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Probing the Structural and Functional Domains of the CFTR Chloride Channel

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The cystic fibrosis transmembrane conductance regulator (CFTR) forms an anion-selective channel involved in epithelial chloride transport. Recent studies have provided new insights into the structural determinants of the channel's functional properties, such as anion selectivity, single-channel conductance, and gating. Using the scanning-cysteine-accessibility method we identified 7 residues in the M1 membrane-spanning segment and 11 residues in and flanking the M6 segment that are exposed on the water-accessible surface of the protein; many of these residues may line the ion-conducting pathway. The pattern of the accessible residues suggests that these segments have a largely α-helical secondary structure with one face exposed in the channel lumen. Our results suggest that the residues at the cytoplasmic end of the M6 segment loop back into the channel, narrowing the lumen, and thereby forming both the major resistance to ion movement and the charge-selectivity filter.

KEY WORDS: Ion selectivity; ion channel; ATP-binding cassette transporter; STE6; MDR; TAP; methanethiosulfonate; cysteine; periplasmic permease.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette/traffic ATPase membrane transporter gene superfamily (Ames *et al.*, 1992; Higgins, 1992; Riordan *et al.*, 1989). CFTR forms a chloride channel that is gated by a combination of phosphorylation and ATP binding and hydrolysis (Welsh *et al.*, 1992; Riordan, 1993; Gadsby *et al.*, 1995). CFTR mediates chloride transport in a variety of epithelia and in cardiac myocytes. Alterations in the activity or expression of CFTR play a role in the pathogenesis of cystic fibrosis (CF) and secretory diarrhea. Mutations in CFTR which reduce its function or level of expression are the molecular basis for CF, the most common lethal genetic disease in Caucasians. CF is characterized by defective chloride transport in a variety of epithelial organs, although many other abnormalities have been described (Boat *et al.*, 1989). Furthermore, abnormal activation of CFTR as a result of the actions of various enterotoxigenic bacterial toxins results in secretory diarrhea, a major cause of infant morbidity and mortality in the third world (Field and Semrad, 1993). Thus, understanding the structural bases for the functional properties of CFTR may provide new insights into the mechanisms of action both of CFTR and of other members of the family of ATP-binding cassette membrane transport proteins as well as provide new avenues for the treatment of CF and secretory diarrhea. The goal of this review is to summarize our current understanding of the structure of the chloride channel of CFTR and of the structural determinants of its functional properties. We will present our current working hypothesis for the structure of the channel based on our own work and the work of many other labs.

MEMBRANE TOPOLOGY OF CFTR

Analysis of the amino acid sequence of CFTR indicated that it contains 1480 amino acids divided

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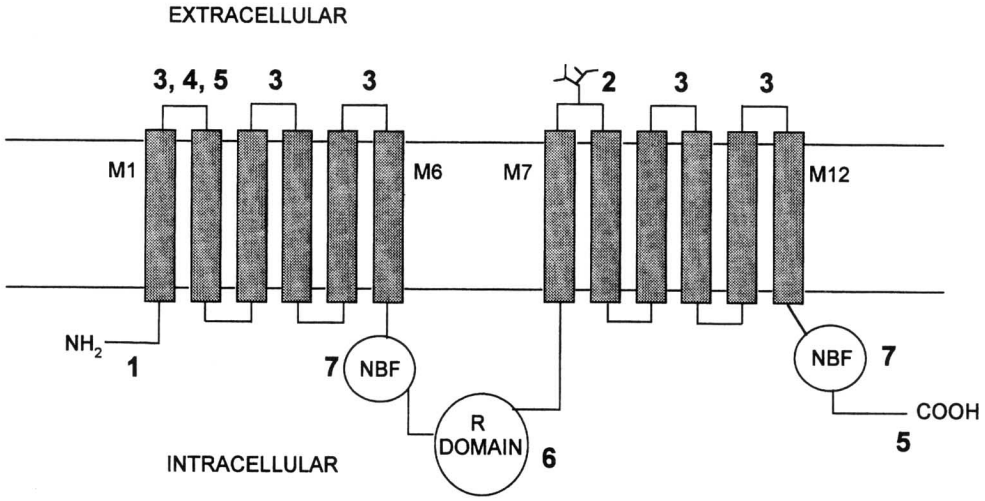

