α-helical (Akabas et al., 1994b). We have previously applied the substituted-cysteine accessibility method to identify the channel-lining residues of several membrane proteins, including the M1 segment of CFTR (Akabas et al., 1994b), the nicotinic acetylcholine receptor (Akabas et al., 1992, 1994a; Akabas and Karlin, 1995), and the GABA receptors (Xu and Akabas, 1993; Xu et al., 1995; Xu and Akabas, 1996). Others have applied this approach to the dopamine D2 receptor (Javitch et al., 1995) and voltage-gated potassium channels (Kurz et al., 1995; Lu and Miller, 1995; Pascual et al., 1995). Site-directed chemical modifications after cysteine mutagenesis has also been used to study other proteins (Todd et al., 1989; Altenbach et al., 1990; Jung et al., 1993; Mindell et al., 1994; Slatin et al., 1994; Steinhoff et al., 1994).

We now report the application of this approach to 24 residues in and flanking the M6 segment of CFTR.

MATERIALS AND METHODS

Oligonucleotide-mediated mutagenesis

The cDNA encoding human CFTR in the pBluescript KS(−) vector (CFTR-pBS) was obtained from Dr. A. E. Smith (Genzyme; Gregory et al., 1990). The cysteine-substitution mutants were generated using the altered-sites mutagenesis procedure (Promega). CFTR-pBS was digested with *XbaI* and *SphI* restriction enzymes, and the resulting 1058-bp fragment was ligated into the pAlter-1 vector (Promega) cut with the same enzymes. Oligonucleotides were synthesized to generate the appropriate cysteine substitutions and to simultaneously introduce silent restriction sites. Mutations were identified by restriction digestion and confirmed by DNA sequencing.

Preparation of mRNA and oocytes

For in vitro mRNA transcription CFTR-pBS was linearized with *SmaI*. Messenger RNA was synthesized, and oocytes from *Xenopus laevis* were prepared and maintained as described previously (Akabas et al., 1994b). One day after the oocytes were harvested, they were injected with 50 nl of mRNA (200 pg/nl). Experiments were performed 1 to 6 days after mRNA injection.

Sulfhydryl reagents

The MTS reagents were synthesized as described previously (Stauffer and Karlin, 1994).

Electrophysiology

CFTR-induced currents were recorded from individual oocytes under two-electrode voltage-clamp as described previously (Akabas et al., 1994b). Electrodes were filled with a 3 M KCl solution and had a resistance of less than 2 MΩ. The ground electrode was connected to the bath via a 3 M KCl/agar bridge. During experiments, the oocytes were maintained in Ca_2^+-free frog Ringer's solution (CFFR) (115 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 10 mM HEPES, pH 7.5, with NaOH) at room temperature. The holding potential was maintained at −10 mV. Periodically the holding potential was ramped from −120 mV to +50 mV over 1.7 s and the current was recorded. From the resulting current-voltage relationship we determined the magnitude of the CFTR-induced current at −100 mV and the corresponding reversal potential. Background currents before the activation of CFTR were less than 10% of the plateau current and were not subtracted from the records. In all cases the leak current at −100 mV was less than 500 nA.

Experimental protocol

We tested the susceptibility of wild-type and mutant CFTR to the MTS reagents using the following protocol. The CFTR chloride current was activated by applying a solution to the extracellular bath to increase the intracellular cAMP concentration of the oocyte and thereby activate protein kinase A. This solution contained 200 μM 8-(4-chlorophenylthio)adenosine cyclic monophosphate (cpt-cAMP), 1 mM 3-isobutyl-1-methylxanthine (IBMX), and 20 μM forskolin in CFFR and is referred to subsequently as cAMP-activating solution. After the CFTR-induced current reached a plateau, a MTS reagent was added to the extracellular bath in the cAMP-activating solution for 8 min. The sulfhydryl reagents were applied to the oocytes at the following equireactive concentrations: 10 mM $MTSES^-$, 2.5 mM $MTSEA^+$, and 1 mM $MTSET^+$ (Stauffer and Karlin, 1994). To determine whether the effects of the MTS reagents were irreversible, the MTS reagents were removed by washing with cAMP-activating solution. Because the magnitude of the plateau current varied between oocytes, the currents recorded from each oocyte were normalized by the current that was measured immediately before the MTS reagents were applied to permit the comparison of effects between oocytes. All mutants were tested in at least two batches of oocytes.

Statistics

Data are presented as means ± SEM. Significance was determined by one-way analysis of variance with the least significant difference post hoc test ($p < 0.05$) provided in the SPSS-PC statistical analysis program.

RESULTS

To identify the residues in and flanking the M6 membrane-spanning segment that are on the water-exposed surface of the protein, we mutated the residues 329 to 353 (except for 343), one at a time, to cysteine (Fig. 1). Residue 343 is a cysteine in wild-type CFTR. We expressed the cysteine-substituted mutants in *Xenopus* oocytes. Application of the cAMP-activating solution stimulated a chloride current in oocytes expressing each mutant. The peak current at −100 mV was −7117 ± 511 nA for the wild type, and ranged from −1709 ± 124 nA for the R347C mutant to −7709 ± 700 nA for the T339C mutant (Fig. 2 *A*). The time for the currents to reach a steady-state level after application of the cAMP-activating solution was 20 ± 1 min for wild type, and ranged from 14 ± 2 min for I344C to 51 ± 4 min for T351C (Fig. 2 *B*).

