Molecular Determinants of Anion Selectivity in the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Pore

Paul Linsdell, Alexandra Evagelidis, and John W. Hanrahan
Department of Physiology, McGill University, Montréal, Québec H3G 1Y6, Canada

ABSTRACT Ionic selectivity in many cation channels is achieved over a short region of the pore known as the selectivity filter, the molecular determinants of which have been identified in Ca^{2+}, Na^+, and K^+ channels. However, a filter controlling selectivity among different anions has not previously been identified in any Cl^- channel. In fact, because Cl^- channels are only weakly selective among small anions, and because their selectivity has proved so resistant to site-directed mutagenesis, the very existence of a discrete anion selectivity filter has been called into question. Here we show that mutation of a putative pore-lining phenylalanine residue, F337, in the sixth membrane-spanning region of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, dramatically alters the relative permeabilities of different anions in the channel. Specifically, mutations that reduce the size of the amino acid side chain present at this position virtually abolish the relationship between anion permeability and hydration energy, a relationship that characterizes the anion selectivity not only of wild-type CFTR, but of most classes of Cl^- channels. These results suggest that the pore of CFTR may indeed contain a specialized region, analogous to the selectivity filter of cation channels, at which discrimination between different permeant anions takes place. Because F337 is adjacent to another amino acid residue, T338, which also affects anion selectivity in CFTR, we suggest that selectivity is predominantly determined over a physically discrete region of the pore located near these important residues.

INTRODUCTION

A defining feature of ion channels is their selectivity, the ability to pass certain ions at a high rate while at the same time effectively excluding others. In voltage-gated cation channels, discrimination between different cations is known to occur over a short region of the pore known as the selectivity filter. Our understanding of how selectivity is achieved has been greatly enhanced by the recent identification of the molecular determinants of the selectivity filters of Ca^{2+} (Yang et al., 1993; Ellinor et al., 1995), Na^+ (Heinemann et al., 1992; Favre et al., 1996), and K^+ channels (Heginbotham et al., 1994; Doyle et al., 1998). Two major mechanisms by which these channels achieve their physiologically crucial selectivity have been suggested. Selectivity may result from selective high-affinity binding of the ion in question (for example, in Ca^{2+} channels; Almers and McCleskey, 1984; Hess and Tsien, 1984; Yang et al., 1993; Ellinor et al., 1995) or from electrostatic ion-channel interactions that effectively allow only the ion in question to enter the selectivity filter (for example, in K^+ channels; Doyle et al., 1998). Of course, there is some degree of overlap between these two mechanisms; both suggest that permeant ions bind within the selectivity filter. In comparison, little is known about the molecular mechanism of anion selectivity in Cl^- channels.

Anion channels are much less selective than cation channels, usually allowing most small anions to permeate to some extent (see below). This low selectivity probably results from the fact that Cl^- is the predominant anion in all biological fluids, such that an evolutionary pressure to establish and maintain strong selectivity does not exist in anion channels. Most classes of Cl^- channels that have been studied in detail show very similar anion selectivity, corresponding to the lyotropic sequence, with weakly hydrated anions (lyotropes) showing a higher permeability than those that bind water molecules more strongly (kosmotropes) (e.g., Bormann et al., 1987; Giraldez et al., 1989; Li et al., 1990; Halm and Frizzell, 1992; Kubo and Okada, 1992; Arreola et al., 1995; Verdon et al., 1995; Jackson et al., 1996; Linsdell and Hanrahan, 1998a). Discrepancies from this sequence, where they have been reported (e.g., Fahlke et al., 1997; Rychkov et al., 1998), are usually small; permeability of the kosmotropic F^- ion is universally low in anion channels. However, the molecular basis of lyotropic anion selectivity has not been determined for any Cl^- channel. The characteristic relationship between permeability and hydration energy suggests that anion dehydration is the limiting factor in anion permeability in Cl^- channels, perhaps reflecting the fact that more work is required to dehydrate an anion than a cation of similar size (Dorman et al., 1996; Marcus, 1997; Dawson et al., 1999).

To date, disruption of lyotropic anion selectivity after mutagenesis of a Cl^- channel has not been reported. In fact, where significant alterations in anion selectivity have been reported, in ClC-1 (Fahlke et al., 1997) and in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Linsdell et al., 1998), the overall effect of mutations was to strengthen the lyotropic nature of the selectivity

Received for publication 29 June 1999 and in final form 28 February 2000.
Address reprint requests to Dr. Paul Linsdell, Department of Physiology and Biophysics, Dalhousie University, Sir Charles Tupper Medical Building, Halifax, Nova Scotia B3H 4H7, Canada. Tel.: 902-494-2265; Fax: 902-494-1685; E-mail: paul.linsdell@dal.ca.

0006-3495/00/06/2973/10 $2.00

sequence. The relative insensitivity of the anion selectivity of CFTR to mutations within the putative pore region led Dawson et al. (1999) to suggest that "anion permeability ratios, which to a first approximation measure how easy it is for an anion to enter the channel, are determined by the difference between the energy required to dehydrate the anion and some stabilization energy that is the consequence of a general interaction of the anion with the pore that is not highly dependent on the details of channel structure." Thus, based on current evidence, the very existence of a discrete anion selectivity filter in the pores of Cl^- channels is questionable, and its necessity for normal channel function is dubious.

Lyotropic anion selectivity occurs not only in Cl^- channels but also in numerous biological and physicochemical systems involving ions in solution (Dani et al., 1983; Collins and Washabaugh, 1985; Cacace et al., 1997; Collins, 1997). Studies with model compounds have suggested that a lyotropic anion binding site requires the combination of an anion-attracting group (a positive charge or dipole) and a neighboring hydrophobic group around which the mobility of water molecules is reduced (Dani et al., 1983).