Fig. 1. Experimental support for the proposed transmembrane topology of CFTR is indicated by the numbers and the relevant references. (1) The N-terminus is predicted to be cytoplasmic due to the lack of a signal sequence (Riordan *et al.*, 1989). (2) The consensus glycosylation sites in the loop between M7 and M8 are glycosylated *in vivo* consistent with the predicted extracellular location of this loop (Gregory *et al.*, 1990; Chang *et al.*, 1994). (3) Removal of the endogenous sites and insertion of N-linked glycosylation sequences individually into each of the predicted extracellular loops yielded glycosylated CFTR (Chang, *et al.*, 1994). (4) The conductance of a CF mutant R117H was dependent on the pH of the extracellular solution (Sheppard *et al.*, 1993). (5) An epitope in the M1–M2 loop was accessible to antibody in nonpermeabilized cells whereas a C-terminal epitope was only accessible in permeabilized cells (Denning *et al.*, 1992a,b). (6) The R-domain is phosphorylated at multiple sites *in vivo* (Cheng *et al.*, 1990; Tabcharani *et al.*, 1991; Berger *et al.*,1993; Dechecchi *et al.*, 1993; Hwang *et al.*, 1994; Seibert *et al.*, 1995). (7) Cytoplasmic ATP is required in the processes of channel opening and closing (Anderson *et al.*, 1991a; Hwang *et al.*, 1993; Smit *et al.*, 1993; Travis *et al.*, 1993; Baukrowitz *et al.*, 1994; Hwang *et al.*, 1994; Winter *et al.*, 1994; Gunderson and Kopito, 1995). Modified from Akabas *et al.* (1994b).

into two homologous domains; each domain contains 6 putative membrane-spanning segments and a nucleotide-binding fold (NBF) (Riordan *et al.*, 1989). The two halves of the protein are connected by a highly charged, cytoplasmic, regulatory domain (R-domain). The R-domain contains multiple consensus sequences for phosphorylation by protein kinases A and C (Riordan *et al.*, 1989). The transmembrane topology predicted by sequence and hydrophobicity analysis (Riordan *et al.*, 1989) is supported by experimental evidence that is summarized in Fig. 1. Based on this transmembrane topology, 80% of the amino acids are located in the cytoplasm, 16% in the membrane-spanning segments, and 4% in the extracellular loops.

WHICH RESIDUES LINE THE CFTR CHLORIDE CHANNEL?

CFTR forms a nonrectifying, Cl^- channel with a small conductance (about 7–10 pS) (Riordan, 1993; Gadsby *et al.*, 1995) and weakly voltage-dependent gating kinetics (Fischer and Machen, 1994). The ion-conducting pathway is lined, at least in part, by residues from the membrane-spanning segments. Thus, in order to elucidate the structural bases for the functional properties of the CFTR chloride channel, we must first identify the residues that line the channel. To accomplish this goal we have applied the scanning-cysteine-accessibility method (SCAM) (Akabas *et al.*, 1992, 1994a) to CFTR. This approach allows the identification of residues that are on the water-accessible surface of the protein; in the case of an ion channel, at least in the open state, the residues that line the channel are part of the water-accessible surface of the protein.

In the scanning-cysteine-accessibility method, we mutate to cysteine consecutive residues, one at a time, in largely hydrophobic, membrane-spanning segments. We express the mutants in *Xenopus* oocytes and examine their functional properties *in situ*. If mutant channels are functional, we test whether the engineered cysteine is on the water-accessible surface of the pro-

tein by the ability of small, charged, hydrophilic, sulfhydryl-specific reagents to react covalently with the engineered cysteine. The reagents we use are derivatives of methanethiosulfonate (MTS): one negatively charged MTS-ethylsulfonate ($MTSES^-$) CH_3SO_2-SCH_2CH_2–SO_3^-, and two positively charged MTS-ethylammonium ($MTSEA^+$) $CH_3SO_2SCH_2CH_2$–NH_3^+, and MTS-ethyltrimethylammonium ($MTSET^+$) CH_2-$SO_2SCH_2CH_2$–$N(CH_3)_3^+$ (Akabas *et al.*, 1992; Stauffer and Karlin, 1994). The reaction between these reagents and a free sulfhydryl couples–SCH_2CH_2–X, the charged portion of the reagent, onto the sulfhydryl via a disulfide bond. The MTS reagents react a billion times faster with the ionized thiolate form of cysteine (RS^-) compared to the un-ionized thiol form (RSH) (Roberts *et al.*, 1986); only cysteines exposed to water will ionize to a significant extent. If the reagents react with a cysteine in the channel lining, it is likely that they will irreversibly alter ion conduction. Thus, we infer that the MTS reagents reacted with an engineered cysteine if conduction is irreversibly altered. Furthermore, for a cysteine mutant that reacted with the MTS reagents we infer that the side chain of the corresponding wild-type residue is exposed on the water-accessible surface of the protein. We assume that for residues in membrane-spanning segments only those residues exposed in the channel will be on the water-accessible surface. While we cannot exclude the possibility that there might be another water-filled crevice, besides the channel, extending into the interior of the protein, such a crevice has not been observed in the integral membrane proteins whose structures have been determined.

CFTR contains 18 endogenous cysteine residues: 4 in putative membrane-spanning segments and the remainder in domains predicted to be cytoplasmic. Following the expression and activation of wild type CFTR in *Xenopus* oocytes, an 8 min application of the MTS reagents to the extracellular bath has no effect on the CFTR-induced current (Akabas *et al.*, 1994b). This suggests that the endogenous cysteine residues are either inaccessible to extracellularly applied MTS reagents or that modification of one or more of the endogenous cysteines has no functional effect.