To test the susceptibility of wild-type CFTR and the cysteine-substitution mutants to the MTS reagents, after the activated CFTR-induced current reached a plateau we applied the MTS reagents to the extracellular bath for 8 min in the continued presence of the cAMP-activating solution, as illustrated in Fig. 3. The MTS reagents were then washed out to determine whether their effects were irreversible. All of the effects of the MTS reagents that we observed were irreversible.

Effects of MTS reagents on wild-type cysteines in CFTR

As reported previously (Akabas et al., 1994b), extracellular applications of the MTS reagents to *Xenopus* oocytes ex-

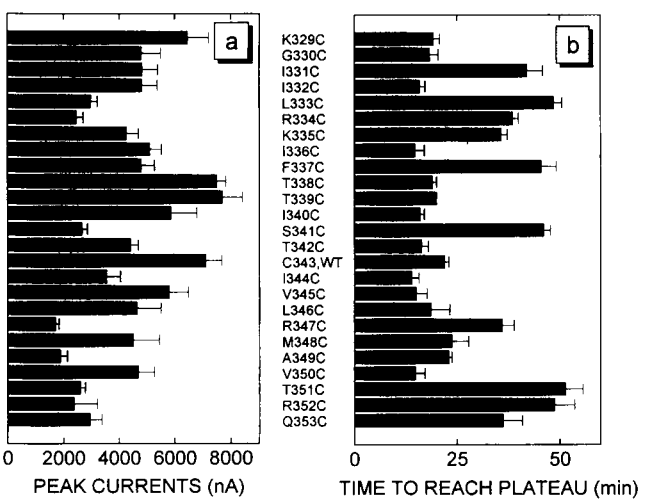

FIGURE 2 Peak CFTR-induced currents and time to reach the plateau current after stimulation with cAMP-activating reagents for 24 cysteine-substitution mutants and wild-type CFTR. (*A*) Average peak CFTR-induced current at −100 mV recorded 2 to 4 days after injection of oocytes with mRNA for each of the cysteine-substitution mutants or wild type (wild-type data are shown at the Cys343 residue and are indicated by WT). In all cases the reversal potential of the current was about −10 mV. (*B*) Average time after addition of the cAMP-activating reagents for the chloride current to reach its plateau level in oocytes expressing wild-type or cysteine-substitution mutant CFTR. CFTR current was activated in oocytes maintained under two-electrode voltage clamp by the addition of 200 μM cpt-cAMP, 20 μM forskolin, and 1 mM IBMX to the bath. For each mutant in (*A* and *B*) the mean ± SEM is shown for six oocytes.

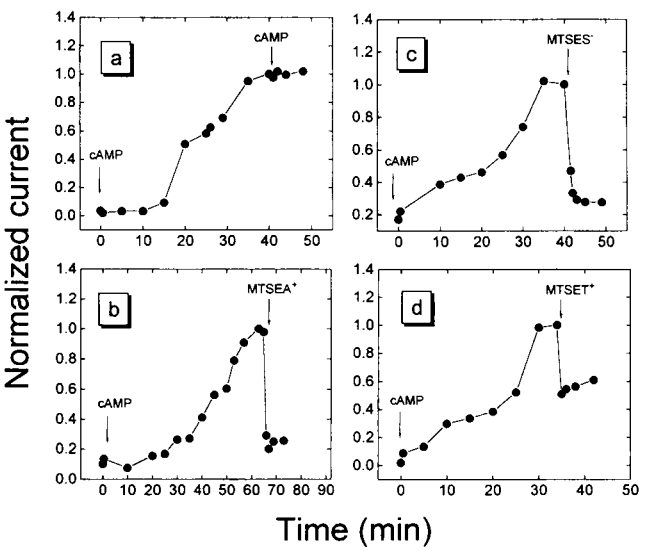

FIGURE 3 Effect of the MTS reagents on the CFTR-induced current in oocytes expressing the R347C mutant. The normalized current at a potential of −100 mV from individual oocytes under two-electrode voltage clamp is plotted as a function of time. At the arrows marked cAMP, 200 μM cpt-cAMP, 20 μM forskolin, and 1 mM IBMX were added to the bath to activate CFTR. After the current reached a plateau, at the arrow, the MTS reagent was added to the bath in the continued presence of cpt-cAMP, forskolin, and IBMX; in *A* only the cAMP reagents were added as a control; (*B*) 2.5 mM $MTSEA^+$; (*C*) 10 mM $MTSES^-$; (*D*) 1 mM $MTSET^+$. The currents were normalized to the value of the current immediately preceding the application of the MTS reagent, as follows: (*A*) 4565 nA; (*B*) 1875 nA; (*C*) 1777 nA; (*D*) 2837 nA.

pressing wild-type CFTR had no significant effects on the CFTR-induced currents. The wild-type CFTR-induced currents increased by less than 15% during an 8-min application of 10 mM $MTSES^-$, 2.5 mM $MTSEA^+$, or 1 mM $MTSET^+$. Because a similar increase [11 ± 6% (N = 3) (data not shown)] was observed when buffer alone, without the MTS reagents, was applied to the oocytes, the increase is presumably due to continued activation of additional CFTR channels. Hence, we inferred that either the 17 wild-type cysteines in CFTR were not accessible to the MTS reagents, or MTS modifications of these cysteines had no effect on the CFTR chloride conductance. In particular, we assume that the lack of effect on wild-type CFTR implies that the sulfhydryl groups of the four wild-type cysteine residues in putative membrane-spanning segments M2, M4, M6 (343), and M7 of CFTR are not facing into the CFTR pore. In this background, we tested the susceptibility of the engineered cysteines to the MTS reagents.

