Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Anion Binding as a Probe of the Pore

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ABSTRACT We compared the effects of mutations in transmembrane segments (TMs) TM1, TM5, and TM6 on the conduction and activation properties of the cystic fibrosis transmembrane conductance regulator (CFTR) to determine which functional property was most sensitive to mutations and, thereby, to develop a criterion for measuring the importance of a particular residue or TM for anion conduction or activation. Anion substitution studies provided strong evidence for the binding of permeant anions in the pore. Anion binding was highly sensitive to point mutations in TM5 and TM6. Permeability ratios, in contrast, were relatively unaffected by the same mutations, so that anion binding emerged as the conduction property most sensitive to structural changes in CFTR. The relative insensitivity of permeability ratios to CFTR mutations was in accord with the notion that anion-water interactions are important determinants of permeability selectivity. By the criterion of anion binding, TM5 and TM6 were judged to be likely to contribute to the structure of the anion-selective pore, whereas TM1 was judged to be less important. Mutations in TM5 and TM6 also dramatically reduced the sensitivity of CFTR to activation by 3-isobutyl 1-methyl xanthine (IBMX), as expected if these TMs are intimately involved in the physical process that opens and closes the channel.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a chloride-selective ion channel (Anderson et al., 1991; Bear et al., 1992; Tabcharani et al., 1993). Hydropathy analysis of the primary amino acid sequence (Riordan et al., 1989) predicted two membrane-spanning domains (MSD1 and MSD2) each containing six transmembrane segments (TM1–12), a topology that was confirmed using N-glycosylation sites to tag intracellular and extracellular loops (Chang et al., 1994). Structure-function studies, including functional characterization of MSD mutations associated with mild cystic fibrosis (CF), pointed to possible roles for specific TM residues in the conduction and gating properties of CFTR (Anderson et al., 1991; Sheppard et al., 1993; Tabcharani et al., 1993; McDonough et al., 1994; Carroll et al., 1995; Akabas and Cheung, 1996), but specific roles for individual TMs in the structure of the anion-selective pore and the molecular basis for the transmission of gating signals to the pore remain to be defined. In at least one instance there are conflicting data regarding a possible role for a TM in anion conduction. Substitution of an aspartic acid for K95 (K95D) in TM1 altered anion selectivity (Anderson et al., 1991), and three cysteine-substituted residues in this TM (G91, K95, and Q98) were identified as being accessible to water-soluble, charged, sulfhydryl-specific reagents (Akabas et al., 1994), but a CFTR construct in which the first 118 amino acids, including those that constitute TM1, were deleted produced channels with conduction properties similar to wild type (Carroll et al., 1995).

The goal of the present study was to use permeation properties to identify transmembrane segments of CFTR that are likely to contribute to the formation of the anion-selective pore. We took as our starting point the assumption that the most reliable probe of the CFTR pore is a permeant anion that must, of necessity, enter, traverse, and interact with the conduction path. The strategy was to compare the permeation of a series of anions in wild-type CFTR (wtCFTR) and in CFTRs bearing amino acid substitutions in TM1, TM5, and TM6. We reasoned that such a comparison would suggest which permeation property (or properties) was the most sensitive to amino acid substitutions in CFTR and that the magnitude of changes in permeation properties might provide an index of the importance of a residue or a TM for the architecture of the pore. Because it is possible that changes in the configuration of one or more of the TMs is involved in gating the channel, we also evaluated the sensitivity of mutant CFTRs to cAMP-dependent activation. The results indicate that the most sensitive index of altered pore properties is the decrease in CFTR conductance seen with anions like Br, NO_3, and thiocyanate (SCN), which, although they are more permeant than Cl, appear also to bind in the pore and slow conduction rates. Apparent anion binding, particularly that of SCN, appeared to be more useful than either permeability ratios or current-voltage (*I-V*) shape for evaluating the possible importance of a residue for the structure of the pore. Using this criterion, TM5 and TM6 were judged likely to contribute significantly to the structure of the pore, whereas TM1 was less important for maintaining the pore architecture. Permeability ratios, obtained from shifts in reversal potential, were

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relatively insensitive to amino acid substitution in CFTR, consistent with the notion that the barrier to entry of anions into the channel is dominated by the energy required to dehydrate the anion. In some cases, mutations that altered anion binding also reduced the sensitivity of the construct to activation by increasing doses of 3-isobutyl 1-methyl xanthine (IBMX), suggesting that TM5 and TM6 may also be involved in the process that opens and closes the channel.

MATERIALS AND METHODS

Choice of mutations

Residues were identified as targets for mutagenesis on the basis of previous structure-function studies, the location of patient mutations, and amino acid conservation across different species. One residue was identified in each of three TMs, 1 (G91), 5 (G314), and 6 (K335), and multiple amino acid substitutions were made at each locus so that we would be more likely to discern the mechanistic basis for any functional effects.

Glycine 91 was one of three residues in TM1 identified as being accessible to water-soluble thiol reagents, and this residue is also the site of a patient mutation, G91R, associated with a pancreatic sufficient phenotype (Guillermit et al., 1993). Another residue in TM1, K95, was implicated as being important for anion selectivity (Anderson et al., 1991), but mutants at this locus did not give rise to robust expression in *Xenopus* oocytes.

TM5 has a relatively high frequency of patient mutations compared with the other 11 putative TMs (Cystic Fibrosis Genetic Analysis Consortium, unpublished data), and two missense patient mutations, G314E (Golla et al., 1994) and G314R (Nasr et al., 1996), have been identified at G314. If modeled as an α-helix, TM5 is relatively amphipathic with respect to the other TMs, and G314 falls on the polar face. G314 is invariant among all cloned species of CFTR (Riordan et al., 1989; Diamond et al., 1991; Marshall et al., 1991; Tata et al., 1991; Tucker et al., 1992), and this glycine was found to be absolutely conserved when aligned with putative pore-forming domains from a wide variety of ion channels (Jan and Jan, 1994), including the H5 domain of inwardly rectifying K^+ channels, the epithelial amiloride-sensitive sodium channel, (γ-rENaC), and the voltage-dependent chloride channel from *Torpedo*, ClC-0.

In previous studies, substitution of a glutamic acid for K335 in TM6 reversed permeability selectivity for Cl and I (Anderson et al., 1991), decreased single-channel conductance, reduced the anomalous mole fraction effects of SCN (Tabcharani et al., 1993), and altered the macroscopic *I-V* relation (McDonough et al., 1994), but because other substitutions were not examined, it is not clear whether these functional changes were due to electrostatic or steric effects.

Preparation of mutant constructs, RNA, and *Xenopus* oocytes

CFTR mutants were generated using the pSelect vector (Promega Biotech, Madison, WI) according to the protocols provided by the manufacturer. Details of the methods describing the generation of wild-type and mutant CFTR constructs and the in vitro transcription of RNA have been previously reported (Drumm et al., 1991; Smit et al., 1993). Incorporation of the mutations was confirmed by sequencing. Details of oocyte preparation and RNA injection have been previously described (Smit et al., 1993; Wilkinson et al., 1996). Briefly, oocytes were removed from anesthetized *Xenopus laevis* toads and manually defolliculated after incubation in a collagenase-containing bath for 2–6 h. The following day, beveled pipettes were used to inject mRNA (50 nl) into oocytes. Oocytes were incubated at 19°C for 3–7 days before electrophysiological recording.