We have substituted cysteine, one at a time, for each of the residues in and flanking the M1 (Arg80 to Arg104) and M6 (Lys329 to Gln353) membrane-spanning segments (Akabas *et al.*, 1994b; Cheung and Akabas, 1996; Akabas, 1998). Following expression in *Xenopus* oocytes, each of these 49 mutants generated cAMP-activated chloride currents with linear current–voltage relationships. The cysteine substitutions were well tolerated by CFTR possibly because the lower temperature (17°C) at which the *Xenopus* oocytes are grown reduces the likelihood that the mutations will cause temperature-sensitive processing defects (Welsh and Smith, 1993).

Of the 49 cysteine-substitution mutants that we have screened to date, the MTS reagents irreversibly altered the currents of 18 mutants: 7 in the M1 segment (Fig. 2A) (Akabas *et al.*, 1994b; Akabas, 1998) and 11 in the M6 segment (Fig. 2B) (Cheung and Akabas, 1996). We infer that the corresponding wild-type residues are on the water-accessible surface of the protein. The accessible residues include 6 charged residues (4 arginines, 2 lysines), 6 noncharged, polar residues (2 glutamines, 2 glycines, 1 serine, 1 threonine), and 6 nonpolar residues (3 leucines, 2 isoleucines, 1 phenylalanine). The fraction of water-accessible, nonpolar residues is consistent with the observation that approximately half of the residues exposed on the surface of globular proteins of known crystal structure are nonpolar (Chothia, 1976).

There is no correlation between amino acid positions at which CF mutations have been identified and whether or not a residue is on the water-accessible surface of CFTR. This suggests that missense mutations in membrane-spanning segments that induce CF alter function by a variety of mechanisms.

SECONDARY STRUCTURE OF THE M1 AND M6 MEMBRANE-SPANNING SEGMENTS

We can infer the secondary structure of membrane-spanning segments from the pattern of the water-accessible residues. When the residues in the M1 segment are plotted on an α-helical wheel or net, the water-accessible residues, except for Gly85, lie on one side of the helix within an arc of 80° (Fig. 2A) (Akabas *et al.*, 1994b; Akabas, 1998), suggesting that the secondary structure of the M1 segment is largely α-helical. Based on the ideal α-helix we would have predicted that Tyr84 should have been accessible rather than Gly85. The experimental results suggest that the α-helix may be interrupted in this region. We cannot rule out the possibility that the cysteine substitutions might distort the local structure and thereby not provide an accurate representation of the water accessibility of the corresponding wild type residues. This seems unlikely, however, because it would require reciprocal effects in two independent cysteine substitution mutants,