Accessibility of substituted cysteines to $MTSES^-$

A 1-min application of 10 mM $MTSES^-$ significantly inhibited the CFTR-induced currents of 9 of the 24 cysteine-substituted mutants (Fig. 4 *A*), L333C, R334C, K335C, F337C, S341C, R347C, T351C, R352C, and Q353C. An 8-min application of 10 mM $MTSES^-$ to these mutants did not markedly increase the inhibitory effects, except for the mutant Q353C, in which the extent of inhibition increased (Fig. 4 *B*); hence the reactions for the other mutants were complete within 1 min.

Accessibility of substituted cysteines to $MTSEA^+$ and $MTSET^+$

All nine of the mutants that reacted with $MTSES^-$ were also significantly inhibited by a 1-min application of 2.5 mM $MTSEA^+$ (Fig. 5 *A*). An 8-min application of 2.5 mM $MTSEA^+$ to these mutants did not markedly increase the inhibitory effects (Fig. 5 *B*); thus the reactions were complete within 1 min. In addition, both a 1-min and an 8-min application of 2.5 mM $MTSEA^+$ significantly inhibited the mutant I331C (Fig. 5). Another mutant, I344C, reacted with $MTSEA^+$ more slowly, requiring an 8-min application of 2.5 mM $MTSEA^+$ to significantly alter the current (Fig. 5 *B*). Unlike its effect on the other mutants, the reaction of $MTSEA^+$ with I344C resulted in potentiation of the subsequent current.

We also examined the ability of a larger, permanently positively charged reagent, $MTSET^+$, to react with three of the mutants, R334C, R347C, and R352C, that were susceptible to $MTSEA^+$. One-minute and 8-min applications of 1 mM $MTSET^+$ inhibited the CFTR-mediated current of the mutants R334C by 53 ± 6% and 52 ± 7% (n = 3); R347C by 44 ± 2% and 36 ± 3% (n = 3) (Fig. 3 D); and R352C by 46 ± 11% and 54.6 ± 10.5% (n = 3). Thus, these reactions were complete within 1 min.

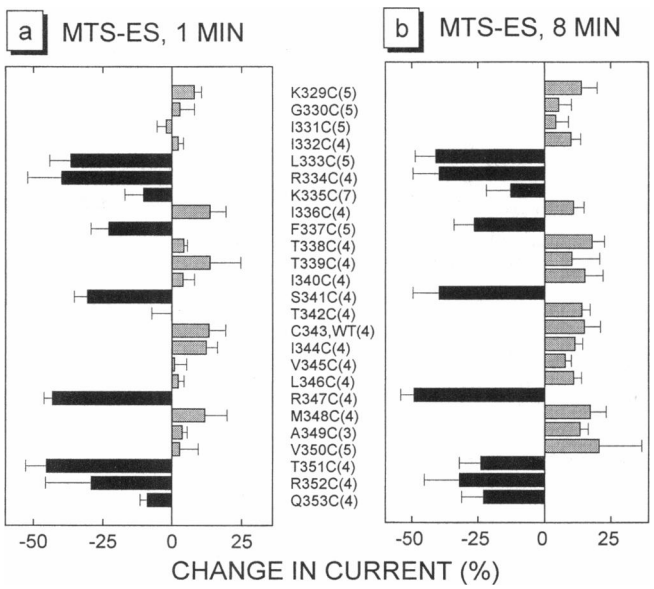

FIGURE 4 The relative effect of 1- and 8-min applications of $MTSES^-$ on wild-type and mutant CFTR-induced currents. The percentage change in the current (*A*) 1 min and (*B*) 8 min after application of 10 mM $MTSES^-$ is shown for wild type (WT) and the 24 cysteine-substitution mutants. The results for wild type are shown for the C343 position. Negative change indicates inhibition of the subsequent currents, positive change indicates potentiation of the subsequent currents. The mean and SEM are shown; the number of oocytes is shown in parentheses next to the mutant name between the two panels. Black bars indicate mutants for which the change was significantly different ($p < 0.05$) from wild type by one-way ANOVA.

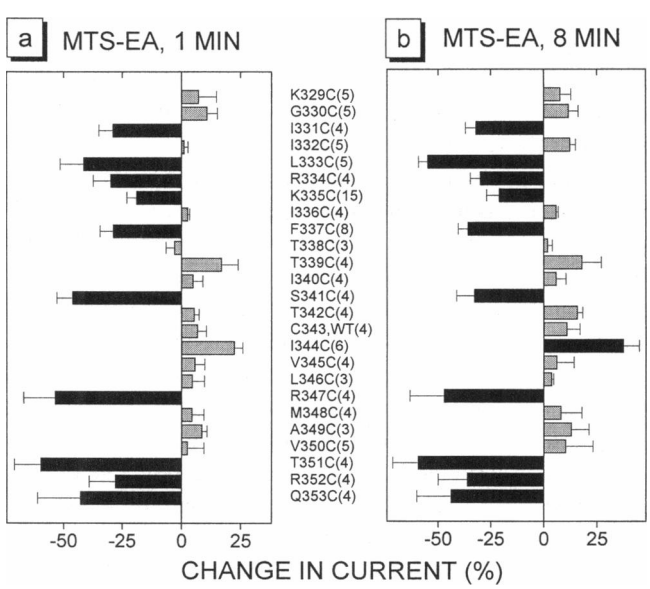

FIGURE 5 The relative effect of 1- and 8-min applications of $MTSEA^+$ on wild-type and mutant CFTR-induced currents. The percentage change in the current (*A*) 1 min and (*B*) 8 min after application of 2.5 mM $MTSEA^+$ is shown for wild type (WT) and the 24 cysteine-substitution mutants. The results for wild type are shown for the C343 position. Negative change indicates inhibition of the subsequent currents; positive change indicates potentiation of the subsequent currents. The mean and SEM are shown; the number of oocytes is shown in parentheses next to the mutant name between the two panels. Black bars indicate mutants for which the change was significantly different ($p < 0.05$) from wild type by one-way ANOVA.