Electrophysiological assays and protocols

Individual oocytes were placed in the recording chamber and continually perfused with amphibian Ringer's (AR). The standard AR contained 98 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (2.5 mM HEPES acid and 2.5 mM sodium HEPES), pH 7.5. For assays that required analysis of conductance at positive membrane potentials, a nominally Ca^{2+}-free AR (NCFAR) was used to reduce background from the endogenous Ca^{2+}-activated chloride channel in the oocytes (Bear et al., 1991). NCFAR contained 98 mM NaCl, 2 mM potassium gluconate, 1 mM $Mg(aspartate)_2$, 1.8 mM barium acetate, and 5 mM HEPES (2.5 mM HEPES acid and 2.5 mM sodium HEPES), pH 7.5. Neither aspartate nor acetate added to a Cl-containing bath in a concentration of 5 mM produced an effect on CFTR conductance. Inclusion of Ba^{2+} also reduced potential contamination from endogenous K channels in the oocyte (Cunningham et al., 1992). Microelectrodes with a resistance of 0.5–2 MΩ were pulled (Sutter P-86, Sutter Instruments, Novato, CA) from borosilicate glass (1.2 mm OD, 0.69 mm ID) and filled with 3 M KCl.

Membrane potentials (V_M) and currents (I) were monitored on-line on a chart recorder (Kipp & Zonen, Bohemia, NY). The membrane potential was clamped using a two-electrode voltage clamp (TEV-200, Dagan Instruments, Minneapolis, MN). After activation of CFTR channels (see below) the oocytes were incubated under open circuit conditions ($V_m \approx$ the chloride equilibrium potential, E_{Cl}). To minimize changes in $[Cl]_i$. A computer-driven ramp command (Clampex, Axon Instruments, Foster City, CA) that changed V_m from -120 to $+50$ mV at a rate of 100 mV per second was used to determine the current-voltage (*I-V*) relationship. After anion substitution, ramps were run every minute until a steady-state conductance was achieved (usually within 3–5 min). After each substitution, the bath was returned to Cl-containing Ringer's to ensure that the level of CFTR activation was maintained and that the effect of the substitution was completely reversible. The *I-V* relationship was constructed from the digitized values of I and V_M. Slope and chord conductances were calculated at chosen potentials on the curve and used to compute wild-type and mutant conductance ratios. In a few cases, slope and chord conductances yielded slightly different values, but in no case did the choice of one measure or the other alter the ratio.

Two reagents were used to activate CFTR: 1) forskolin (RBI, Natick, MA, or Sigma Chemical Co., St. Louis, MO), an activator of adenylate cyclase, and 2) IBMX (RBI or Sigma), a phosphodiesterase inhibitor. The IBMX concentration-response assay measured the steady-state activation levels of wild-type and mutant constructs at increasing concentrations (0.02–5.0 mM) of IBMX in the continued presence of 10 μM forskolin. The data for each oocyte were normalized to the maximal obtained cAMP-dependent chloride conductance, $g_{Cl(max)}$, and the $K_{1/2(IBMX)}$ was defined as the concentration of IBMX that gave rise to 50% activation. For some constructs, maximal activation was not achieved at the maximal concentration of IBMX used in this study (5 mM). Hence a rectangular hyperbola was fitted to each data set to estimate the $K_{1/2}$ and g_{max} (see Table 5 footnote). In previous studies we corrected the activated CFTR conductance for block by IBMX ($K_I \approx 10$ mM), but because the effect of the correction is small, it was omitted in the present study (Wilkinson et al., 1996).

Permeability ratios were calculated using the Goldman-Hodgkin-Katz equation as follows:

$$\frac{P_s}{P_{Cl}} = \exp\left\{\frac{\Delta E_{rev}}{(RT/zF)}\right\} \quad (1)$$

Where P_S is the permeability of the substitute anion, P_{Cl} is the Cl permeability, ΔE_{rev} is the shift in reversal potential and R, T, z, and F have their usual meaning.

Expression levels

Wild-type and 11 mutant CFTR constructs were used in this study: G91A, G91E, G91R, G314A, G314D, G314E, G314Q, K335R, K335A, K335D,

and K335E. Maximal expression levels varied both between different CFTR constructs and between different oocytes for a given construct. To reduce the differences in expression levels, the mass of RNA injected for each mutant was adjusted (0.1–15 ng) so that for oocytes activated with 1 mM IBMX and 10 μM forskolin, the slope conductance at −60 mV was typically between 10 and 100 μS. For the calculation of concentration-dependent attenuation of CFTR-mediated current by SCN or concentration-dependent activation of CFTR by IBMX, the results for each oocyte were normalized and presented as a percent of the maximal value.

Statistical comparisons

The results were compared by one-way analysis of variance against wtCFTR using a Dunnett's or Dunn's test as appropriate after tests of normality and variance.

RESULTS

Evidence for anion binding in CFTR

Fig. 1 contains the results of representative experiments in which the macroscopic *I-V* relation for wtCFTR was determined first in the presence of 98 mM $[Cl]_o$ and again after complete replacement of $[Cl]_o$ by one of five ions: formate (HCOO), bromide (Br), nitrate (NO_3), iodide (I), or thiocyanate (SCN). Formate substitution shifted the reversal potential (V_r) to the right indicating that $P_{HCOO}/P_{Cl} < 1.0$. The conductance ratio, g_{HCOO}/g_{Cl} was also less than unity, so that both the permeability ratio and the conductance ratio portrayed HCOO as an ion that permeates CFTR less readily than Cl. Substitution with I also resulted in a shift in V_r to more positive values and reduced membrane conductance, as reported by Anderson et al. (1991) and Tabcharani et al. (1992). The results obtained with Br, NO_3, and SCN, however, were distinctly different. In each case, replacement of $[Cl]_o$ by the substitute anion shifted V_r to the left ($P_s/P_{Cl} > 1$) but reduced the conductance. For all substitute anions except I, the response was rapidly and completely reversed when the chloride was returned to the extracellular bath. The recovery from exposure to I, however, was often slower such that attenuation of inward currents could persist for up to 30 min. The results of a series of such experiments are compared in Fig. 1 *F* and demonstrate that Br, NO_3, and SCN, although judged by their effect on V_r to be highly permeant relative to chloride, also reduced the conductance. This behavior is consistent with a model for anion permeation that includes anion binding in the channel where the relative affinities are: SCN > Br > NO_3 > Cl (Dawson, 1996; Dawson et al., 1996).

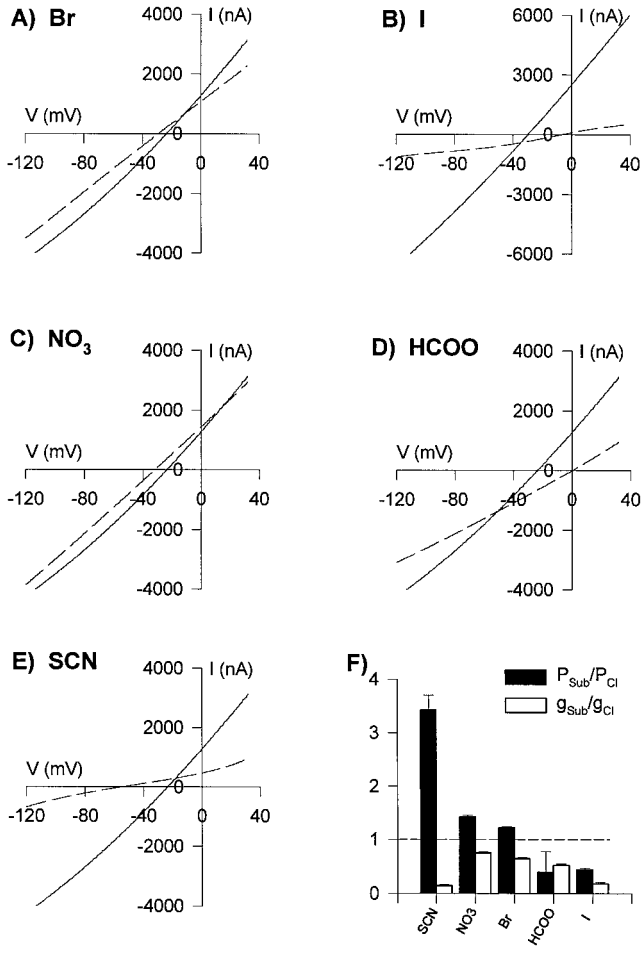

FIGURE 1 Representative current-voltage relations in the presence of substitute anions for wtCFTR expressed in *Xenopus* oocytes. (*A–E*) Macroscopic *I-V* plots after complete replacement of $[Cl]_o$ for each of the substitute anions used in this study. (*F*) Permeability ratios (■), measured from the shift in reversal potential, and conductance ratios (□ measured from the chord conductance at +25 mV (see also Table 2).