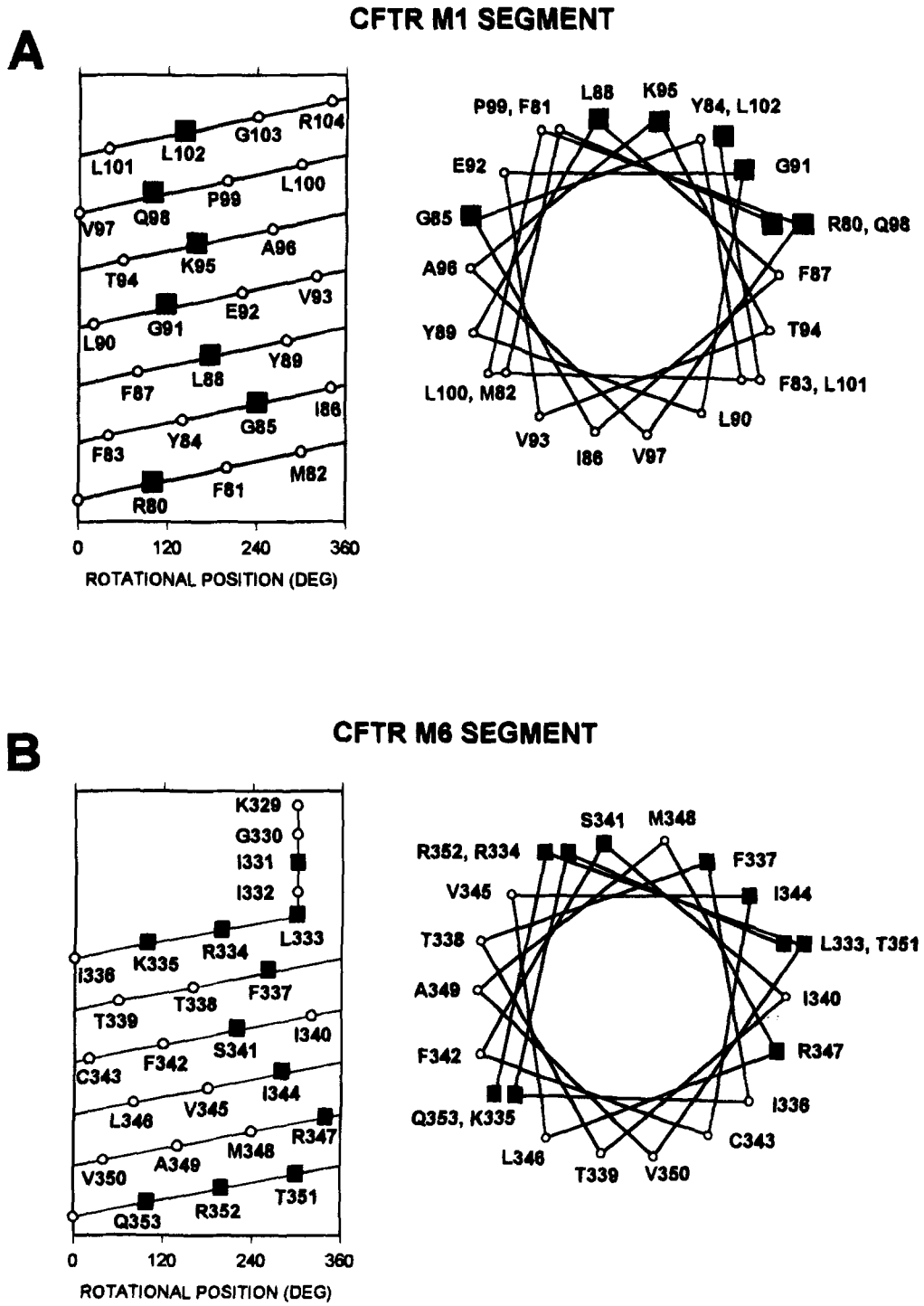

Fig. 2. Helical wheel (right) and net (left) representations of the residues in and flanking the (A) M1 and (B) M6 membrane-spanning segments. MTS-accessible residues are indicated by a black square, and inaccessible residues are indicated by an open circle. In the helical net representations extracellular is at the top, intracellular at the bottom, and 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. (Modified from Cheung and Akabas, 1997; Akabas, 1998.)

decreasing the accessibility at residue 84 and increasing the accessibility of residue 85.

When the residues in and flanking the M6 membrane-spanning segment are plotted on an α-helical representation (Fig. 2B), the pattern generated by the accessible residues is also largely consistent with an α-helical secondary structure (Cheung and Akabas, 1996). There are, however, some notable exceptions. Seven of the 11 accessible residues lie within an arc of 100°; however, Met348 lies within this arc but is not accessible.[3] The position of the Cα carbon of Arg347 does not lie within the accessible arc; however, given the long hydrophobic side chain of arginine the guanidinium group could still reach around to the accessible surface of the helix. Two accessible residues, Lys335 and Gln353, lie on the opposite side of the helix (Fig. 2B). The accessibility of Lys335, near the extracellular end of the M6 segment, does not preclude this portion of the M6 segment from being α-helical. The M6 segment may extend beyond the hydrophobic region of the membrane with Lys335 on the back side of the helix, away from the channel entrance but accessible to the extracellular solution. The effect of modification of the K335C mutant by the MTS reagents on CFTR current would be indirect.

The same explanation cannot be invoked for Gln353 at the cytoplasmic end of the M6 segment. Based on the accessibility of three consecutive residues, Thr351, Arg352, and Gln353, we inferred that the secondary structure of this region is not α-helical (Cheung and Akabas, 1996). By measuring the voltage dependence of the rates of reaction of the MTS reagents with exposed cysteines, we were able to calculate the electrical distance to these residues from the extracellular end of the channel. These results indicate that Arg352 is closer to the extracellular end than either of the adjacent residues (Fig. 3A), which is not consistent with an α-helical conformation (Cheung and Akabas, 1997). This suggests that this region flanking the cytoplasmic end of the M6 segment may loop back into the channel lumen (Fig. 4) forming a channel reentrant loop analogous to the L3 loop in porin (Weiss *et al.*, 1991; Cowan *et al.*, 1992; Schirmer *et al.*, 1995), the P-loop in voltage-gate ion channels (MacKinnon, 1995), or the M2 segment in the glutamate receptors (Hollman *et al.*, 1994; Kuner *et al.*, 1996).

[3] It is tempting to assume that the residues for which the sulfhydryl reagents had no effect are not exposed in the channel lumen. Although this is the likely explanation, it may not be correct in all cases. We have measured the effects of modification using macroscopic currents; thus, it is possible that modification might increase open probability but decrease single-channel conductance or *vice versa*, resulting in an unaltered macroscopic current. Alternatively, local steric constraints may prevent access of the reagents to an engineered cysteine residue in the channel lining or, as with any experiment using site-directed mutagenesis, the mutation may alter the protein structure so that the accessibility of the engineered cysteine does not accurately reflect the accessibility of the corresponding wild type residue.