DISCUSSION

Water-exposed residues in and flanking the M6 segment

The CFTR-induced currents of 11 of 24 cysteine-substitution mutants in and flanking the M6 membrane-spanning segment were irreversibly altered by extracellular application of the MTS reagents (Figs. 4 and 5), which, therefore, reacted with these engineered cysteines. We assume that the MTS reagents will only react with engineered cysteine residues that are on the water-accessible surface of the protein, because these reagents are highly polar and because the rate of reaction of the MTS reagents with an ionized thiolate anion is 5×10^9 times faster than the rate of reaction with the unionized thiol (Roberts et al., 1986); only sulfhydryls exposed to water will ionize to a significant extent. We infer that the structure of each mutant is similar to the structure of wild type because we observed CFTR-induced currents comparable to wild type in oocytes expressing each of the 24 cysteine-substitution mutants. Therefore, for those mutants that reacted with the MTS reagents, we infer that the corresponding wild-type residues Ile331, Leu333, Arg334, Lys335, Phe337, Ser341, Ile344, Arg347, Thr351, Arg352, and Gln353 are on the water-accessible surface of the protein. We assume that the only water-accessible surface for residues in membrane-spanning segments is the surface exposed in the ion channel lumen. However, residues at the extracellular end of a membrane-spanning segment or in an extracellular loop may be on the water-accessible surface of the protein, and therefore may be accessible to the MTS reagents, but are not part of the channel lining. As is discussed below, this may be the situation for residues Ile331 and Lys335.

Our inference that a residue is exposed if it reacts with the MTS reagents is independent of whether the reaction results in potentiation or inhibition of the subsequent currents. Elucidation of the mechanism of channel block by the MTS reagents will require the determination of the effects of modification on single-channel conductance and open probability. Nevertheless, our observation that reaction of both negatively and positively charged MTS reagents with susceptible residues caused inhibition implies that the effects of modification may not be due solely to local electrostatic effects. The slow rate of reaction of $MTSEA^+$ with the I344C mutant and the inability of the larger, anionic $MTSES^-$ to react with I344C suggests that access of the reagents to this residue may be sterically limited, possibly by neighboring residues on other membrane-spanning segments.

It is important to recognize that lack of effect of the MTS reagents with a particular engineered cysteine does not necessarily imply that the residue is buried. Local steric factors might prevent reaction, or the chemical modification may alter single-channel conductance and open probability in a reciprocal manner, resulting in little or no change in the macroscopic current.

Extent of the M5 to M6 loop

The extracellular location of the N-terminal end of the M6 segment has been elegantly demonstrated by glycosylation site insertion (Chang et al., 1994); however, the precise residue(s) that begins the membrane-spanning segment has not been defined. In the original hydrophobicity analysis the extracellular loop between M5 and M6 was predicted to consist of a single residue, Lys329 (Riordan et al., 1989). At least three residues, however, are required for a 180° turn (Creighton, 1984); thus the loop may include additional residues. Because Ile331 does not lie on the side of the helix that is exposed at more cytoplasmic positions in the M6 segment (Fig. 6; see below) and because CFTR can be glycosylated when six residues containing a glycosylation consensus sequence are inserted between Lys329 and Gly330, the loop is likely to extend at least as far as Ile331.

Secondary structure of the M6 segment

Based on the observation that the channel-lining residues in the extracellular half of the M1 segment lay on one side of the helix when plotted on an α-helical wheel, we inferred that the secondary structure was α-helical (Akabas et al., 1994b). In contrast, the overall pattern of the channel-lining residues in the M6 segment is not consistent with an α-helical secondary structure (Fig. 6). Although most of the MTS-accessible residues lie on what would be one side of an α-helix, there are

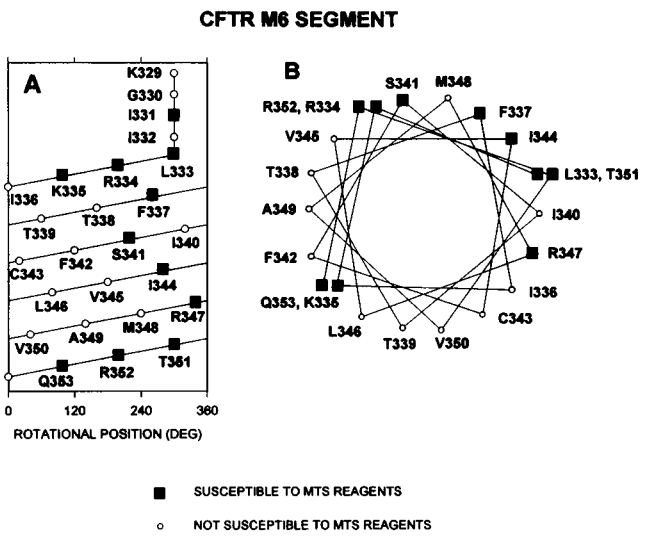

FIGURE 6 α-Helical representations of the residues in and flanking the M6 membrane-spanning segment of CFTR. Black squares indicate residues that are susceptible to the MTS reagents; open circles indicate residues that were not susceptible to the MTS reagents. (*A*) Helical net representation. The extracellular end is at the top; the intracellular end is at the bottom. The *x* axis represents the position on the circumference of the helix. Residues that are aligned vertically are on the same face of the helix. The residues K329 to I332 are not drawn as a helix, to indicate that they are likely to be part of the loop connecting M5 and M6. (*B*) Helical wheel representation of the residues from L333 to Q353. Note that K335 and Q353 lie on the opposite side of the helix from the exposed face.