The disparity between P_S/P_{Cl} and g_S/g_{Cl} was most pronounced for SCN, suggesting that, although this ion permeates the CFTR channel, its movement is slowed by relatively tight binding in the pore, consistent with the observations of Tabcharani et al. (1993) who studied the blocking effects of SCN applied to the cytoplasmic side of detached patches. If anion binding is a part of the permeation process, then Cl and SCN might be expected to compete. As a test of Cl/SCN competition in wtCFTR, we compared the attenuation of CFTR-mediated conductance produced by 5 mM $[SCN]_o$ in the presence of either 98 mM or 9.8 mM $[Cl]_o$. Table 1 contains the results of several such experiments. In the presence of 98 mM $[Cl]_o$, the attenuation produced by 5 mM $[SCN]_o$ (~40%) was identical at −60 mV and + 30 mV. Reducing $[Cl]_o$ to 9.8 mM altered the efficacy of $[SCN]_o$ in a voltage-dependent fashion. At a potential of +30 mV, which would favor the access of extracellular anions to an intrachannel binding site, the

TABLE 1 Extracellular $[Cl^-]$ influences the efficacy of inhibition by 5 mM $[SCN^-]_o$ on wtCFTR

		$g_{(+SCN)}/g_{(-SCN)}$	
$[Cl]_o$ (mM)	*n*	V_m = −60 mV	V_m = +30 mV
98	6	0.59 ± 0.03	0.61 ± 0.02*
9.8	6	0.66 ± 0.04	0.40 ± 0.03*

*Significant difference between the conductance ratio ($g_{(+SCN)}/g_{(-SCN)}$) at +30 mV (inward anion movement) for 98 mM $[Cl^-]_o$ compared with 9.8 mM $[Cl^-]_o$ ($p < 0.05$). The conductance ratios were measured from the slope conductance at −60 mV and +30 mV (±10 mV).

attenuation produced by 5 mM $[SCN]_o$ was significantly enhanced when $[Cl]_o$ was reduced by 10-fold. At a potential of −60 mV, which would favor the access of intracellular chloride to the site, the efficacy of $[SCN]_o$ was unaffected by changing $[Cl]_o$. This result is consistent with competition of Cl and SCN for a site in the permeation path, which binds SCN more tightly than Cl.

SCN^- effects are primarily on the conduction properties of CFTR

Several observations suggested that the blocking effect of SCN was predominantly on the conduction properties of CFTR. The action of SCN was rapid with respect to other reagents that attenuate CFTR currents (e.g., glybenclamide (Sheppard and Welsh, 1992) and diphenylamine-2-carboxylate, DPC (McCarty et al., 1993)) and was readily reversible. For example, after replacement of 10% of the $[Cl]_o$ in the bath with $[SCN]_o$, the conductance of wtCFTR channels declined to a stable value with a half-time of 15 s and returned to its pre-SCN value with a half-time of ~40 s (data not shown). This response is consistent with the notion that SCN acts by binding in the pore and that it is more difficult to get the ion out than to get it in.

As a test for possible effects of SCN on the macroscopic gating of CFTR, we compared the concentration-dependent activation of CFTR Cl currents by IBMX (Drumm et al., 1991; Smit et al., 1993; Wilkinson et al., 1996) in the presence of 0, 2, 5, and 10% $[SCN]_o$ (0, 1.98, 4.9, and 9.8 mM, respectively). The respective values for the $K_{1/2}$ for activation by IBMX were (in mM) 0.51 ± 0.06 (n = 5), 0.56 ± 0.11 (n = 4), 0.28 ± 0.05 (n = 3), and 0.34 ± 0.04 (n = 3) and did not differ significantly (p = 0.09). In addition, the efficacy of inhibition by $[SCN]_o$ was independent of the level of activation. That is, the attenuation measured when 10% of extracellular Cl was replaced with SCN was the same for wtCFTR stimulated with 200 μM, 500 μM, or 1 mM IBMX (data not shown). Finally, dose-dependent inhibition of CFTR conductance by $[SCN]_o$ (see below) was identical in wtCFTR and a CFTR mutant exhibiting highly altered gating, K1250A (Wilkinson et al., 1996).

Effects of amino acid substitutions on permeation properties

Based on the results of ion substitution studies conducted with wtCFTR, we compared the permeation properties of a series of CFTR constructs bearing mutations in TM1, TM5, and TM6. For each construct we examined the permeability ratios obtained from shifts in the reversal potential, the conductance ratios seen in the presence of substitute ions, the concentration-dependent attenuation of CFTR conductance by $[SCN]_o$, and the shape of the macroscopic I-V relation.

Permeability and conductance ratios

Table 2, *A* and *B*, contains the permeability and conductance ratios, respectively, obtained from anion substitution experiments conducted with the G91, G314, and K335 mutants. Shown are the values for the apparent permeability ratio, P_S/P_{Cl}, obtained from the shift in reversal potential upon complete replacement of the $[Cl]_o$ with the substitute anion and the ratios for the chord conductances, g_S/g_{Cl}, measured at +25 mV under the same condition. Across the panel of 11 mutations considered in this study changes in anion permeability ratios were, for the most part, unremarkable. Only one mutation, K335E, significantly altered the sequence of permeability ratios derived from shifts in reversal potential. This substitution increased P_I/P_{Cl} such that I preceded Cl in the selectivity sequence, confirming the report of Anderson et al. (1991). In this construct only the ratio for I was altered significantly, however. The aspartic acid substitution (K335D) altered the permeability ratios for Br, HCOO, and I, but the average values did not differ markedly from those seen with wtCFTR, and the sequence was not changed. Neither the arginine (K335R) nor the alanine substitution (K335A) resulted in any substantial change in the permeability ratios (with the exception of SCN for K335A). Substitutions for G91 were similarly without any systematic impact on permeability ratios, although the arginine-substituted construct had higher ratios for SCN and Br. Substitutions for G314 did not alter the permeability sequence, although the values for NO_3 and Br were slightly increased. Thus, the overall impression conveyed by a comparison of the apparent permeability ratios is the general lack of any systematic change brought about by amino acid substitutions in TM1, TM5, or TM6.

In contrast, the conductance values (Table 2 *B*) seen with complete $[Cl]_o$ replacement suggested a systematic alteration in anion permeation consistent with decreased binding of substitute ions relative to $[Cl]_o$. The conductance ratios for anions judged by shifts in reversal potential to be most highly permeant (SCN, NO_3, and Br) were all increased significantly by substituting a negatively charged residue (D or E) for K335, whereas mutants in which the charge was conserved (K335R) or removed (K335A) were identical to wtCFTR. The K335D and K335E constructs also exhibited increased conductance ratios for I, a result previously reported by Anderson et al. (1991) for K335E CFTR. Likewise, substitutions for G314 tended to increase conductance ratios. Most dramatic here was the substitution of glutamic acid (G314E), which raised conductance ratios for SCN, NO_3, and Br. It was of particular interest that the introduction of a glutamine (G314Q) also produced increased conductance ratios for the highly permeant anions and I, whereas the aspartic-acid-substituted construct (G314D) was not different from wtCFTR. In contrast to the readily apparent effects of substitutions for G314 and K335, neither charged nor neutral substitutions for G91 altered conductance ratios.