DIAMETER OF THE CHANNEL LUMEN

$MTSET^+$ can penetrate from the extracellular end to react with Q353C (Cheung and Akabas, 1996). $MTSET^+$ is roughly cylindrical in shape and fits 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 Q353C, flanking the cytoplasmic end of the M6 segment, must be at a minimum 6 Å.

The size of the narrowest point in the channel can be inferred from the size of the largest permeant anion. NO_3^- (Anderson *et al.*, 1991c), SCN (Tabcharani *et al.*, 1993), and glutamate (Linsdell and Hanrahan, 1996) are reported to permeate the CFTR channel. Gluconate has been reported to be permeant by some investigators (Kartner *et al.*, 1991) but impermeant by others (Linsdell and Hanrahan, 1996). ATP (Grygorczyk *et al.*, 1996; Li *et al.*, 1996; Reddy *et al.*, 1996), aspartate (Anderson *et al.*, 1991c), and SO_4^{2-} (Reisin *et al.*, 1994) are reported to be impermeant through CFTR. Depending on the extent of hydration of these ions as they pass through the narrowest point in the channel, these results suggest that the minimum diameter at the narrowest region is about 5 to 6 Å. The reported permeability of CFTR to nonelectrolytes is consistent with this diameter; water and urea are permeant, but methylglucose and sucrose are not (Hasegawa *et al.*, 1992).

LOCATION OF THE CHARGE-SELECTIVITY FILTER

CFTR forms an anion-selective channel; however, the site and mechanism of charge selectivity is unknown. The channel is not ideally anion selective; chloride is 10 to 20 times more permeable than Na^+ (Anderson *et al.*, 1991b; Bear *et al.*, 1991). CFTR displays anomalous mole-fraction effects, indicating that there are multiple anion binding sites within the channel (Tabcharani *et al.*, 1993). Mutation of Arg347,

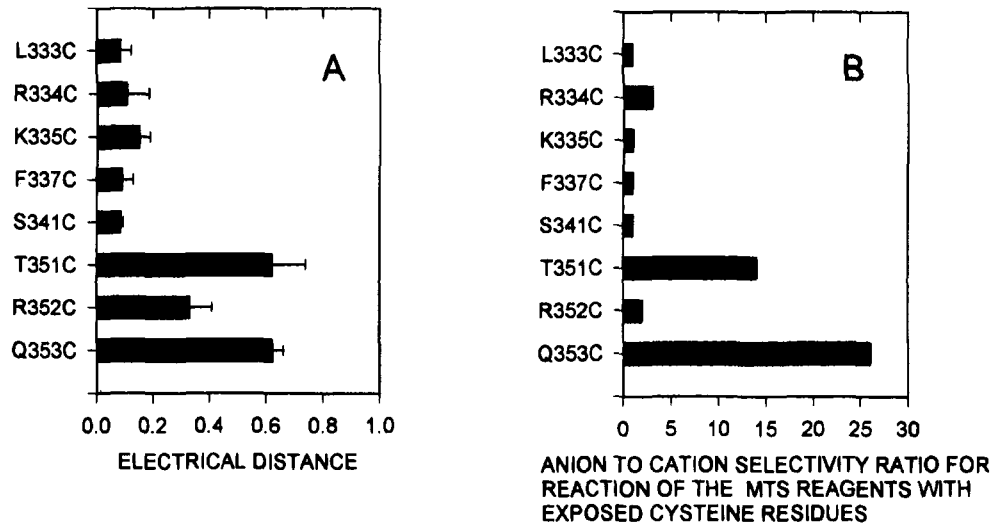

Fig. 3. (A) Average electrical distances from the extracellular end of the channel to the water-accessible cysteine residues in the M6 segment. Electrical distances determined from the voltage dependence of the reaction rates of $MTSES^-$ and $MTSET^+$ with the indicated cysteine residues. (Modified from Cheung and Akabas, 1997.) (B) Anion-to-cation selectivity ratio for the reaction of the $MTSES^-$ and $MTSET^+$ with the water-accessible cysteine residues in the M6 segment. A ratio of 1 indicates no selectivity; the larger the ratio, the greater the anion selectivity. (Modified from Cheung and Akabas, 1997.)

in the M6 membrane-spanning segment, eliminated the anomalous mole-fraction effects, suggesting that it was at or near an anion binding site (Tabcharani *et al.*, 1993). The Cl^- to Na^+ permeability ratio, however, was not altered by the mutation of Arg347 or of Lys95, Lys335, or Arg1030 to negatively charged residues (Anderson *et al.*, 1991b).