Previously we showed that the anion selectivity of the CFTR Cl^- channel could be altered by mutation of a threonine residue (T338) in the sixth transmembrane region (TM6) (Linsdell et al., 1998). Because the amino acid side chain at this position is not thought to be in contact with the aqueous lumen of the channel pore (based on substituted cysteine accessibility mutagenesis experiments; Cheung and Akabas, 1996), we suggested that mutations at this position may affect channel permeation properties via a change in the orientation of the TM6 α-helix. Based on the proposed importance of hydrophobic residues in lyotropic anion binding outlined above, we have now examined the effects of mutating two large, hydrophobic amino acid residues in TM6 (F337 and I344), the side chains of which are thought to be in contact with the aqueous pore lumen (Cheung and Akabas, 1996). We find that mutations that reduce the size of the side chain present at position 337 lead to a loss of the characteristic lyotropic relationship between anion permeability and hydration energy. The effects of these point mutations suggest that the CFTR Cl^- channel pore may indeed contain a localized region at which lyotropic anion selectivity is predominantly determined.

MATERIALS AND METHODS

Mutagenesis and expression of CFTR

Mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Fifty nanograms of pNUT-CFTR plasmid DNA (Tabcharani et al., 1991) was incubated with two synthesized complementary oligonucleotides containing the desired mutation, deoxynucleoside triphosphates at a final concentration of 250 μM each, and *Pfu* reaction buffer included in the kit. Temperature cycling was then performed with a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA) as follows. After a hot start at 95°C, 2.5 U of *Pfu* DNA polymerase was added, and the mixture was overlaid with 30 μl of mineral oil. Denaturation was performed at 95°C for 30 s, annealing at 55°C for 1 min, and elongation at 68°C for 20 min, for a total of 16 cycles. When the temperature cycling was completed, the mixture was treated with *Dpn*I for 2 h at 37°C to digest methylated and hemimethylated DNA, thereby removing the DNA template. Competent XL-1 Blue *Escherichia coli* was transformed with the mutated DNA, and several colonies were obtained. DNA was isolated from at least three colonies, and the mutated region was sequenced with the T7 Sequenase kit (Amersham Life Science, Baie D'Urfe, QC, Canada) to verify the presence of the mutation.

Subconfluent baby hamster kidney (BHK) cells were transfected with mutated pNUT-CFTR DNA by the calcium phosphate precipitation method, as described previously (Linsdell et al., 1998). Transfected cells were selected using 500 μM methotrexate (Faulding (Canada) Inc., Vaudreuil, QC, Canada) after 48 h. Individual colonies were picked and amplified after approximately 1 week of growth in methotrexate-containing medium. Whole-cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting, using the M3A7 anti-CFTR monoclonal antibody to verify expression of CFTR, as described previously (Linsdell et al., 1998).

Electrophysiological recording

Macroscopic CFTR current recordings were made using the excised, inside-out configuration of the patch-clamp technique, as described previously (Linsdell and Hanrahan, 1996, 1998a; Hanrahan et al., 1998). Channels were activated after patch excision by exposure of the cytoplasmic face of the patch to 40–60 nM protein kinase A catalytic subunit (PKA) (prepared in the laboratory of Dr. M. P. Walsh, University of Calgary, Alberta, Canada, as described previously; Tabcharani et al., 1991) plus 1 mM MgATP (Sigma Chemical Co., Oakville, ON, Canada). Solutions contained (in mM) 150 NaCl, 2 $MgCl_2$, 10 *N*-tris-(hydroxymethyl) methyl-2-aminoethanesulfonic acid; or 154 NaX (where X is the anion being tested), 2 $Mg(OH)_2$, 10 *N*-tris-(hydroxymethyl) methyl-2-aminoethanesulfonic acid. All solutions were adjusted to pH 7.4 by the addition of NaOH. Where the pipette solution did not contain any Cl^- (Table 3), the Ag/AgCl wire inside the pipette was protected by a NaCl-containing agar bridge. Given voltages have been corrected for measured liquid junction potentials of up to 6 mV between dissimilar pipette and bath solutions (Hanrahan et al., 1998). All chemicals were obtained from Sigma, except $NaClO_4$ and sodium methane sulfonate (Aldrich Chemical Co., Milwaukee, WI). Experiments with different anions were carried out on different patches.

Macroscopic current-voltage (*I-V*) relationships were constructed using depolarizing voltage ramp protocols, with a rate of change of voltage of 37.5–100 mV s^{-1} (see Linsdell and Hanrahan, 1996, 1998a). All *I-V* relationships shown have had the background (leak) current recorded before the addition of PKA subtracted digitally as described previously (Linsdell and Hanrahan, 1996, 1998a). Current traces were filtered at 100 Hz, using an eight-pole Bessel filter, digitized at 250 Hz, and analyzed using pCLAMP6 computer software (Axon Instruments, Foster City, CA). The current reversal potential, V_{rev}, was estimated by fitting a polynomial function to the *I-V* relationship and was used to estimate the permeability of different anions relative to that of Cl^- (P_X/P_{Cl}) according to the equation

$$P_X/P_{Cl} = \exp(\Delta V_{rev} F/RT), \quad (1)$$

where ΔV_{rev} is the difference between V_{rev} measured under biionic conditions with a test anion X^- and that measured with symmetrical Cl^--containing solutions, and *F*, *R*, and *T* have their usual thermodynamic meanings.

Experiments were carried out at room temperature (20–23°C). Throughout, mean values are presented as mean ± SEM. For graphical presentation of mean values, error bars represent ± SEM; where no error bars are shown, ±SEM is smaller than the size of the symbol.

RESULTS

The CFTR molecule consists of 12 TM regions, two cytoplasmic nucleotide binding domains (NBDs), and a cytoplasmic regulatory (R) domain (Fig. 1 *A*). We mutated two putative pore-lining hydrophobic amino acids in TM6, Phe^{337} (to alanine, serine, leucine, tyrosine, and tryptophan) and Ile^{344} (to alanine) (Fig. 1 *B*). All mutants constructed produced mature, fully glycosylated (band C) CFTR protein after stable expression in BHK cells, as judged by Western blotting (data not shown). In all cases, expression in BHK cells led to the appearance of PKA- and ATP-dependent anion currents (e.g., Fig. 2).