TABLE 2 Summary of permeability and conductance ratios from anion substitution experiments

	n	SCN	NO3	Br	HCOO	I
A. Permeability Ratios						
Wild type	4–9	3.42 ± 0.28	1.42 ± 0.04	1.22 ± 0.02	0.39 ± 0.01	0.44 ± 0.03
G91A	3–6	3.24 ± 0.26	1.53 ± 0.04	1.27 ± 0.02	0.37 ± 0.04	0.40 ± 0.04
G91E	3–7	3.50 ± 0.54	1.59 ± 0.04	1.27 ± 0.01	0.35 ± 0.01	0.51 ± 0.04
G91R	3–4	**5.26 ± 0.46***	1.60 ± 0.03	**1.40 ± 0.01***	0.32 ± 0.04	**0.64 ± 0.04***
G314A	3–4	2.87 ± 0.17	1.45 ± 0.03	1.19 ± 0.02	0.31 ± 0.03	0.33 ± 0.03
G314D	4	3.42 ± 0.34	1.44 ± 0.05	1.25 ± 0.04	0.33 ± 0.03	0.51 ± 0.05
G314E	3–4	3.72 ± 0.56	**1.65 ± 0.09***	**1.35 ± 0.03***	0.49 ± 0.04	0.53 ± 0.04
G314Q	3–4	3.89 ± 0.37	1.62 ± 0.11	1.27 ± 0.04	0.36 ± 0.03	0.62 ± 0.05
K335R	3–5	3.44 ± 0.29	1.35 ± 0.04	1.22 ± 0.03	0.40 ± 0.05	0.41 ± 0.07
K335A	5–6	**5.34 ± 0.58***	1.48 ± 0.06	1.28 ± 0.04	0.37 ± 0.03	0.60 ± 0.06
K335D	4–6	3.02 ± 0.19	1.50 ± 0.03	**1.10 ± 0.02***	**0.54 ± 0.04***	**0.65 ± 0.06***
K335E	5–8	3.64 ± 0.21	1.48 ± 0.06	1.29 ± 0.03	0.46 ± 0.04	**1.10 ± 0.04***
B. Conductance Ratios						
Wild type	4–9	0.14 ± 0.02	0.75 ± 0.02	0.64 ± 0.02	0.52 ± 0.03	0.18 ± 0.03
G91A	3–6	0.14 ± 0.01	0.77 ± 0.02	0.61 ± 0.02	0.47 ± 0.02	0.19 ± 0.02
G91E	3–7	0.15 ± 0.03	0.73 ± 0.02	0.60 ± 0.01	0.50 ± 0.04	0.30 ± 0.02
G91R	3–4	0.14 ± 0.00	0.84 ± 0.01	0.63 ± 0.01	**0.32 ± 0.01***	0.14 ± 0.01
G314A	3–4	0.30 ± 0.09	**0.89 ± 0.01***	0.66 ± 0.01	0.48 ± 0.09	0.24 ± 0.01
G314D	4	0.28 ± 0.05	0.82 ± 0.01	0.70 ± 0.02	0.49 ± 0.06	0.27 ± 0.03
G314E	3–4	**0.62 ± 0.07***	**1.18 ± 0.04***	**0.84 ± 0.05***	0.42 ± 0.05	0.29 ± 0.09
G314Q	3–4	**0.63 ± 0.02***	**1.01 ± 0.04***	**0.82 ± 0.03***	0.50 ± 0.02	**0.42 ± 0.02***
K335R	3–5	0.14 ± 0.01	0.76 ± 0.03	0.61 ± 0.02	0.59 ± 0.06	0.16 ± 0.03
K335A	6	0.20 ± 0.03	0.77 ± 0.02	0.61 ± 0.02	0.45 ± 0.03	0.21 ± 0.02
K335D	4–6	**0.65 ± 0.04***	**1.25 ± 0.02***	**0.89 ± 0.02***	0.61 ± 0.06	**0.58 ± 0.06***
K335E	5–8	**0.50 ± 0.06***	**1.19 ± 0.03***	**0.89 ± 0.02***	0.53 ± 0.03	**0.48 ± 0.03***

(*A*) The apparent permeability ratios (P_S/P_{Cl}) for each substitute anion were calculated from the shift in reversal potential using the Goldman-Hodgkin-Katz relation (noted in Materials and Methods). (*B*) The conductance ratios (g_S/g_{Cl}) for each substitute anion were calculated from the chord conductance measured at +25 mV.

*Significant differences from wtCFTR ($p < 0.05$).

The marked reduction in wtCFTR conductance seen when $[Cl]_o$ was replaced with SCN suggested that this ion would be the most sensitive probe of the effect of mutations on anion binding. Thus we used the concentration-dependent attenuation of CFTR conductance by $[SCN]_o$ to obtain a more quantitative measure of changes in anion binding induced by G91, G314, and K335 mutations. As an example, the *I-V* plots shown in Fig. 2, *A* and *B*, illustrate the effect of $[SCN]_o$ substitution on wild-type and G314E CFTR. Substitution of as little as 2% (~2 mM) of $[Cl]_o$ by $[SCN]_o$ attenuated CFTR conductance in a voltage-independent manner, and substitution of 90% (~88 mM) of $[Cl]_o$ by $[SCN]_o$ shifted the reversal potential to the left (as expected if $P_{SCN} > P_{Cl}$) and further attenuated the conductance. In the G314E mutant the attenuation of g_{Cl} by 2% $[SCN]_o$ was virtually abolished. Substitution of 90% of $[Cl]_o$ shifted V_r to the left, but the attenuation of conductance was significantly less than that seen with wtCFTR.

Constructs were compared by subjecting oocytes to incremental, equimolar replacement of $[Cl]_o$ by $[SCN]_o$, and results for wild-type and the G91, G314, and K335 mutants are shown in Fig. 3, *A*, *B*, and *C*, respectively. Increasing the mole fraction of SCN in the perfusate resulted in a monotonic decrease in wtCFTR conductance without any tendency toward anomalous mole fraction effects. The data were quantitated by fitting a rectangular hyperbola to the data points by means of an Eadie-Hofstee plot. This analysis yielded values for the $K_{1/2}$ for the attenuation of CFTR conductance by external SCN and the maximal fractional attenuation. The value of $K_{1/2}$ cannot be equated with a binding constant because SCN, a permeant ion, also contributes to the conductance. Nevertheless, these parameters yielded a quantitative comparison of the efficacy of SCN on wild-type and mutant CFTRs. For wtCFTR this analysis yielded an apparent $K_{1/2}$ of 4.56 ± 0.26 mM ($n = 15$) and predicted a maximal fractional attenuation of 0.88 ± 0.02. The behavior of G314D CFTR was virtually identical to that of wtCFTR. The alanine substitution reduced the apparent affinity for SCN, but the maximal inhibition was not affected. The affect of the alanine substitution for G314 was actually greater than is at first apparent from Fig. 3 *B*. Attenuation by SCN was reduced by ~50% at 5 mM $[SCN]_o$. Both the glutamic acid and glutamine substitutions virtually abolished attenuation by $[SCN]_o$, such that an Eadie-Hofstee analysis was not possible. There was no readily discernible voltage dependence to the dose-dependent attenuation of wtCFTR conductance by SCN; and similar results were obtained for G314D and G314A. In the case of the G314E and G314Q mutants, however, the SCN effect appeared to be moderately voltage dependent (Fig. 2