In order to identify the location of the charge-selectivity filter we measured the reaction rates of negatively and positively charged MTS reagents with channel-lining engineered cysteine residues in the M6 segment (Cheung and Akabas, 1997). There are two major determinants of the reaction rates at a membrane potential of 0 mV. First, the intrinsic electrostatic potential in the channel due to the protein and specific interactions between permeating ions, waters of hydration, and channel-lining residues will determine the ion selectivity of the access pathway from the extracellular solution through the channel to the engineered cysteine (Andersen and Koeppe, 1992). Second, because the MTS reagents react with the ionized thiolate form (RS^-) of cysteine (Roberts *et al.*, 1986; Stauffer and Karlin, 1994), the cationic MTS reagents react faster with small sulfhydryls in solution, such as 2-mercaptoethanol, than does the anionic reagent; the ratio of the reaction rates of $MTSES^-/MTSET^+$ with 2-mercaptoethanol is 0.08 (Stauffer and Karlin, 1994). The ratio of the reaction rates of $MTSES^-/MTSET^+$ with L333C, the most extracellular residue tested, is also 0.08, suggesting that there is no charge selectivity for the movement of the MTS reagents from the extracellular solution to L333C. Thus, in order to normalize for the difference in the intrinsic reaction rates of the two MTS reagents we divide the ratio of the rates at a given residue by the ratio of the reaction rates with L333C. In addition, this factors out differences in the extent of ionization of each engineered cysteine which might vary due to the local environment (Honig and Sharp, 1995). Thus, the ratio of the ratios of the reaction rate constants (Stauffer and Karlin, 1994) gives a measure of the anion selectivity of the pathway from the extracellular solution to each engineered cysteine (Fig. 3B). An anion selectivity ratio of 1 indicates no selectivity for anions over cations; the larger the ratio, the greater the anion selectivity. The anion selectivity ratio is about 1 for the residues in the extracellular half of the M6 segment (Fig. 3B). This implies that entry into the extracellular end of the channel is nonselective, i.e., both anions and cations can enter the extracellular portion of the channel.

The major site of charge selectivity appears to be in the region of T351C and Q353C where the anion-to-cation selectivity rises to between 15 and 25 (Fig. 3B) (Cheung and Akabas, 1997). It is interesting to note that at these two residues the rates of reaction of the negatively charged $MTSES^-$ are at least 2 times

larger than the rates with the other channel-lining residues. This region may form an anion-binding site where the residence time of the $MTSES^-$ is increased leading to the observed increase in the reaction rate. Furthermore, the reaction rate of the positively charged $MTSET^+$ with these two residues is decreased compared to the reaction rates with the other channel-lining residues. Because the size of these two reagents is similar, we believe that the selectivity is due to the opposite charge of the reagents.

Arg352, which is between T351C and Q353C, appears to be a major determinant of the anion selectivity in this region. Thus, when cysteine is substituted for Arg352 the charge selectivity drops to the same low level that is observed in the more extracellular portion of the channel (Fig. 3B). If other residues in this region were the main determinants of anion selectivity, then, the anion selectivity of the R352C mutant should have been similar to that of the adjacent residues. Moreover, based on our measurements of electrical distance, R352C is closer to the extracellular end of the channel than is T351C or Q353C (Fig. 3A). Thus, ions passing from the extracellular end of the channel would first encounter Arg352, which we infer forms part of the charge-selectivity filter, before they could reach T351C or Q353C, thereby accounting for the greater anion selectivity observed at these residues.

These measurements of anion selectivity reflect the location of the selectivity filter for charged MTS reagents. Although the reagents are able to penetrate from the extracellular end as far as Gln353, we do not know whether they are able to permeate through the entire CFTR channel. The MTS reagents fit into a right cylinder 6 Å in diameter and 10 Å in length. They are larger than a typical permeating anion such as Cl^-, which is about 3.6 Å in diameter. It remains to be shown that selectivity for small monovalent ions such as Cl^- and Na^+ is determined by the same residues that determine selectivity for the MTS reagents, and experiments are in progress to address this issue.

CFTR displays modest selectivity between monovalent halides. The permeability sequence is $Br^- > Cl^- > I^- > F^-$ (Anderson *et al.*, 1991b; Bear *et al.*, 1991; Kartner *et al.*, 1991; Cunningham *et al.*, 1992; Tabcharani *et al.*, 1992). This sequence is consistent with a binding site in the channel with moderately high affinity for anions (Wright and Diamond, 1977; Eisenman and Horn, 1983). The mutations K95D and K335E altered the halide permeability sequence, leading to the suggestion that these residues might be involved in anion binding (Anderson *et al.*, 1991b). The mutation K335E, however, did not alter the SCN^--induced anomalous mole fraction effects (Tabcharani *et al.*, 1993), and our data imply that Lys335 is on the opposite side of the helix from the channel-lining face (Fig. 2B) (Cheung and Akabas, 1996), making it unlikely that this residue is part of an anion binding site. More likely, the K335E mutation induces a conformational change in residues that form an anion-binding site, possibly at quite a distance from this residue. A similar mechanism is likely involved in the alteration of the halide permeability sequence by the P99L mutation (Sheppard *et al.*, 1996) because Pro99 is also not a channel-lining residue (Akabas *et al.*, 1994b).