several notable exceptions: Met348 is predicted to be on the exposed side of the helix but was unaffected by the MTS reagents, Ile340 is at the edge of the exposed side of the helix but was unaffected by the MTS reagents, and the exposed residues Lys335 and Gln353 are on the opposite side of the helix from the channel-exposed face.

Based on the accessibility of the residues from Phe337 to Ile344, we would predict that Met348 should be accessible and Arg347 should be inaccessible if the secondary structure of the region from Phe337 to Met348 were α-helical (Fig. 6). Because the opposite pattern of exposure was observed, i.e., Arg347 is accessible and Met348 is not, a possible explanation is that the secondary structure of the segment from Val345 to Met348 might be a 3^{10} helix rather than an α helix. In a 3_{10} helix the rotation around the helix axis would be 120° per residue, and Arg347 would be on the exposed face, not Met348. The last turn of an α-helical segment often assumes a 3_{10} helix conformation (Creighton, 1984). Thus, the secondary structure of the M6 segment might be α-helical above Ile344 and terminate in a 3_{10} helix from Val345 to Met348.

The two exposed residues, Lys335 and Gln353, that are predicted to be on the opposite side of an α helix from the channel-exposed face (Fig. 6 *B*), are each in regions where three consecutive residues are accessible. At the extracellular end, in the region of Lys335, this could be consistent with an α-helical secondary structure, although it could also be consistent with an extended secondary structure. If the extracellular end of the M6 segment (333 to 335) is α-helical and extends above the plane of the membrane, then Lys335 could be exposed to the extracellular bath on the back side of the helix but not in the channel. In this case the effects of modification on the K335C mutant by the MTS reagents would have to be indirect. Alternatively, there could be an extended secondary structure in this region (333 to 335), exposing all three residues in the channel lining.

The accessibility of three residues at the cytoplasmic end of M6, in the region of Gln353, is inconsistent with an α-helical secondary structure. There must be an extended structure at least in the region of Gln353. This extended structure might possibly be part of a membrane-embedded, cytoplasmic loop extending into the channel lumen, as in the L3 loop of porins (Weiss et al., 1991; Cowan et al., 1992; Schirmer et al., 1995) and proposed for the P-region of voltage-gated channels (Kurz et al., 1995; Lu and Miller, 1995; MacKinnon, 1995; Pascual et al., 1995; Sun et al., 1996) and the M2 segment of the glutamate receptors (Hollmann et al., 1994). In CFTR, as in the other channels, such a channel-embedded loop might be important in forming the charge selectivity filter, the size selectivity filter, or the gate.

Alternatively, in the cases of Lys335 and Gln353, we do not know that all of the residues are exposed in the same state of the channel. All of our experiments have been performed on channels in the activated state, in which the channel undergoes rapid transitions between the open and closed states. If the conformation or the orientation of the M6 segment is different in the open and closed states, then our pattern of exposed residues would be a composite of the two conformations.

Although we favor the explanations given above, there are several other explanations for the accessibility or lack of accessibility of any given residue that we cannot exclude and which would alter the interpretation of secondary structure: 1) Although it is tempting to assume that residues for which the MTS reagents had no effect are not exposed in the channel lumen, we cannot rule out the possibility that Ile340 and/or Met348 might be exposed in the channel but did not react, because of local steric factors. 2) Because we have measured the effects of modification using macroscopic currents, it is possible that, for example, modification might increase open probability but decrease single-channel conductance or vice versa, thereby resulting in an unaltered macroscopic current. Finally, a cysteine mutation might distort the local structure, altering the accessibility of the cysteine relative to the corresponding wild-type residue.

Diameter of the channel lumen

The ability of $MTSET^+$ to react with the R352C mutant indicates that it can penetrate from the extracellular end to this level. $MTSET^+$ is roughly cylindrical in shape and would fit into a right cylinder 6 Å in diameter and 10 Å in length. Therefore, the channel diameter from the extracellular end to the position of R352C, near the cytoplasmic end of the M6 segment, must be at least 6 Å. It is important to recognize that this is a minimum diameter for the channel from the extracellular end to the level of Arg352. If the size selectivity filter were located at a more cytoplasmic position than Arg352, then the channel might only be permeant to smaller anions. Based on osmotic permeability and tracer flux measurements, water and urea were permeable through CFTR, but methylglucose, about 6 Å in diameter, and sucrose were impermeant (Hasegawa et al., 1992). Several groups have suggested that ATP is permeant through the CFTR channel (Reisen et al., 1994; Schwiebert et al., 1995). If ATP is permeant, the channel diameter must be at least 10 Å.