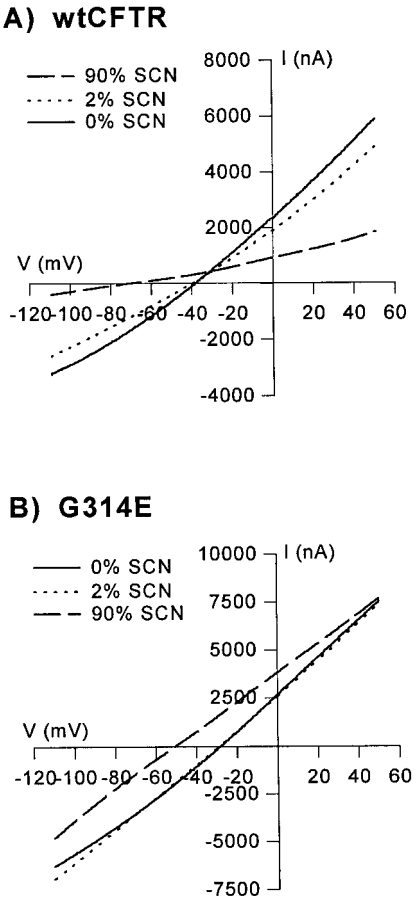

FIGURE 2 The effect of replacement of $[Cl^-]_o$ by $[SCN^-]_o$ is shown for wtCFTR (*A*) and G314E (*B*). ——, current seen with 100% $[Cl^-]_o$; - - - and · · ·, 2% and 90% replacement of $[Cl^-]_o$ with $[SCN^-]_o$, respectively.

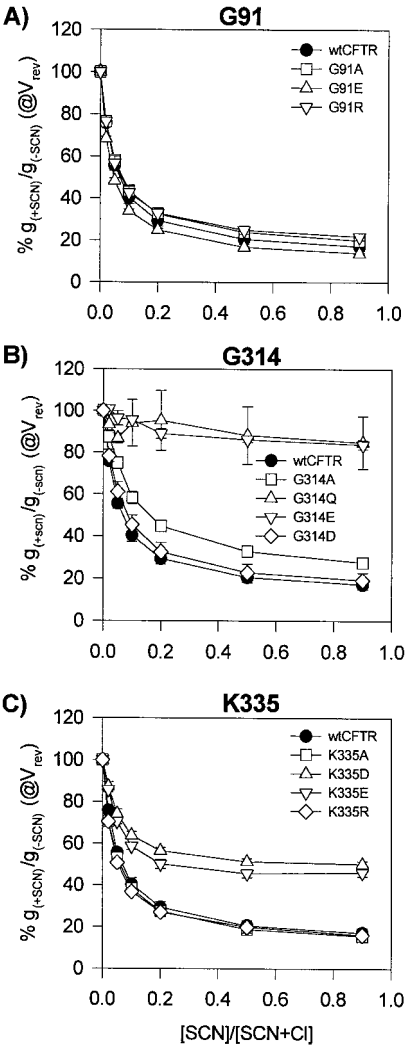

FIGURE 3 The fractional conductance remaining after replacement of $[Cl^-]_o$ with $[SCN^-]_o$, $(g_{(+SCN)}/g_{(-SCN)})$ is plotted against the mole fraction of $[SCN^-]$. Activating conditions were held constant (10 μM forskolin plus 1 mM IBMX) as an increasing fraction of $[Cl^-]_o$ was replaced with $[SCN-]_o$ (0.02–0.9). The oocyte was maintained under open circuit conditions between data collection events. Conductance ratios (means ± SEM) were calculated from the slope conductance measured at the reversal potential (±10 mV). (*A*) wtCFTR compared with the G91 variants; (*B*) wtCFTR compared with the G314 variants; (*C*) wtCFTR compared with the K335 variants.

B). The measurements of conductance at the reversal potential or at +20 mV indicated that the attenuation by $[SCN]_o$ was virtually abolished by the mutation. In contrast at −60 mV, the conductance was actually enhanced slightly by the presence of external SCN.

Concentration-dependent attenuation of CFTR conductance by $[SCN]_o$ was not altered by substitutions for G91. Similar negative results were obtained for the K335R and K335A constructs, but in the K335D and K335E mutants maximal attenuation by $[SCN]_o$ was reduced by nearly 50%, although the apparent binding affinity was unaffected. The result is consistent with the notion that the affinity of anion binding was not diminished in the K335D and K335E constructs, but the impact of binding on conduction was reduced. Sensitivity to $[SCN]_o$ was identical to wtCFTR in a construct bearing a mutation in NBF2, K1250A (data not shown), which exhibits severely altered activation in the form of a highly stabilized active state (Wilkinson et al., 1996). The data in Table 3 show that for wtCFTR and G314Q and G314E, two of the most severely affected constructs, P_{SCN}/P_{Cl} calculated from the shift in V_r was independent of the fractional abundance of $[SCN]_o$.

Shape of the macroscopic *I-V* curve

To quantify the shape of the macroscopic *I-V* curves for CFTR constructs bearing mutations in TM1, TM5, and TM6, we measured the ratio of slope conductances at a fixed potential difference on either side of the reversal potential. Table 4 contains the rectification ratios (RRs), defined as the slope conductance 30 mV positive to the reversal potential divided by the slope conductance 30 mV negative to the reversal potential.

Of the three substitutions for G91, the arginine (G91R) altered the RR most dramatically, increasing it nearly sevenfold, although the negatively charged glutamate (G91E)

TABLE 3 The permeability ratio (P_{SCN}/P_{Cl}) is independent of the mole fraction of $[SCN]_o$ for wtCFTR and the G314 variants

		P_{SCN}/P_{Cl}					
[SCN]/{[SCN]+[Cl]}	*n*	0.02	0.05	0.10	0.20	0.50	0.90
Wild type	12	3.82 ± 0.50	4.43 ± 0.57	4.58 ± 0.48	4.69 ± 0.43	4.66 ± 0.38	4.44 ± 0.35
G314A	9	4.32 ± 0.73	3.78 ± 0.53	3.81 ± 0.47	3.79 ± 0.34	3.82 ± 0.29	3.72 ± 0.25
G314D	3	2.99 ± 0.26	2.56 ± 1.05	2.82 ± 1.07	2.68 ± 0.97	2.87 ± 0.65	2.89 ± 0.43
G314E	6	4.48 ± 1.05	4.01 ± 0.69	4.17 ± 0.62	4.15 ± 0.59	3.96 ± 0.41	3.82 ± 0.40
G314Q	3	5.39 ± 0.57	4.49 ± 0.58	4.69 ± 1.26	4.05 ± 1.26	3.86 ± 1.47	3.68 ± 1.51

The permeability ratios were calculated from the shift in reversal potential using the Goldman-Hodgkin-Katz equation. None of the values are statistically different from one another.

also significantly increased the RR. At the K335 locus, substitution by either glutamate (K335E) or aspartate (K335D) significantly reduced the mean value of RR by 25% and 38%, respectively. The alanine substitution at G91, the alanine and glutamate substitutions at G314, and the alanine and arginine substitutions at K335 were without effect.

In principle, changes in the shape of the macroscopic *I-V* plot could include a contribution due to a change in voltage-dependent gating, but several observations suggest that the mutant-dependent changes reported here are not voltage-dependent gating effects, but rather reflect changes in the conduction pathway. First, the shapes of the *I-V* plots were identical at different levels of activation of CFTR achieved by using either 0.2, 1.0, or 5.0 mM IBMX in the presence of 10 μM forskolin. Second, in the course of ion substitution studies we noted that the shape of the *I-V* curve was not altered by the shift in the reversal potential. Finally, the mutant that exhibited the most striking shape change (G91R) did not differ from wild type in its dose-dependent activation by IBMX (see below).