Anion selectivity was examined under biionic conditions, with Cl^--containing solutions in the extracellular (pipette) solution and test anions in the intracellular (bath) solution, as described previously for wild-type (Linsdell and Hanrahan, 1998a) and T338-mutated CFTR (Linsdell et al., 1998). Example leak-subtracted *I-V* relationships obtained with different intracellular anions are shown in Fig. 2. Because both control (pre-PKA stimulation) and stimulated currents were recorded under the same ionic conditions (to obtain a valid leak current), only one *I-V* relationship was obtained per patch. Therefore, experiments with different anions were carried out on different patches, and as such no information is contained in the relative current amplitudes in Fig. 2 (Linsdell and Hanrahan, 1998a; Linsdell et al., 1998). Mean relative permeabilities for different anions, estimated from the current reversal potential according to Eq. 1 (see Materials and Methods), are given in Table 1. F337W gave only small currents after expression in BHK cells, such that its full anion selectivity sequence could not be determined. The mutants F337L, F337Y, and I344A gave only modest alterations in anion permeability (Table 1) that led to only slight changes in the anion selectivity sequence (Table 2). In contrast, both F337A and F337S showed dramatically altered anion selectivity (Fig. 2 and Tables 1 and 2), characterized by large reductions in the relative permeability of lyotropic anions (Br^-, I^-, SCN^-, NO_3^-) and greatly increased permeability of the small, kosmotropic F^- anion. The effects of these mutations on the permeabilities of ClO_4^-, formate, and acetate were less striking (Table 1). Overall, the effect of these changes was to decrease the apparent ability of the CFTR channel to discriminate between different anions (Fig. 2), suggesting a reduction in anion selectivity in these mutants.

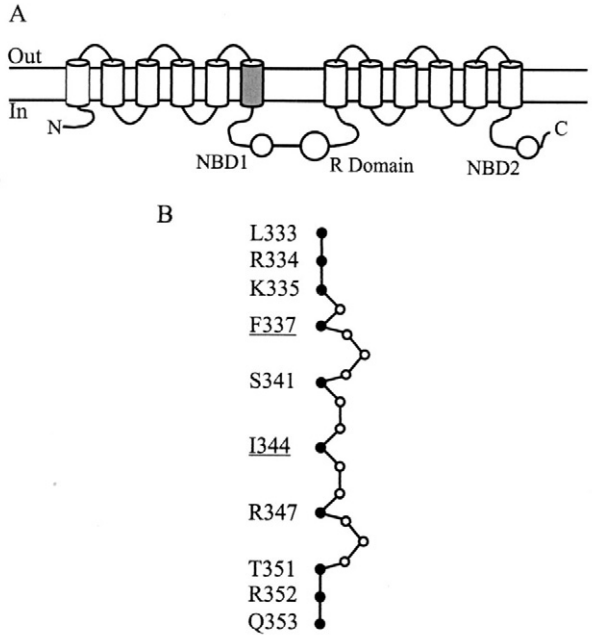

FIGURE 1 Cartoon showing the primary structure of the TM6 region of CFTR. (*A*) Proposed overall topology of CFTR, comprising 12 TM regions, two cytoplasmic NBDs, and the cytoplasmic R domain. The shaded area indicates TM6. (*B*) Primary sequence of TM6, after Cheung and Akabas (1996). Filled circles indicate those amino acids with side chains that have been proposed, on the basis of substituted cysteine accessibility mutagenesis experiments, to be in contact with the aqueous lumen of the pore (Cheung and Akabas, 1996). These include the two residues mutated in the present study, F337 and I344 (*underlined*).

As described previously for wild-type CFTR (Linsdell and Hanrahan, 1998a), the mutants F337A, F337S, and F337Y all showed negligible Na^+ permeability (Table 1). Sodium permeability was estimated from the change in reversal potential when 75% of NaCl in the intracellular solution was replaced by sucrose, as described previously (Linsdell and Hanrahan, 1998a).

The altered anion selectivity of F337A and F337S led to a disruption of the relationship between anion permeability and hydration energy in these mutants (Fig. 3). Both wild-type and F337Y (Fig. 3), as well as F337L, F337W, and I344A (not shown; see Table 2), were able to select for anions that bound water molecules less strongly, consistent with the lyotropic selectivity sequence common to most classes of Cl^- channels (see Introduction). In contrast, for both F337A and F337S, there was no obvious correlation between anion permeability and energy of hydration (Fig. 3), suggesting that lyotropic selectivity is greatly diminished in these mutants.

Initially we had set out to examine the role of hydrophobic amino acid side chains in CFTR anion selectivity. However, although the results summarized in Table 1 suggest that the large, hydrophobic residue F337 does contribute to lyotropic anion selectivity in the CFTR pore, the effects of different mutations indicate that it is side-chain size, not hydrophobicity, at position 337 that determines channel selectivity. Thus the mutant F337Y, which substitutes a similarly sized but polar side chain, has a selectivity that is almost identical to that of wild type. In contrast, the two mutations that strongly affect selectivity, F337A and F337S, both involve a substantial reduction in amino acid side-chain volume. The hypothesis that the effects of mutations at F337 are independent of side-chain polarity is supported