Position of the charge selectivity filter

We found that all of the channel-lining residues are accessible to the cationic reagent $MTSEA^+$. To rule out the possibility that $MTSEA^+$ was entering the channel as an uncharged amine, we tested the ability of the permanently charged reagent, $MTSET^+$, to react with several channel-lining residues. $MTSET^+$ reacted with residues as cytoplasmic as Arg352, confirming that positively charged ions can enter the channel. This implies that cations can penetrate from the extracellular end of this anion-selective channel at least as far as Gln353. Either the charge selectivity filter is near the cytoplasmic end of the channel, or, because of the lack of ideal anion selectivity in the CFTR channel, the permeability ratio of Cl^-/Na^+ is about 10 (Anderson et al., 1991b), cations can enter the channel. Measurement of the rates of reaction will be necessary to determine the position of the charge selectivity filter.

Relationship to previous studies of residues in the M6 segment

The functional roles of residues in the M6 segment have been studied by other investigators. The CFTR channel displays anomalous mole fraction effects, which suggests that the channel contains multiple ion-binding sites. The multiple ion occupancy effects were eliminated by mutation of Arg347 to Asp or His, and the single-channel conductance was reduced (Tabcharani et al., 1993). These authors concluded that Arg347 must be near an anion-binding site in the channel; consistent with their conclusions, we have shown that Arg347 is exposed in the channel lumen. Curiously, the mutation R347E was reported to have little effect on the relative halide permeability or conductance sequences of the channel (Anderson et al., 1991b). The mutation K335E, a water-accessible residue, altered the relative halide permeability and conductance sequences (Anderson et al., 1991b). It did not, however, affect multiple ion occupancy effects, although it did reduce the single-channel conductance to about 4 pS (Tabcharani et al., 1993). Another mutation in M6, S341A, caused a fourfold reduction in the affinity for the voltage-dependent channel blocker diphenylamine-2-carboxylate (McDonough et al., 1994); we have shown that Ser341 is exposed in the channel lumen and thus could interact with a channel blocker.

Mutations of several residues in the M6 segment have been identified in patients with cystic fibrosis, including Arg334, Thr338, and Arg347 (Dean et al., 1990; Nunes et al., 1991; Tsui, 1992; Audrézet et al., 1993; Férec et al., 1993; Saba et al., 1993). Some of these are channel-lining residues, but others are not (Fig. 6). The mutations R334W and R347P have been shown to reduce single-channel conductance and open probability (Sheppard et al., 1993).

CFTR may provide insights into structure-function relationships in other ABC transport proteins

CFTR is a member of the ABC superfamily (Ames et al., 1992) that includes transporters such as the multiple drug resistance P-glycoprotein (Gottesman and Pastan, 1993), the transporter for antigen presentation (Heemels and Ploegh, 1995), and the STE6 transporter in yeast. Little is known about the structure of the membrane-spanning segments and their functional roles in other ABC transporters. Based on the homology with CFTR, our studies may provide insights into the structure of their membrane-spanning domains.

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REFERENCES

Akabas, M. H., and A. Karlin. 1995. Identification of acetylcholine receptor channel-lining residues in the M1 segment of the α-subunit. *Biochemistry.* 34:12496–12500.

Akabas, M. H., C. Kaufmann, P. Archdeacon, and A. Karlin. 1994a. Identification of acetylcholine receptor channel-lining residues in the entire M2 segment of the α subunit. *Neuron.* 13:919–927.

Akabas, M. H., C. Kaufmann, T. A. Cook, and P. Archdeacon. 1994b. Amino acid residues lining the chloride channel of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 269: 14865–14868.

Akabas, M. H., D. A. Stauffer, M. Xu, and A. Karlin. 1992. Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Science.* 258:307–310.

Altenbach, C., T. Marti, H. G. Khorana, and W. L. Hubbell. 1990. Transmembrane protein structure: spin labeling of bacteriorhodopsin mutants. *Science.* 248:1088–1092.

Ames, G. F-L., C. S. Mimura, S. R. Holbrook, and V. Shyamala. 1992. Traffic ATPases: a superfamily of transport proteins operating from *Escherichia coli* to humans. *Adv. Enzymol.* 65:1–47.

Anderson, M. P., H. A. Berger, D. P. Rich, R. J. Gregory, A. E. Smith, and M. J. Welsh. 1991a. Nucleotide triphosphates are required to open the CFTR chloride channel. *Cell.* 67:775–784.

Anderson, M. P., R. J. Gregory, S. Thompson, D. W. Souza, S. Paul, R. C. Mulligan, A. E. Smith, and M. J. Welsh. 1991b. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science.* 253:202–205.

Audrézet, M. P., B. Mercier, H. Guillermit, I. Quéré, C. Verlingue, G. Rault, and C. Férec. 1993. Identification of 12 novel mutations in the CFTR gene. *Hum. Mol. Genet.* 2:51–54.

Baukrowitz, T., T.-C. Hwang, A. C. Nairn, and D. C. Gadsby. 1994. Coupling of CFTR Cl^- channel gating to an ATP hydrolysis cycle. *Neuron.* 12:473–482.

Bear, C. E., F. Duguay, A. L. Naismith, N. Kartner, J. W. Hanrahan, and J. R. Riordan. 1991. Cl^- channel activity in *Xenopus* oocytes expressing the cystic fibrosis gene. *J. Biol. Chem.* 266:19142–19145.

Berger, H. A., S. M. Travis, and M. J. Welsh. 1993. Regulation of the cystic fibrosis transmembrane conductance regulator Cl^- channel by specific protein kinases and protein phosphatases. *J. Biol. Chem.* 268: 2037–2047.

Carson, M. R., S. M. Travis, and M. J. Welsh. 1995. The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. *J. Biol. Chem.* 270:1711–1717.