TABLE 4 Quantitative analyses of the macroscopic *I-V* shape changes

Mutant	Δ Net charge	*n*	RR $g(+30)/g(-30)$	RR/RR_{WT}
Wild type		5	1.220 ± 0.06	1.00
G91A	0	4	1.293 ± 0.06	1.06
G91E	−1	5	**1.512 ± 0.10***	1.24
G91R	1	4	**8.041 ± 0.87***	6.59
G314A	0	4	1.201 ± 0.09	0.98
G314D	−1	4	1.362 ± 0.08	1.12
G314E	−1	7	1.405 ± 0.08	1.15
G314Q	0	5	1.376 ± 0.10	1.13
K335R	0	4	1.209 ± 0.06	0.99
K335A	−1	4	1.295 ± 0.07	1.06
K335D	−2	5	**0.762 ± 0.02***	0.62
K335E	−2	4	**0.919 ± 0.02***	0.75

The slope conductance was measured at +30 mV and −30 mV with respect to the reversal potential.
*Significantly different from wtCFTR ($p < 0.05$).

Substitutions for G314 and K335 but not G91 altered the cAMP-dependent activation properties of CFTR

Concentration-dependent activation of the G91, G314, and K335 mutants by IBMX was compared with that of wtCFTR, and the values for $K_{1/2(IBMX)}$ are compiled in Table 5. Concentration-dependent activation of G91A, G91E, and G91R CFTR was not discernibly different from that of wtCFTR. Similarly, neither the alanine (K335A) nor the arginine (K335R) substituted K335 constructs exhibited an alteration in sensitivity to activating conditions. In contrast, values for the $K_{1/2(IBMX)}$ for both K335D and K335E were significantly greater than that for wtCFTR, an indication of diminished sensitivity to activating conditions. As with the effect of SCN, the acidic side chain appeared to be critical for the change in $K_{1/2(IBMX)}$ produced by substitutions for K335.

TABLE 5 Concentration-dependent activation of wtCFTR, G91, G314, and K335 variants by IBMX in the presence of 10 μM forskolin

Mutant	*n*	$K_{1/2(IBMX)}$ (mM)
Wild type	15	0.35 ± 0.04
G91A	5	0.42 ± 0.06
G91E	8	**0.51 ± 0.06***
G91R	5	0.49 ± 0.09
G314A	10	**1.21 ± 0.11***
G314D	3	**1.35 ± 0.16***
G314E	8	**6.39 ± 1.35***
G314Q	4	**14.26 ± 6.64***
K335R	4	0.46 ± 0.04
K335A	2	0.35 ± 0.15
K335D	7	**0.87 ± 0.13***
K335E	3	**0.95 ± 0.07***

The steady-state slope conductance was measured at −60 mV as increasing concentrations of IBMX (0.02–5.0 mM) were added to the perfusate in the continued presence of 10 mM forskolin. Results were fit to the following equation: $g_{Cl} = g_{Cl(max)} \times ([IBMX]/[IBMX]+K_{1/2})$. Note that this fitting procedure allows us to estimate $K_{1/2}$ values for even the most insensitive constructs, G314E and G314Q, despite the fact that these values were greater than the highest concentration of IBMX used in this study.
*Significant differences from wtCFTR ($p < 0.05$).

All of the substitutions for G314 resulted in a significant shift in the steady-state concentration-dependent response relation indicative of reduced sensitivity to activation. The $K_{1/2}$ seen with either G314E or G314Q was comparable to that seen with nucleotide-binding mutations such as G551D that are associated with severe CF (Wilkinson et al., 1996). The alanine and aspartic acid substitutions resulted in less dramatic but nevertheless significant decreases in sensitivity comparable to that produced by G551S (Wilkinson et al., 1996), a CF mutation associated with a pancreatic sufficient phenotype (Strong et al., 1991). It is noteworthy that, although the activation of G314D CFTR was significantly compromised, SCN binding was virtually identical to that seen with wtCFTR.

DISCUSSION

In these experiments we compared the conduction and activation properties of wtCFTR and 11 constructs bearing mutations in G91 (TM1), G314 (TM5), and K335 (TM6). At each site the consequences of multiple substitution were evaluated so that some insight into the mechanistic basis for any change in function could be derived. The results provide a partial portrait of the functional role of these portions of the protein that is summarized in Table 6. This survey, although necessarily limited, suggests that 1) of the conduction properties determined here, the ability of the CFTR pore to bind permeant anions is the most sensitive to structural alterations in the protein, so that the relative binding of permeant anions by CFTR is the most reliable indicator of the importance of a residue or a TM for the structure of the pore, 2) by this criterion, G314 and K335 in TM5 and TM6, respectively, are judged to be more important than G91 in TM1 for the architecture of the putative anion binding site or sites, and 3) TMs 5 and 6 appear to play a role in the conformational changes that gate the pore, whereas TM1 is less important for this process.

Anion binding defines the pore of CFTR

The results of the ion substitution experiments presented here are consistent with the hypothesis that a signal feature of the conduction path of the wtCFTR chloride channel is the ability to bind permeant anions. The most compelling evidence for anion binding was provided by a comparison of permeability ratios and conductance ratios for three highly permeant substitute ions: Br, NO_3, and SCN. For each of these ions the permeability ratio inferred from the shift in V_r was greater than unity whereas the ratios of the slope or chord conductances were less than unity. The most striking discrepancy was seen with SCN for which $P_{SCN}/P_{Cl} = 3.42$ whereas $g_{SCN}/g_{Cl} = 0.14$. In addition, the present results suggest that Cl and SCN compete for a binding site in the pore. These results are consistent with the hypothesis that SCN entering the pore from the extracellular side can bind to a site in the pore with an affinity sufficient to slow net anion flow so that SCN acts like a channel blocker.

Although we cannot exclude subtle effects of substitute ions on gating, several observations suggest that it is unlikely that altered gating played a significant role in the attenuation of macroscopic conductance. First, SCN, the anion that produced the most pronounced attenuation of Cl conductance, acted rapidly and the effect was readily reversed, suggesting an effect on the permeation path. Second, the concentration-dependent activation of wtCFTR by IBMX was unaffected by substitution of as much as 10% of $[Cl]_o$ by $[SCN]_o$, although the conductance was reduced by ~50%. Third, the efficacy of inhibition by $[SCN]_o$ was independent of the level of activation. Finally, in at least one mutant, G314D, the effect of $[SCN]_o$ on conductance was unaffected despite the fact that sensitivity to activation by IBMX was severely compromised. The pattern of the effect of anion substitution was identical for Br, NO_3, and SCN, and the conductance ratio for all three ions was increased in G314E and G314Q CFTR channels. A variety of compounds have been identified as blockers of CFTR, e.g., diphenylamine-2-carboxylate (McCarty et al., 1993; McDonough et al., 1994), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (Linsdell and Hanrahan, 1996a), gluconate and glutamate (Linsdell and Hanrahan, 1996b), and the sulfonyl ureas tolbutamide and glybenclamide (Sheppard and Welsh, 1992), but the site of action of these compounds has yet to be determined. In contrast, it seems highly likely that SCN, a permeant anion, blocks CFTR by binding within the pore, so that changes in the binding of this anion are likely to reflect changes in pore architecture.