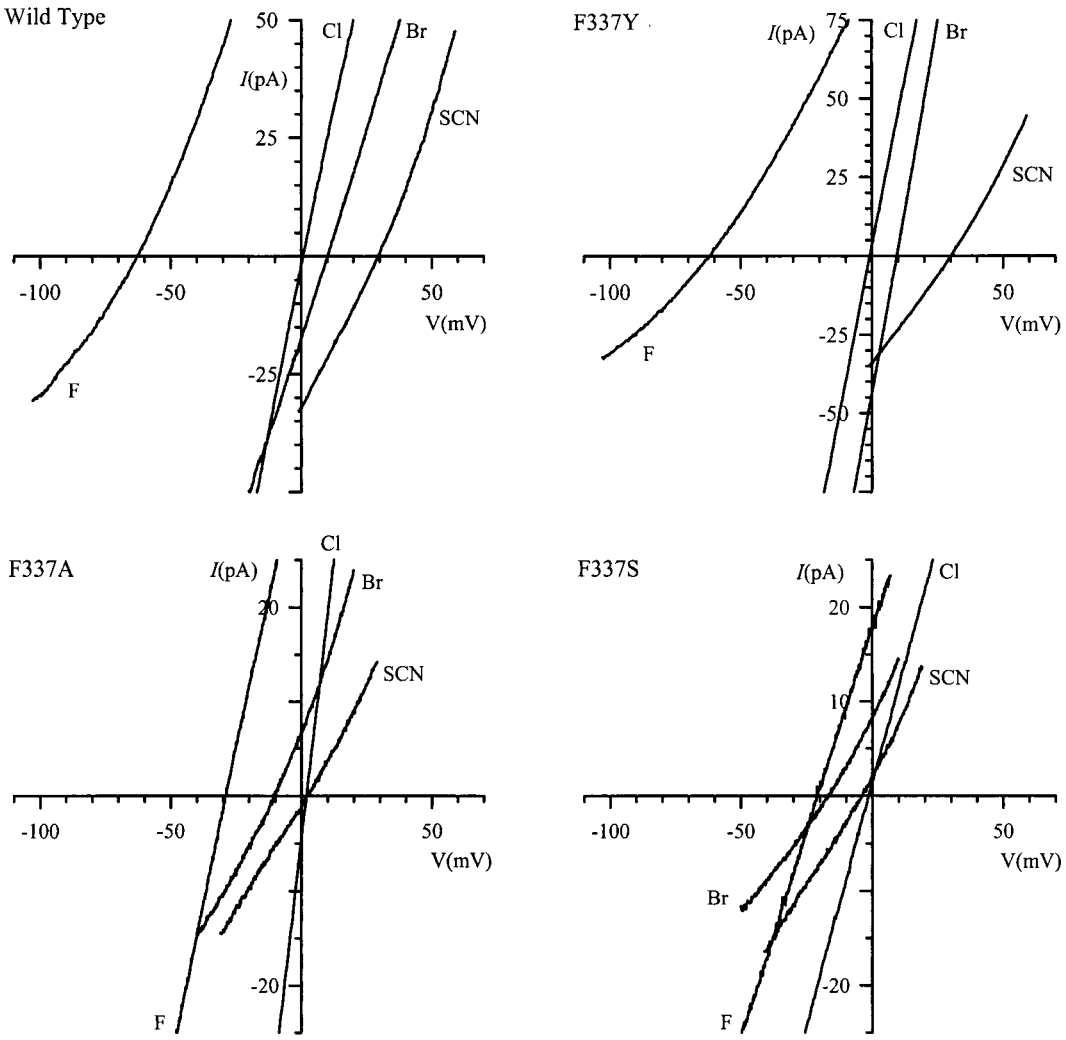

FIGURE 2 Anion selectivity of macroscopic CFTR currents. Leak-subtracted macroscopic *I-V* relationships were generated using depolarizing voltage ramp protocols as described in Materials and Methods. Each *I-V* relationship is from a different patch (see text). Currents were recorded under biionic conditions, with Cl^- in the extracellular solution and the named anion present in the intracellular solution. Note that the range of reversal potentials with different anions is greatly reduced in both F337A and F337S, indicating a reduced ability of the channel to discriminate between different anions.

by the fact that replacement of F337 by a small, hydrophobic alanine or by a small, polar serine gave rise to channels with identical anion selectivity sequences (Table 2).

The importance of side-chain size at position 337 in determining anion selectivity is directly illustrated in Fig. 4. The permeabilities of the two halides with lower hydration energies than Cl^- (Br^- and I^-), as well as that of the lyotropic anion with the highest permeability in wild type CFTR (SCN^-), increase with increasing side-chain volume at position 337, whereas the permeability of the kosmotropic halide F^- decreases with side-chain volume. A similar effect on the permeability of the lyotrope NO_3^- (not shown) is seen, whereas the permeabilities of the larger ClO_4^-, formate, and acetate anions were less strongly affected by the mutation of F337. Thus the presence of a large amino acid side chain at this position favors the permeability of small lyotropic anions and reduces the permeability of small kosmotropic anions.

One way in which the size of an amino acid side chain might affect selectivity is via a change in the physical dimensions of the narrowest part of the pore. Indeed, the selectivity filter is thought to be located at the most constricted region of the pore in both Na^+ (Sun et al., 1997) and K^+ (Doyle et al., 1998) channels. It was recently suggested that different classes of cation channels might achieve selectivity between monovalent cations based purely on the dimensions of the narrowest pore region (Laio and Torre, 1999). Estimation of the functional diameter of the CFTR pore is difficult and ambiguous because of the highly asymmetrical permeability of large organic anions (Linsdell and

TABLE 1 Relative permeability of intracellular ions in wild-type and mutant CFTR Cl^- channels

	Wild type	F337A	F337S	F337L	F337Y	F337W	I344A
Cl	1.00 ± 0.01 (10)	1.00 ± 0.04 (6)	1.00 ± 0.08 (3)	1.00 ± 0.02 (5)	1.00 ± 0.02 (6)	1.00 ± 0.03 (5)	1.00 ± 0.01 (9)
Br	1.37 ± 0.07 (8)	0.60 ± 0.04 (4)**	0.50 ± 0.04 (4)**	1.22 ± 0.04 (5)	1.39 ± 0.04 (3)	1.12 ± 0.05 (4)*	1.74 ± 0.01 (3)*
I	0.83 ± 0.03 (6)	0.23 ± 0.04 (5)**	0.23 ± 0.02 (4)**	0.39 ± 0.01 (3)**	0.69 ± 0.03 (7)*	—	0.99 ± 0.05 (4)*
F	0.103 ± 0.007 (9)	0.35 ± 0.01 (4)**	0.43 ± 0.02 (4)**	0.15 ± 0.02 (3)*	0.095 ± 0.009 (3)	0.081 ± 0.009 (3)	0.075 ± 0.012 (5)*
SCN	3.55 ± 0.26 (7)	0.97 ± 0.05 (4)**	0.93 ± 0.10 (5)**	2.85 ± 0.20 (4)	3.05 ± 0.29 (4)	4.42 ± 0.56 (4)	3.27 ± 0.30 (5)
NO_3	1.58 ± 0.04 (10)	1.30 ± 0.03 (3)*	1.08 ± 0.02 (4)**	1.38 ± 0.03 (4)*	1.43 ± 0.04 (3)	1.62 ± 0.03 (3)	1.71 ± 0.06 (4)
ClO_4	0.25 ± 0.01 (8)	0.19 ± 0.00 (3)*	0.17 ± 0.03 (4)*	0.23 ± 0.04 (3)	0.15 ± 0.01 (4)**	—	0.24 ± 0.02 (3)
Formate	0.24 ± 0.01 (9)	0.27 ± 0.02 (3)	0.33 ± 0.03 (4)*	0.35 ± 0.02 (3)*	0.24 ± 0.01 (3)	—	0.28 ± 0.01 (3)
Acetate	0.091 ± 0.003 (10)	0.073 ± 0.004 (3)*	0.12 ± 0.02 (5)	—	0.092 ± 0.014 (4)	—	0.076 ± 0.007 (3)
Na^+	0.007 ± 0.010 (24)	0.001 ± 0.018 (3)	0.001 ± 0.021 (5)	—	0.002 ± 0.004 (3)	—	—