ELECTRICAL DISTANCE TO THE EXPOSED RESIDUES AND THE ELECTRICAL POTENTIAL PROFILE WITHIN THE CHANNEL

There has been considerable speculation about the electrical field within an ion channel. Many theories have assumed that a transmembrane potential creates a constant field within the channel, i.e., the potential falls linearly along the length of the channel. The validity of this assumption is uncertain. By measuring the electrical distance to each channel lining residue we measure the fraction of the electrical potential through which the reagent passes from the extracellular end of the channel to the engineered cysteine residue (Cheung and Akabas, 1997). The lack of significant electrical distance to the residues from L333C to S341C (Fig. 3A) implies that the MTS reagents do not pass through the electrical field to reach these residues. There are several possible interpretations of this result. One, if there is no significant resistance to ion entry into the extracellular end of the channel the potential may not fall in this region of the channel. In this case the fall in potential, and the rise in the corresponding fractional electrical distance, would occur in the region of high resistance to ion movement, in the cytoplasmic half of the M6 segment (Fig. 3A). Two, if there are no binding sites for ions in this portion of the channel, then there would be little voltage dependence to the rate of reaction with residues in this region because voltage would equally effect both the rate of entry into and exit from this region of the channel. Under this circumstance, i.e., the absence of ion binding sites, the equilibrium Boltzmann analysis of the voltage dependence would not provide the correct electrical distance. Finally, we cannot exclude the possibil-

ity that the extracellular half of the M6 segment is not normal to the plane of the membrane, in which case access to these residues would not involve movement through the transmembrane electric field.

The electrical distance increases dramatically from S341C to T351C (Fig. 3A), suggesting that most of the electrical potential falls in this region of the channel. Therefore, this is likely to be the major site of resistance to ion movement in the channel. Thus, the channel appears to have a low-resistance extracellular end and a high-resistance cytoplasmic end. The anion-selectivity filter is located in the high-resistance region of the channel. The largest electrical distances that we measured, to T351C and Q353C, was only 0.6. This suggests that the channel extends beyond the level of these residues to involve residues that were predicted on the basis of hydrophobicity analysis to be cytoplasmic (Cheung and Akabas, 1997).

INHIBITORS OF CFTR-INDUCED CURRENTS

CFTR is blocked in a voltage-dependent manner by the arylaminobenzoates, diphenylamine-2-carboxylate (DPC) and flufenamic acid (Anderson *et al.*, 1991c; McCarty *et al.*, 1993; McDonough *et al.*, 1994), but it is not blocked by other Cl^- channel blockers such as DIDS and anthracene-9-carboxylate (Kartner *et al.*, 1991; Cunningham *et al.*, 1992). Ser341, in the M6 segment, has been implicated in the DPC binding site; mutation of this residue to alanine increased the K_d by a factor of 4 (McDonough *et al.*, 1994). Glibenclamide and tolbutamide, sulfonylureas that bind to the homologous sulfonylurea receptor and stimulate insulin secretion in pancreatic β cells (Aguilar-Bryan *et al.*, 1995), also inhibit the CFTR Cl^- channel (Sheppard and Welsh, 1992), reportedly causing open channel block (Venglarik *et al.*, 1996). Whether these inhibitors act by physically occluding the pore or whether the binding of the inhibitors induces a conformational change that drives the channel into a closed state is currently unknown.

GATING OF THE CFTR CHANNEL

There are two independent events which are necessary to open the CFTR channel. First, the protein must be phosphorylated. The extent of phosphorylation regulates the channel open probability (Hwang *et al.*, 1993, 1994) although the identification of the specific residues in the R-domain, the phosphorylation of which alters gating, remains uncertain (Cheng *et al.*, 1991; Chang *et al.*, 1993; Seibert *et al.*, 1995).

The second event required for channel activation is the binding and hydrolysis of ATP at one or both of the nucleotide-binding folds. The exact relationship between channel opening and closing and the timing of ATP binding and hydrolysis at the two NBF's remains a subject of intense investigation (Anderson *et al.*, 1991a; Baukrowitz *et al.*, 1994; Winter *et al.*, 1994; Carson *et al.*, 1995; Gadsby *et al.*, 1995; Gunderson and Kopito, 1995; Schultz *et al.*, 1995). To study the structure of the nucleotide-binding folds, the DNA encoding each of the nucleotide-binding folds has been engineered into fusion proteins with the maltose-binding protein, overexpressed in *E. coli* and purified. The purified fusion proteins appear to fold appropriately and to bind and hydrolyze ATP (Ko *et al.*, 1993, 1994; Ko and Pedersen, 1995). Potential structural relationships of the NBF's to both ATPases (Hyde *et al.*, 1990; Ko and Pedersen, 1995) and GTPases (Carson and Welsh, 1995) have been discussed.