The results presented here are consistent with the notion that the binding of anions within the CFTR pore is a sensitive indicator of changes in pore structure whereas permeability ratios appear to be rather insensitive to similar

TABLE 6 Qualitative summary of the functional consequences of mutations at G91, G314, and K335

	G91 (TM1)			K335 (TM6)				G314 (TM5)			
Property	G91A	G91E	G91R	K335R	K335A	K335D	K335E	G314A	G314D	G314E	G314Q
I-V shape	−	−	+++	−	−	++	+	−	−	−	−
P_{sub}/P_{Cl}	−	−	−	−	−	−	++	−	−	−	−
g_{sub}/g_{Cl}	−	−	−	−	−	+++	+++	++	−	+++	+++
SCN^- binding	−	−	−	−	−	+++	+++	++	−	++++	++++
Activation	−	−	−	−	−	++	++	+++	+++	++++	++++

Results are expressed as follows: −, function of the CFTR construct with the indicated substitution was indistinguishable from wild type; + to ++++, semiquantitative indication of the magnitude of the change in the function compared with wild type.

changes. This result is consistent with the hypothesis that the permeability ratios, because of their sensitivity to the barrier height (in rate theory permeation models) are reporting the relative ease with which anions leave the bulk solution and enter the pore, a process that is likely to be strongly influenced by the energy required to dehydrate the anions. Thus permeability ratios, which measure the difference in energy barrier heights, appear to depend more on differences in anion-water interactions than differences in anion-pore interactions. In this regard it is perhaps noteworthy that SCN, the most easily dehydrated of the anions used here, is also the most tightly bound. Weak anion-water interactions may favor association with the binding site (Collins, 1995, 1997).

Experiments on K channels provide evidence for dramatic effects of point mutations on cation binding within the pore. For example, Kirsch et al. (1992) and Taglialatela et al. (1993) identified a leucine in the so-called p loop thought to line the pore of K channels, which, when substituted by a valine, produced a dramatic increase in the ratio of rubidium to potassium conductance from 0.3 to 2.8, whereas the permeability ratio (P_{Rb}/P_K) derived from reversal potentials increased from 0.6 to 0.9.

SCN^-: a probe of anion binding in channels and other proteins

The results presented here are consistent with those obtained with a variety of Cl-selective conductances, indicating that SCN can bind with relatively high affinity within Cl channels (Harris, 1958; Hutter and Padsha, 1959; Takeuchi and Takeuchi, 1971; Epstein et al., 1973; Hagiwara and Takahashi, 1974; Vaughan, 1987). In these studies, substitution of $[SCN]_o$ for $[Cl]_o$ produced changes in reversal potential or membrane potential consistent with $P_{SCN} > P_{Cl}$ but resulted in reduced membrane conductance. Bormann et al. (1987) studied anion permeation through single glycine and GABA receptor channels and found that P_{SCN}/P_{Cl} was $\sim$7 but that conductance ratios were 0.54 and 0.75, respectively. White and Miller (1981) found that SCN blocked the Cl-selective channel from *Torpedo electroplax*. The block was voltage dependent and competitive with Cl, and the affinity was sufficiently high that SCN currents could not be detected. In an apical Cl channel from T84 cells, Halm and Frizzell (1992) found that $P_{SCN}/P_{Cl} = 2$, but at a mole fraction of 0.2, SCN reduced single-channel conductance.

Tabcharani et al. (1993) found that 1–10 mM SCN, substituted for Cl on the cytoplasmic side, produced a voltage-dependent attenuation of single-channel currents in detached patches containing CFTR channels. SCN block was abolished or reduced by the substitution of a neutral or acidic side chain at the site of the arginine at position 347 (R347) in TM6. In the present study, attenuation of wtCFTR conductance by extracellular application of SCN was independent of membrane potential except when $[Cl]_o$ was reduced. Interpretation of the voltage independence of attenuation by SCN is complicated by the fact that SCN, the blocking ion, is permeant, so that both the on rate and the off rate are expected to be voltage dependent. Predicting the net effect of voltage on the efficacy of block, therefore, is not straightforward. Analysis of voltage dependence may be complicated further by the possibility that the pore can accommodate more than one ion at a time so that the voltage dependence of SCN block will be influenced by the occupancy of the channel by other permeating ions (Tabcharani et al., 1993). The data presented here provide no direct evidence for multiple ion occupancy of wtCFTR. As the mole fraction of external SCN was increased, P_{SCN}/P_{Cl} did not vary and CFTR conductance declined monotonically. Overholt and colleagues (1993, 1995) proposed that anion binding in the pore of CFTR was, in part, responsible for the rectification of whole-cell currents seen with epithelial and cardiac isoforms of CFTR. Specifically, they suggested that the less permeant anion, glutamate, when used as a substitute for cytoplasmic chloride, competed with Cl for binding to a site in the pore. Linsdell and Hanrahan (1996b) reported block of single-channel CFTR currents by cytoplasmic glutamate and gluconate.

Tight binding of SCN is also characteristic of other biological and physicochemical systems (Wright and Diamond, 1977; Dani et al., 1983), including carbonic anhydrase (Davenport, 1940), serum albumin (Pande and McMenamy, 1970; Norne et al., 1975a,b; Record et al., 1978), and certain ion-selective electrodes (Brown et al., 1989; Yim et al., 1993). Observations on these systems provide the basis for some speculations about the nature of a SCN binding site. The binding of SCN to albumin was proposed to depend in part on the presence of arginines, but a defined three-dimensional architecture for the binding site was implicated because anion binding to a small peptide fragment containing the presumed binding site was markedly reduced (Pande and McMenamy, 1970; Norne et al., 1975a,b; Record et al., 1978). Highly SCN-selective ionophores include positively charged metal centers (e.g., Brown et al., 1989), but selectivity for SCN over Cl is enhanced by sterically hindering the accessibility of anions to the metal center (Brown et al., 1989; Yim et al., 1993). SCN is an effective inhibitor of carbonic anhydrase (Davenport, 1940), which contains a zinc ion in the catalytic center, but structural studies of carbonic anhydrase and acetoacetic decarboxylase (Wright and Diamond, 1977) suggested that the architecture of the binding sites favored linear anions such as SCN over large spherical anions. For the apical Cl channel of T84 cells, Halm and Frizzell (1992) suggested that the electrostatic configuration of polyatomic anions such as SCN might allow them to interact more favorably than a comparably sized spherical halide ion within the pore. One possibility, based on the results of Tabcharani et al. (1993), is that an arginine, e.g., R347, could serve as a positive center for an anion binding site in CFTR. Hipper et al. (1995) reported that the mutations R334E, R334H, K335E, K335H, R347E, and R347H did not alter CFTR conduction properties, but careful inspection of the data

presented revealed that the level of CFTR expression was very low so that altered properties of mutant CFTRs might have been easily obscured.

G314 is important for the architecture of an anion binding site in the pore of CFTR

The striking effects of substitutions for G314 on the blockade of CFTR by SCN suggest that this residue is important for the structural integrity of an anion binding site in the pore. The glutamine and glutamic acid substitutions dramatically reduced the apparent binding of SCN but had little or no effect on permeability ratios or the shapes of the macroscopic *I-V* plots, suggesting that neither mutation resulted in a gross alteration in protein structure. If the CFTR permeation path is envisioned as consisting of two barriers separated by an energy well that represents the putative binding site, then the conductance ratios seen for wtCFTR with Br, NO_3, and SCN, i.e., $g_S/g_{Cl} < 1$, could be attributed to a higher-affinity binding for these substitute ions and, accordingly, would be represented by a deeper well. The increased conductance ratios seen in G314E, G314Q, and to a lesser extent, G314A channels are compatible with the hypothesis that substitution for G314 distorted an anion binding site such that the affinities for Br, NO_3, and SCN were all reduced relative to Cl. It is important to emphasize that these mutations reduced, but did not eliminate, SCN binding. If binding were eliminated, the conductance ratio at voltages positive to V_r would be expected to approach the ratio of the permeabilities (Dawson, 1996).