Relative permeabilities for different anions present in the intracellular solution under biionic conditions were calculated from macroscopic current reversal potentials (e.g., Fig. 2), according to Eq. 1 (see Materials and Methods). Sodium permeability was estimated from the change in reversal potential when 75% of the NaCl in the intracellular solution was replaced by sucrose, as described previously (Linsdell and Hanrahan, 1998a). For all mutants, the current reversed close to 0 mV in symmetrical Cl^--containing solutions. Numbers in parentheses indicate the number of patches examined in each case. Asterisks indicate a significant difference from the corresponding value in wild type. *$P < 0.05$; **$P < 0.001$, two-tailed *t*-test.

Hanrahan, 1998a,b). Previously we have used the permeability of extracellular organic anions, the permeability of which does appear to be limited by steric factors, to gain some estimate of the functional dimensions of the pore (Linsdell et al., 1997, 1998; Linsdell and Hanrahan, 1998a). We used a similar approach to determine whether the altered anion selectivity of F337A and F337S was associated with any change in functional pore diameter (Table 3). For these experiments, the intracellular solution contained Cl^- and the extracellular solution contained the test anion under biionic conditions. The permeabilities of F337A and F337S to extracellular formate, acetate, and propanoate ions were not significantly different from those observed in wild-type CFTR, and both pyruvate and methane sulfonate were not measurably permeant in wild type, F337A, or F337S. Thus, given the experimental caveats outlined above, we find no evidence for any alteration in the functional dimensions of the pore associated with these mutations.

DISCUSSION

Numerous point mutations within the transmembrane regions of CFTR have been shown to affect pore properties such as unitary Cl^- conductance (Sheppard et al., 1993, 1996; Tabcharani et al., 1993; McDonough et al., 1994; Linsdell et al., 1998) and anion binding (Tabcharani et al., 1993; McDonough et al., 1994; Linsdell and Hanrahan, 1996; Mansoura et al., 1998). However, most mutations that have been studied to date have had little or no effect on the anion selectivity of the channel (Anderson et al., 1991; Hipper et al., 1995; Sheppard et al., 1996; Mansoura et al., 1998; Vankeerberghen et al., 1998). This has led to the suggestions that CFTR selectivity may be determined over the length of the pore rather than at a single site (Dawson et al., 1999) and that CFTR is not an ion channel (Hipper et al., 1995). However, the effects of the mutations F337A and F337S, which virtually abolish the normal lyotropic anion selectivity sequence (Tables 1 and 2 and Fig. 3) by decreasing the relative permeability of lyotropic anions and increasing that of kosmotropic anions (Fig. 4), support an alternative explanation, namely that selectivity is determined at a discrete region unaffected by previously studied mutations. This region may be somewhat analogous to the well-defined selectivity filters of cation channels (see Introduction). However, the physiological significance of an anion "selectivity filter" in anion channels that are so poorly selective is unclear. Because Cl^- is the only lyotropic anion in biological fluids (Collins, 1997), selectivity for lyotropic anions may be a simple way to ensure high Cl^- permeability.

As with all mutagenesis studies carried out in the absence of direct structural information, it is possible that the effects of mutations at F337 are due to a conformational change in the protein at some distance from the site of the mutation. Although we cannot rule out this possibility, we feel that the fact that mutations at two adjacent TM6 residues, F337 (this study) and T338 (Linsdell et al., 1998), significantly affect

TABLE 2 Anion selectivity sequences for wild-type and mutant CFTR Cl^- channels

Wild-type	$SCN^- > NO_3^- > Br^- > Cl^- > I^- > ClO_4^- \approx$ form $> F^-$ > ace
F337A	$NO_3^- > Cl^- \geq SCN^- > Br^- > F^-$ > form $\geq I^- > ClO_4^-$ > ace
F337S	$NO_3^- > Cl^- \geq SCN^- > Br^- > F^-$ > form $> I^- > ClO_4^-$ > ace
F337L	$SCN^- > NO_3^- > Br^- > Cl^- > I^-$ > form $> ClO_4^- > F^-$
F337Y	$SCN^- > NO_3^- \geq Br^- > Cl^- > I^-$ > form $> ClO_4^- > F^- \approx$ ace
I344A	$SCN^- > Br^- \geq NO_3^- > Cl^- \approx I^-$ > form $> ClO_4^-$ > ace $\approx F^-$

Sequences were derived from the relative anion permeabilities given in Table 1. form, formate; ace, acetate.