Chang, X. B., Y. X. Hou, T. J. Jensen, and J. R. Riordan. 1994. Mapping of cystic fibrosis transmembrane conductance regulator membrane topology by glycosylation site insertion. *J. Biol. Chem.* 269:18572–18575.

Chang, X-B., J. A. Tabcharani, Y.-X. Hou, T. J. Jensen, N. Kartner, N. Alon, J. W. Hanrahan, and J. R. Riordan. 1993. Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. *J. Biol. Chem.* 268:11304–11311.

Cheng, S. H., D. P. Rich, J. Marshall, R. J. Gregory, M. J. Welsh, and A. E. Smith. 1991. Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR chloride channel. *Cell.* 66: 1027–1036.

Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius, and J. P. Rosenbusch. 1992. Crystal structures explain functional properties of two *E. coli* porins. *Nature.* 358:727–733.

Creighton, T. E. 1984. Proteins: Structures and Molecular Properties. W. H. Freeman, New York.

Dean, M., M. B. White, J. Amos, B. Gerrard, C. Steward, K. T. Khaw, and M. Leppert. 1990. Multiple mutations in highly conserved residues are found in mildly affected cystic fibrosis patients. *Cell.* 61:863–870.

Férec, C., C. Verlingue, H. Guillermit, I. Quéré, O. Raguénés, J. Feigelson, M. P. Audrézet, P. Moullier, and B. Mercier. 1993. Genotype analysis of adult cystic fibrosis patients. *Hum. Mol. Genet.* 2:1557–1560.

Gottesman, M. M., and I. Pastan. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62: 385–427.

Gregory, R. J., S. H. Cheng, D. P. Rich, J. Marshall, S. Paul, K. Hehir, L. Ostergaard, K. W. Klinger, M. J. Welsh, and A. E. Smith. 1990. Expression and characterization of the cystic fibrosis transmembrane conductance regulator. *Nature.* 347:382–386.

Gunderson, K. L., and R. R. Kopito. 1995. Conformational states of CFTR associated with channel gating: the role of ATP binding and hydrolysis. *Cell.* 82:231–239.

Hasegawa, H., W. Skach, O. Baker, M. C. Calayag, V. Lingappa, and A. S. Verkman. 1992. A multifunctional aqueous channel formed by CFTR. *Science.* 258:1477–1479.

Heemels, M. T., and H. Ploegh. 1995. Generation, translocation and presentation of MHC class I-restricted peptides. *Annu. Rev. Biochem.* 64:463–492.

Hollmann, M., C. Maron, and S. Heinemann. 1994. N-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. *Neuron.* 13:1331–1343.

Hwang, T. C., M. Horie, and D. C. Gadsby. 1993. Functionally distinct phospho-forms underlie incremental activation of protein kinase-regulated Cl^- conductance in mammalian heart. *J. Gen. Physiol.* 101: 629–650.

Hwang, T. C., G. Nagel, A. C. Nairn, and D. C. Gadsby. 1994. Regulation of the gating of cystic fibrosis transmembrane conductance regulator Cl channels by phosphorylation and ATP hydrolysis. *Proc. Natl. Acad. Sci. USA.* 91:4698–4702.

Javitch, J. A., D. Fu, J. Chen, and A. Karlin. 1995. Mapping the binding-site crevice of the dopamine D2 receptor by the substituted-cysteine accessibility method. *Neuron.* 14:825–831.

Jung, K., H. Jung, J. Wu, G. G. Prive, and H. R. Kaback. 1993. Use of site-directed fluorescence labelling to study proximity relationships in the lactose permease of *Escherichia coli*. *Biochemistry.* 32: 12273–12278.

Kurz, L. L., R. D. Zuhlke, H. J. Zhang, and R. H. Joho. 1995. Side-chain accessibilities in the pore of a K^+ channel probed by sulfhydryl-specific reagents after scanning-cysteine mutagenesis. *Biophys. J.* 68:900–905.

Lu, Q., and C. Miller. 1995. Silver as a probe of pore-forming residues in a potassium channel. *Science.* 268:304–307.

MacKinnon, R. 1995. Pore loops: an emerging theme in ion channel structure. *Neuron.* 14:889–892.

McDonough, S., N. Davidson, H. A. Lester, and N. A. McCarty. 1994. Novel pore-lining residues in CFTR that govern permeation and open-channel block. *Neuron.* 13:623–634.

Mindell, J. A., H. Zhan, P. D. Huynh, R. J. Collier, and A. Finkelstein. 1994. Reaction of diphtheria toxin channels with sulfhydryl-specific reagents: observation of chemical reactions at the single molecule level. *Proc. Natl. Acad. Sci. USA.* 91:5272–5276.

Nunes, V., P. Gasparini, G. Novelli, A. Gaona, A. Bonizzato, F. Sangiuolo, A. Balassopoulou, F. J. Giménez, M. Dognini, M. Ravnik-Glavac, M. Cikuli, V. Mokini, R. Komel, B. Dallapiccola, P. F. Pignatti, D. Loukopoulos, T. Casals, and X. Estivil. 1991. Analysis of 14 cystic fibrosis mutations in five south European populations. *Hum. Genet.* 87:737–738.

Pascual, J. M., C. C. Shieh, G. E. Kirsch, and A. M. Brown. 1995. K^+ pore structure revealed by reporter cysteines at inner and outer surfaces. *Neuron.* 14:1055–1063.

Reisin, I. L., A. G. Prat, E. H. Abraham, J. F. Amara, R. J. Gregory, D. A. Ausiello, and H. F. Cantiello. 1994. The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel. *J. Biol. Chem.* 269:20584–20591.