The location of the gate and the conformational change involved in the transition from the closed to the open state is unknown. The mutation of residues in many regions of the protein, both in membrane-spanning segments and in cytoplasmic domains, has been shown to alter gating kinetics (Sheppard *et al.*, 1993, 1995; Cotten *et al.*, 1996; Seibert *et al.*, 1996; Wilkinson *et al.*, 1996). The changes in gating kinetics by mutations in the membrane-spanning domains suggest that the membrane-spanning segments undergo a conformational change during gating. In the nicotinic acetylcholine receptor, we showed that the surface accessibility of residues in membrane-spanning segments changes during gating (Akabas *et al.*, 1994a); as a result of these conformational changes, the mutation of residues in membrane-spanning segments of the acetylcholine receptor induce changes in the gating kinetics. Thus, phosphorylation and/or the binding and hydrolysis of ATP at the NBF's must induce a conformational change in the membrane-spanning segments of CFTR.

Removal of a large portion of the R-domain results in expression of a chloride current whose gating is regulated by cytoplasmic ATP; phosphorylation still causes small increases in Cl^- flux (Rich *et al.*, 1991). It was hypothesized that the R-domain may function as the channel gate (Rich *et al.*, 1991, 1993); however, the remaining requirement for ATP to open and close

the channel suggests that a significant portion of the gate is formed by another part of the protein.

A MODEL OF THE CFTR CHANNEL OR OUR WORKING HALLUCINATION

Based on the results summarized above we have developed a model of the CFTR channel (Fig. 4): It is important to emphasize that this is an evolving model based on considerable speculation but it provides a framework for the design of further experiments. We believe that the diameter of the channel lumen is large over most of its length and that it narrows near the cytoplasmic end of the channel. The residues Thr351, Arg352, and Gln353, flanking the cytoplasmic end of the M6 segment, form part of the channel lining in this narrow region, possibly as part of a reentrant loop into the channel lumen. Other residues predicted to be cytoplasmic may also form reentrant loops and participate in lining the narrow portion of the channel. Based on our results this narrow region is the site of high resistance to ion movement and the location of both the anion and the size selectivity filters.

The wider portion of the channel, from the extracellular side of the narrow region to the extracellular end of the channel, is accessible to both anions and cations (Fig. 3B). Based on the lack of voltage dependence of the reaction of the MTS reagents with cysteines substituted for residues lining this region of the channel (Fig. 3A) there appears to be little resistance to the movement of ions from the extracellular solution into this portion of the channel and/or little ion binding in this region of the channel. The membrane-spanning segments, with primarily α-helical secondary structure, form the walls of this wide portion of the channel.

RELATIONSHIP TO OTHER ATP-BINDING-CASSETTE MEMBRANE TRANSPORTERS

Unlike most members of this gene family, CFTR is not an ATP-driven membrane transporter. Nevertheless, the structure and mechanisms of action of CFTR may be similar to other members of the gene family. If we assume that the structure of CFTR is similar to the structure of other members of the ATP-binding-cassette, membrane-transporter gene superfamily (ABC transporters) then the model presented above may provide insights into how some members of this

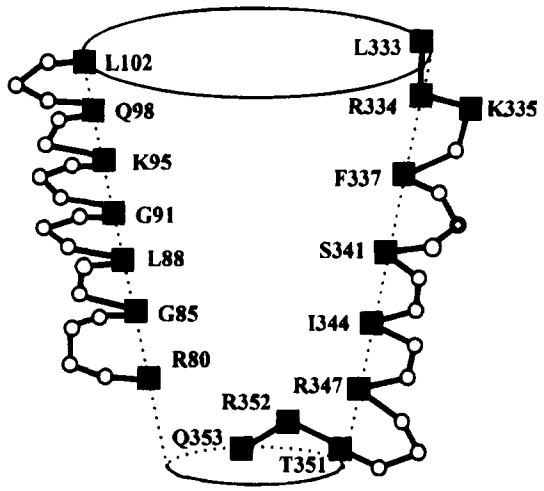

Fig. 4. Cartoon of the CFTR channel illustrating the residues of the M1 and M6 segments lining part of the channel wall. The residues flanking the cytoplasmic end of the M6 segment are shown looping back into the channel lumen; this narrows the lumen and forms the anion-selectivity filter. Black squares indicate the MTS-accessible residues, and open circles indicate the MTS-inaccessible residues. (Modified from Cheung and Akabas, 1997.)

superfamily, such as the yeast STE6 transporter (Michaelis, 1993) and the transporter for antigen presentation (TAP) (Heemels and Ploegh, 1995) are able to transport molecules as large as peptides, which in an extended conformation would be about 15 Å across. If we assume that in the ABC transporters the arrangement of the membrane-spanning segments is similar and creates a pore with a diameter sufficiently large to accommodate an extended peptide, then the specificity of the channel or transporter would mainly be determined by the "narrow" region of the pore, lined by the reentrant loops at the cytoplasmic end of the transport pathway. These loops, possibly formed by residues flanking the cytoplasmic end of the M6 and perhaps the M12 segments or other putative cytoplasmic residues, would be the sites of substrate binding. The hydrolysis of ATP would result in conformational changes in these reentrant loops that would drive movement of the substrate through the pore.

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