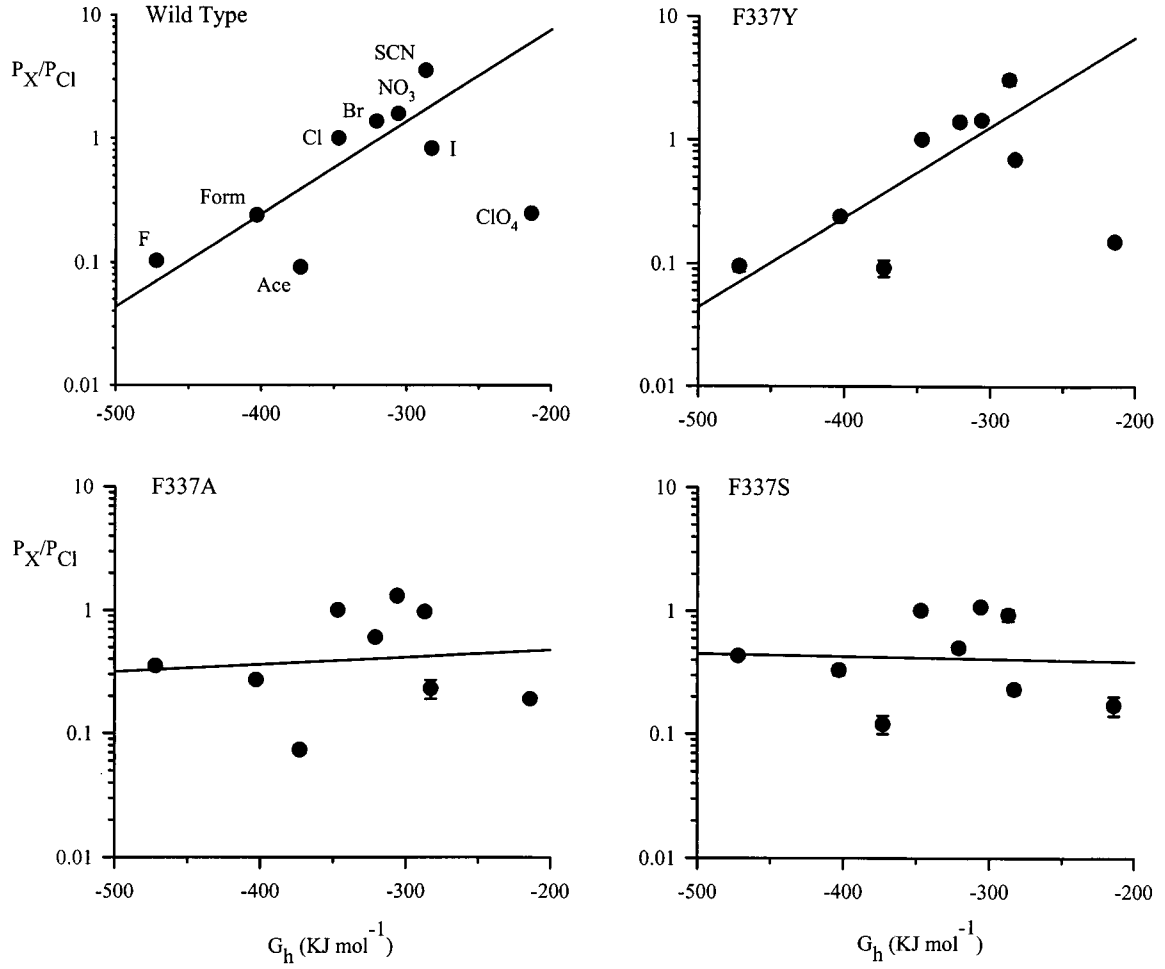

FIGURE 3 Relationship between relative anion permeability and hydration energy for wild-type and F337-mutated CFTR. Relative permeabilities (P_X/P_{Cl}) are as given in Table 1. Free energies of hydration (G_h) were taken from Marcus (1997). The anions illustrated are (*left to right*) F^-, formate, acetate, Cl^-, Br^-, NO_3^-, SCN^-, I^-, and ClO_4^-. Note that the permeability of ClO_4^-, the most weakly hydrated anion studied, is anomalously low in wild-type CFTR and is relatively unaffected by mutation of F337 (see Discussion).

anion selectivity suggests that anion selectivity is determined mainly over this region of the pore. A mutation in TM6 that greatly reduced the size of a more distant putative pore-lining hydrophobic amino acid residue, I344A, had no strong effect on selectivity (Tables 1 and 2). Furthermore, the mutations F337A and F337S altered selectivity between different anions without disrupting the ability of the channel to select for Cl^- over Na^+ (Table 1), supporting the hypothesis that the CFTR pore uses different mechanisms to determine lyotropic anion selectivity and anion:cation selectivity (Linsdell et al., 1998; Guinamard and Akabas, 1999).

The lyotropic anion selectivity sequence of wild-type CFTR, like that of most classes of Cl^- channels, illustrates the central role of anion dehydration in determining anion selectivity. This is consistent with the traditional view of ion permeability in channels, that the permeating ion is at least partially dehydrated as it passes through the channel, with the electrostatic interactions between the ion and its waters of hydration in free solution being replaced by interactions with polar groups on the walls of the pore (Hille, 1992; Dawson et al., 1999). Because the energy required to dehydrate the anion seems to control its permeability in Cl^- channels, electrostatic interactions between anions and Cl^- channel pores are presumed to be relatively weak compared to those between anions and water molecules, such that Cl^- channel anion selectivity sequences are usually "weak field strength," according to the nomenclature of Eisenman (Wright and Diamond, 1977; Eisenman and Horn, 1983). Nevertheless, it is clear that in CFTR, interactions between permeating anions and the pore do influence anion selectivity, because point mutations in the channel (F337A and F337S) disrupt the selectivity sequence.