Simply introducing a negative charge at G314 (G314D) was not sufficient to reduce SCN binding, suggesting that the destabilizing effect was not electrostatic in nature, but rather that the role of TM5 may be to provide some element of conformation necessary for the tight binding of anions to another residue, such as R347 in TM6 (Tabcharani et al., 1993). Furthermore, the dramatic reduction in SCN binding seen in G314Q CFTR demonstrates that the presence of arginines, such as R347, is not sufficient to induce tight anion binding in the pore. As reported previously (Nasr et al., 1996), low expression of the G314R construct made it impossible to assess its properties.

Substituting either glutamine or glutamate for G314 produced striking changes in anion binding, but changes in the permeability ratios ranged from small to negligible. The observation that a structural change that has such a profound effect on anion binding can be virtually without effect on permeability ratios strengthens the conclusion that permeability ratios are likely to be determined more by ion-water than by ion-channel interactions. In a simple, symmetric two-barrier, one-site model, the permeability ratios measure the relative ease with which anions leave water and enter the channel. The sequence of relative permeabilities for wtCFTR, $SCN > NO_3 > Br > Cl > I$, is, according to the Eisenman model, indicative of a weak ion-channel interaction. Were it not for the position of iodide, the sequence would be the lyotropic or Hoffmeister series, which is ordered by ease of dehydration of the anions (Dani et al., 1983; Yim et al., 1993; Linsdell et al., 1997). The fact that I falls out of this series may point to some relatively unusual interaction of I with CFTR that is not seen in other Cl-selective channels (Hanrahan et al., 1993; Tabcharani et al., 1997).

K335 is near the conduction path but is not an anion binding site

In contrast to the results seen with substitutions for G314, the introduction of a negative charge at the K335 locus produced a consistent change in the effect of SCN on CFTR conductance, whereas simply eliminating the positive charge was without effect. The fact that the apparent affinity of SCN binding was not affected by the addition of a negative charge but the maximal effect was reduced suggests that SCN binding in K335D or K335E CFTR is similar to that seen with wtCFTR but that the bound anion is not as effective in obstructing the conduction path. This observation, taken together with the fact that eliminating the positive charge did not alter anion binding, is compatible with the hypothesis that K335 does not form a positive center for an anion binding site. The conclusion is consistent with the results of Tabcharani et al. (1993) who found that introducing a glutamic acid at K335 reduced, but did not eliminate, the anomalous mole fraction dependence of CFTR conductance on $[SCN]_o$.

Several other observations support the hypothesis that the 335 residue is positioned such that it can influence anion permeation via purely electrostatic effects. The addition of negative charge at this position produced a tendency toward inward rectification of the macroscopic *I-V* plot, which is consistent with a selective reduction of inward anion flux (outward current) such as might be expected if the charge at this position influenced the local ion concentration at the mouth of the pore. Although the addition of negative charge altered *I-V* shape, the removal of positive charge was without effect. This result could be a reflection of the saturable nature of the anion conductance; i.e., the effect of the positive change to increase the local anion concentration could be negligible, although the effect of a negative change to reduce the concentration attenuates inward anion flow. These electrostatic effects would be expected to be nonselective, an expectation that is generally confirmed by the lack of effect of substitutions on permeability ratios calculated from reversal potential shifts. One important exception to this is the permeability selectivity of CFTR for I. We found, as did Anderson et al. (1991), that the sequence for Cl and I was reversed in K335E CFTR. In the present study, however, the sequence for the mutant was not that predicted solely by hydration energies, suggesting that I interacts in a unique way with both wtCFTR and K335E. I permeation properties in CFTR have been reported to be complex in Chinese hamster ovary (CHO) cells where Tabcharani et al.

(1997) reported that the apparent value of P_I/P_{Cl} could vary between 1.8 and <0.4 depending on the protocol. These authors proposed that in wtCFTR I binding alters P_I/P_{Cl}.

G91 is not critical for the architecture of the anion binding site

The results of the substitutions at G91 support the hypothesis that this residue does not play a critical role in the anion conduction path of CFTR. With one exception (*I-V* shape, see below), substitutions were without effect on conduction properties or cAMP-dependent activation. In view of the results obtained with substitutions for K335 and G314, it is particularly noteworthy that the concentration-dependent inhibition of CFTR conductance by $[SCN]_o$ was unaffected by mutations of G91, regardless of the nature of the substituted residues. Thus, neither alanine, which would be expected to dramatically alter helical structure in TM1, nor the addition of negatively or positively charged side chains exerted even a small influence on the apparent binding of SCN in the pore. Although a limited sample, this result is consistent with the notion that TM1 may not contribute directly to the lining of the pore and that G91 is sufficiently distant from the anion binding site so as not to exert electrostatic effects on the interactions of permeating anions with the pore. These findings are consistent with the results obtained by Carroll et al. (1995) with a deletion construct lacking TM1. This alteration resulted in a small decrease in single-channel conductance (6%) and no change in ion selectivity. One of the most interesting mutants surveyed in this study was the G91R CFTR, which exhibited a dramatic, nearly sevenfold, increase in outward rectification (Table 4) but was identical to wtCFTR with regard to the apparent binding of SCN and dose-dependent activation by IBMX. In contrast, G314E CFTR exhibited a dramatic decrease in anion binding and sensitivity to activation by IBMX but no change in *I-V* shape. These results suggest that, whatever the mechanism for rectification may be, the structural basis for this behavior is in some way separate from that which determines the affinity of anion binding.

Akabas et al. (1994) implicated TM1 as a pore-lining transmembrane segment based on the accessibility of cysteines inserted for G91, K95, and Q98 to highly polar sulfhydryl reagents derived from methanethiosulfonate (MTS). Specifically, they found that G91, predicted on the basis of a presumed α-helix to lie about halfway across the membrane, was accessible to a cationic reagent $MTSEA^+$ (MTS-ethylammonium) applied extracellularly and suggested that cations might penetrate into the pore of CFTR to this level. There are several alternative explanations for this result, however. First, thiol reagents may penetrate into a hydrophilic crevice between TM1 and its nearest neighbors, so that accessibility need not necessarily imply that the access was via the anion-conducting pore (Yang et al., 1996). Second, the engineered cysteines may have altered the packing of TM (or relation to neighbors) so as to create a hydrophilic pocket that bound $MTSEA^+$. In fact, the data presented by Akabas et al. (1994) suggest that the activation of the G91C mutant was markedly slowed compared with that of wtCFTR. Finally, Holmgren et al. (1996) recently showed that $MTSEA^+$ rapidly penetrates a lipid bilayer so that it is expected to enter oocytes.

TM5 and TM6 play a role in cAMP-dependent activation of CFTR

One of the most striking results obtained with TM1, TM5, and TM6 mutations was the change in the $K_{1/2}$ for activation by IBMX. Most of the mutations in TM5 and TM6 that compromised SCN binding also increased $K_{1/2(IBMX)}$, and the decrease in sensitivity to activating conditions was similar to that seen with nucleotide-binding fold mutations associated with severe and mild CF, respectively. In G314D CFTR, however, apparent SCN binding was similar to that seen with wtCFTR, but activation was significantly impaired, suggesting that 1) sensitivity to activation by IBMX is, if anything, even more sensitive than anion binding to alterations in the structure of TM5 and 2) there is no obligatory relation between anion binding and activation.

In the absence of detailed structural information pertaining to the configuration of the transmembrane domains of CFTR, we can only speculate about the origin of the activation deficit seen with G314 and K335 substitutions. One possibility is that such substitutions interfere with the final step in the activation process, a conformational change that allows ions to translocate through the pore. For example, the conformational flexibility afforded by G314 may be required to effect the transduction of signals from the cytoplasmic domains to the pore of CFTR. These observations, however, suggest that caution is in order when interpreting the effects of amino acid substitution in the TMs on CFTR function because both conduction and gating may be significantly altered.

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