Riordan, J. R., J. M. Rommens, B. S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou, M. T. Drumm, M. C. Iannuzzi, F. S. Collins, and L. C. Tsui. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science.* 254:1066–1073.

Roberts, D. D., S. D. Lewis, D. P. Ballou, S. T. Olson, and J. A. Shafer. 1986. Reactivity of small thiolate anions and cysteine-25 in papain towards methyl-methanethiosulfonate. *Biochemistry.* 25:5595–5601.

Saba, L., G. B. Leoni, A. Meloni, V. Faà, A. Cao, and M. C. Rosatilli. 1993. Two novel mutations in the transmembrane domains of the CFTR gene in subjects of Sardinian descent. *Hum. Mol. Genet.* 2:1739–1740.

Schirmer, T., T. A. Keller, Y.-F. Wang, and J. P. Rosenbusch. 1995. Structural basis for sugar translocation through maltoporin channel at 3.1 Å resolution. *Nature.* 267:512–514.

Schultz, B. D., C. J. Venglarik, R. J. Bridges, and R. A. Frizzell. 1995. Regulation of CFTR Cl^- channel gating by ADP and ATP analogues. *J. Gen. Physiol.* 105:329–361.

Schwiebert, E. M., M. E. Egan, T. H. Hwang, S. B. Fulmer, S. A. Allen, G. R. Cutting, and W. B. Guggino. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell.* 81:1063–1073.

Seibert, F. S., J. A. Tabcharani, X. B. Chang, A. M. Dulhanty, C. Mathews, J. W. Hanrahan, and J. R. Riordan. 1995. cAMP-dependent protein kinase-mediated phosphorylation of cystic fibrosis transmembrane conductance regulator residue Ser-753 and its role in channel activation. *J. Biol. Chem.* 270:2158–2162.

Sheppard, D. N., D. P. Rich, L. S. Ostedgaard, R. J. Gregory, A. E. Smith, and M. J. Welsh. 1993. Mutations in CFTR associated with mild-disease-form Cl^- channels with altered pore properties. *Nature.* 362: 160–164.

Slatin, S. L., X. Q. Qiu, K. S. Jakes, and A. Finkelstein. 1994. Identification of a translocated protein segment in a voltage-dependent channel. *Nature.* 371:158–161.

Smit, L. S., D. J. Wilkinson, M. K. Mansoura, F. S. Collins, and D. C. Dawson. 1993. Functional roles of the nucleotide-binding folds in the activation of the cystic fibrosis transmembrane conductance regulator. *Proc. Natl. Acad. Sci. USA.* 90:9963–9967.

Stauffer, D. A., and A. Karlin. 1994. Electrostatic potential of the acetylcholine binding sites in the nicotinic receptor probed by reactions of binding-site cysteines with charged methanethiosulfonates. *Biochemistry.* 33:6840–6849.

Steinhoff, H.-J., R. Mollaaghababa, C. Altenbach, K. Hideg, M. Krebs, H. Gobind Khorana, and W. L. Hubbell. 1994. Time-resolved detection of strucutral changes during the photocycle of spin-labeled bacteriorhodopsin. *Science.* 266:105–107.

Sun, Z. P., M. H. Akabas, E. H. Goulding, A. Karlin, and S. A. Siegelbaum. 1996. Exposure of residues in the cyclic-nucleotide-gated channel pore: P region structure and function in gating. *Neuron.* 16:141–149.

Tabcharani, J. A., X. B. Chang, J. R. Riordan, and J. W. Hanrahan. 1991. Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature.* 352:628–631.

Tabcharani, J. A., J. M. Rommens, Y. X. Hou, X. B. Chang, L. C. Tsui, J. R. Riordan, and J. W. Hanrahan. 1993. Multi-ion pore behavior in the CFTR chloride channel. *Nature.* 366:79–82.

Todd, A. P., J. Cong, F. Levinthal, C. Levinthal, and W. L. Hubbell. 1989. Site-directed mutagenesis of colicin E1 provides specific attachment sites for spin labels whose spectra are sensitive to local conformation. *Proteins.* 6:294–305.

Travis, S. M., M. R. Carson, D. R. Ries, and M. J. Welsh. 1993. Interaction of nucleotides with membrane-associated cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* 268:15336–15339.

Tsui, L-C. 1992. Mutations and sequence variations detected in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: a report from the cystic fibrosis genetic analysis consortium. *Hum. Mutat.* 1:197–203.

Weiss, M. S. U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G. E. Schulz. 1991. Molecular architecture and electrostatic properties of a bacterial porin. *Science.* 254:1627–1630.

Winter, M. C., D. N. Sheppard, M. R. Carson, and M. J. Welsh. 1994. Effect of ATP concentration on CFTR Cl^- channels: a kinetic analysis of channel regulation. *Biophys. J.* 66:1398–1403.

Xu, M., and M. H. Akabas. 1993. Amino acids lining the channel of the γ-aminobutyric acid type A receptor identified by cysteine substitution. *J. Biol. Chem.* 268:21505–21508.

Xu, M., and M. H. Akabas. 1996. Identification of channel-lining residues in the M2 membrane-spanning segment of the $GABA_A$ receptor α_1 subunit. *J. Gen. Physiol.* 107:195–205.

Xu, M., D. F. Covey, and M. H. Akabas. 1995. Interaction of picrotoxin with $GABA_A$ receptor channel-lining residues probed in cysteine mutants. *Biophys. J.* 69:1858–1867.