Both F337A and F337S compromise the relationship between anion permeability and hydration energy (Fig. 3), suggesting a reduction in the relative importance of anion

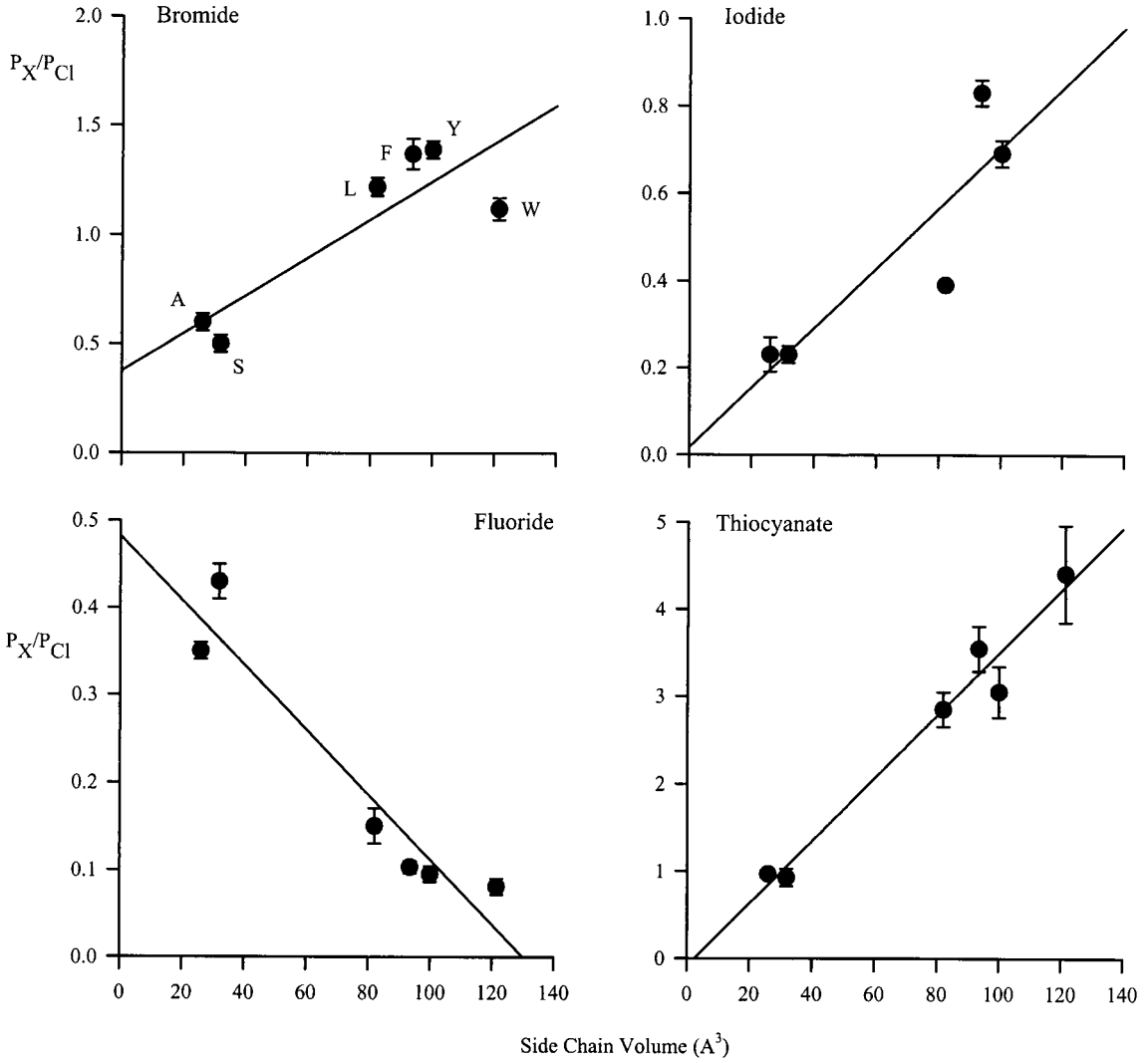

FIGURE 4 Relationship between the relative permeability of selected anions (P_X/P_{Cl}) and the size of the amino acid side chain at position 337. The permeabilities of the lyotropic anions Br^-, I^-, and SCN^- are positively correlated with side-chain size at this position, while the permeability of the kosmotrope F^- decreases as a function of side-chain size. Relative permeabilities are as given in Table 1. The side-chain volume of the amino acid present at position 337 was estimated according to the method of Richards (1974). The amino acids present at this position, in order of increasing side chain size, were alanine (A), serine (S), leucine (L), phenylalanine (F; wild-type), tyrosine (Y), and (except for I^-) tryptophan (W).

dehydration in determining permeability in these mutants. However, the permeability of ClO_4^-, the most weakly hydrated anion studied, is anomalously low in wild-type CFTR and is relatively unaffected by mutation of F337. The reason for the low ClO_4^- permeability of CFTR is unknown, as discussed previously (Linsdell et al., 1998). The permeability of ClO_4^- may be limited by factors other than anion dehydration; these other factors are apparently not affected by mutation of F337.

The suggestion that the amino acid side chain at position 337 is in contact with the aqueous lumen of the pore is based on the substituted cysteine accessibility mutagenesis study of Cheung and Akabas (1996). We have made no attempt to independently verify this work, which is known to be subject to certain experimental caveats (see, e.g., Dawson et al., 1999). Our own results, which suggest that the size of the side chain at this position plays a more important role in determining anion selectivity than its chemical nature, would be consistent with mutagenesis of either a pore-lining side chain (Cheung and Akabas, 1996) or an inaccessible side chain, which, when mutated, indirectly alters pore shape (Linsdell et al., 1998).

One possible explanation for the loss of the relationship between anion permeability and hydration energy in F337A and F337S is that anions are able to pass through the pores of these mutants with more of their associated waters of hydration intact than in wild type, so reducing the degree of anion dehydration required for permeation. This could re-

TABLE 3 Relative permeability of extracellular organic anions in wild-type and mutant CFTR Cl^- channels

	Wild type	F337A	F337S
Formate	0.129 ± 0.007 (4)	0.157 ± 0.013 (3)	0.112 ± 0.002 (3)
Acetate	0.038 ± 0.007 (4)	0.026 ± 0.008 (4)	0.029 ± 0.013 (3)
Propanoate	0.022 ± 0.003 (4)	0.024 ± 0.001 (3)	0.024 ± 0.002 (3)
Pyruvate	<0.011 (4)	<0.011 (3)	<0.011 (3)
Methane sulfonate	<0.011 (3)	<0.011 (3)	<0.011 (2)

Relative permeabilities for different organic anions present in the extracellular solution under biionic conditions were calculated as described in the legend to Table 1. Maximum permeabilities are given for ions that were not measurably permeant over the voltage range used. None of these values are significantly different from the corresponding value in wild type ($P > 0.05$ in each case, two-tailed *t*-test).

sult, for example, from a widening of the narrowest part of the pore due to replacement of the bulky phenylalanine side chain at position 337 with a smaller alanine or serine, which might allow more highly hydrated anions to fit through the pore. Although this is an attractive possibility, we feel that several factors argue against this explanation. Our own recent work has shown that the pore of CFTR is very wide (Linsdell and Hanrahan, 1998a,b), much larger than the diameter of an unhydrated Cl^- anion, such that it seems unlikely that steric factors contribute greatly to the permeability of small anions. In fact, for intracellular anions, the mutations F337A and F337S had a much stronger effect on the permeability of small anions (halides, SCN^-, NO_3^-) than on larger anions (ClO_4^-, formate, acetate), suggesting that removal of a steric barrier is not the primary effect of these mutations (Table 1). Furthermore, neither F337A nor F337S showed greatly altered permeability to extracellular organic anions (Table 3), the permeabilities of which do appear to be limited by unhydrated anion size (Linsdell et al., 1997, 1998; Linsdell and Hanrahan, 1998a). Although the relationship between the permeability of such organic anions, when present in the extracellular solution, and the actual physical dimensions of the pore, is unclear (Linsdell and Hanrahan, 1998a), the results summarized in Table 3 do not suggest a strong alteration in the functional dimensions of the pore in F337A or F337S.

A reduction in the relative importance of anion dehydration in determining permeability, as is suggested in F337A and F337S, could result not only from a decrease in the degree of anion dehydration, but also from an increase in the strength of the interaction between permeating anions and the channel pore. In terms of halide permeability, wild-type CFTR (permeability sequence $Br^- > Cl^- > I^- > F^-$) shows a moderately weak field strength selectivity sequence (Eisenman sequence III; Wright and Diamond, 1977). Under other experimental conditions, this sequence becomes $I^- > Br^- > Cl^- > F^-$ (Eisenman sequence I) (Tabcharani et al., 1997), a discrepancy that we suggested was due to a unique interaction between Cl^- and I^- ions within the pore. Under the macroscopic current recording conditions used here, the mutation T338A changes the halide selectivity from Eisenman sequence III to sequence I, consistent with the strengthening of lyotropic selectivity in this mutant (Linsdell et al., 1998). While the mutants F337L, F337Y, and I344A maintain Eisenman sequence III, both F337A and F337S convert the channel to a relatively strong field strength sequence ($Cl^- > Br^- > F^- > I^-$; Eisenman sequence V) (Table 2). This increase in field strength might imply that permeating anions interact more strongly with the pores of F337A and F337S than with wild-type CFTR. Consistent with this, CFTR single-channel conductance (measured with symmetrical 154 mM Cl^-) is reduced from 7.6 ± 0.1 pS ($n = 12$) for wild type to 1.8 ± 0.0 pS ($n = 7$) for F337S (P. Linsdell, unpublished observations).

The effects of mutations at position 337 on anion selectivity are clearly correlated with the size of the amino acid present at this position (Fig. 4); in contrast, they show no correlation with the polarity of the side chain. Thus (assuming that the side chain of F337 is pore lining; see above), we find no evidence to support the hypothesized role of hydrophobic groups in contributing to lyotropic anion selectivity (see Dani et al., 1983), although such hydrophobic groups may be contributed by other TM regions. There is evidence that TMs 1, 3, 5, and 12 may also contribute to the pore of CFTR (Anderson et al., 1991; Akabas et al., 1994; McDonough et al., 1994; Akabas, 1998; Mansoura et al., 1998; Dawson et al., 1999).

Thiocyanate in particular is well known to interact with hydrophobic environments (see Dawson et al., 1999). However, SCN^- permeability is reduced to a similar extent in F337A (hydrophobic) and F337S (polar), but is not altered in F337Y (polar) (Table 1), suggesting that SCN^- permeability is not influenced by hydrophobic interactions with the large, hydrophobic side chain of F337.

How, then, might we explain the effects of the mutations F337A and F337S on anion selectivity? One way in which amino acid side-chain size might influence the strength of the interaction between permeating anions and the pore is by controlling how close the ion may come to a positive charge or dipole on the pore wall. Thus in wild-type CFTR (and to a similar extent F337L and F337Y), the bulky side chain at position 337 might impede the approach of permeating anions to a nearby anion-attracting group, ensuring relatively weak, long-distance interactions between the anion and this positive site. Reduction of this steric effect in both F337A and F337S would allow the permeating anion

to more closely approach, and thereby interact more strongly with, the anion-attracting group. The nature of such an anion-attracting group in this region of the pore is not known; it may be contributed by the amide dipole of the peptide backbone of TM6 (Linsdell et al., 1998) or by nearby polar amino acid side chains. The lack of correlation between anion permeability and amino acid side-chain polarity at position 337 suggests that the polar groups of serine, tyrosine, or tryptophan residues introduced at this position do not interact with permeating anions in a way that might influence anion selectivity.

In contrast to what is known about the way in which interactions between permeating ions and the selectivity filter controls the selectivity of cation channels (see Introduction), the molecular mechanisms underlying anion channel selectivity are poorly understood. However, irrespective of the precise mechanism of anion selectivity, the effects of mutations at residue F337 in TM6 of CFTR support the existence of a spatially localized region within a Cl^- channel pore at which selectivity is mainly determined. Based on the effects of mutations at F337 (this study) and T338 (Linsdell et al., 1998) on anion selectivity, compared to the lack of effect of a number of mutations at other sites throughout the pore (see above), we suggest that such a "lyotropic selectivity filter" may be located close to these TM6 residues, although a more remote effect of mutations cannot be ruled out. The characteristic lyotropic anion selectivity of CFTR may result from a balance between anion dehydration and electrostatic interactions between permeating anions and this region of the pore, rather than a nonspecific weak interaction between anions and the pore walls in general.

We thank Jie Liao for maintaining the cell cultures.

This work was supported by the Medical Research Council of Canada (MRC), the Canadian Cystic Fibrosis Foundation (CCFF), and the National Institute of Diabetes and Digestive and Kidney Diseases. PL was supported by an MRC postdoctoral fellowship and is currently a CCFF scholar. JWH is an MRC senior scientist.

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