

CFTR: A Cysteine at Position 338 in TM6 Senses a Positive Electrostatic Potential in the Pore

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ABSTRACT We investigated the accessibility to protons and thiol-directed reagents of a cysteine substituted at position 338 in transmembrane segment 6 (TM6) of CFTR to test the hypothesis that T338 resides in the pore. *Xenopus* oocytes expressing T338C CFTR exhibited pH-dependent changes in g_{Cl} and I-V shape that were specific to the substituted cysteine. The apparent pK_a of T338C CFTR was more acidic than that expected for a cysteine or similar simple thiols in aqueous solution. The pK_a was shifted toward alkaline values when a nearby positive charge (R334) was substituted with neutral or negatively charged residues, consistent with the predicted influence of the positive charge of R334, and perhaps other residues, on the titration of a cysteine at 338. The relative rates of chemical modification of T338C CFTR by MTSET⁺ and MTSES[−] were also altered by the charge at 334. These observations support a model for CFTR that places T338 within the anion conduction path. The apparent pK_a of a cysteine substituted at 338 and the relative rates of reaction of charged thiol-directed reagents provide a crude measure of a positive electrostatic potential that may be due to R334 and other residues near this position in the pore.

INTRODUCTION

The anion selective pore of the CFTR chloride channel has been probed by means of site-directed mutagenesis and covalent modification of engineered cysteines (Akabas, 1998; Cheung and Akabas, 1996, 1997; Smith et al., 2001). Based on the permeation of halides and tighter-binding, polyatomic anions (often referred to as *pseudohalides*; Douglas et al., 1994) we proposed that the CFTR channel could be envisioned as an hourglass-shaped pore comprising a narrow region, where anions bind, coupled to the external and internal solutions by two wider vestibules (Smith et al., 1999). Modification of engineered cysteines by pH and thiol-directed reagents were consistent with the hypothesis that R334 in transmembrane segment 6 (TM6) lies in the outer vestibule of the pore, where it establishes a positive electrostatic potential that increases the local concentration of Cl[−], thereby enhancing pore conductance (Smith et al., 2001). Here we present evidence that T338, which lies one (predicted) helical turn cytoplasmic to R334 (Fig. 1), also resides within the pore. Furthermore, we propose that the titration of a cysteine residue at 338, as well as the rate of modification of this

cysteine by charged thiol-directed reagents, provides a crude measure of a positive electrostatic potential in this region of the pore that may be attributable to R334 and perhaps other residues in the outer vestibule.

Evidence for the existence of a positive electrostatic potential in the outer vestibule of the CFTR pore was derived from two sources. The first was an analysis of the apparent pK_a of a cysteine thiolate at position 338. The pK_a was more than one pH unit more acidic than that expected for a cysteine or other simple thiols in free solution. We interpreted the shift in pK_a using a simplified model that relates the shift in pK_a to the local electrostatic potential produced by nearby positive charges, one of which may be the arginine at position 334. A complementary approach, similar to that first used by Stauffer and Karlin (1994) and Pascual and Karlin (1998), was based on an analysis of the relative rates of modification of a cysteine at 338 by the charged disulfides, MTSET⁺ and MTSES[−]. These two independent approaches yielded similar estimates for the electrostatic potential due to nearby charges and provide support for a conduction model for CFTR that features a positively charged outer vestibule.

MATERIALS AND METHODS

Mutagenesis and in vitro transcription

The methods used for mutagenesis and in vitro transcription were the same as those reported previously (Smith et al., 2001; Liu et al., 2001). CFTR mutants were generated using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The sequences in the region of the mutation and in the whole PCR-generated region were confirmed by direct DNA sequencing.

The CFTR cRNAs for *Xenopus* oocyte injection were synthesized by using the in vitro transcription kit, mMessage mMachine (Ambion, Austin, TX). The transcription products were purified and the quality and quantity of the transcripts were assessed on an agarose gel.

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Abbreviations used: CFTR, cystic fibrosis transmembrane conductance regulator; cRNA, complementary ribonucleic acid; DTT, dithiothreitol; IBMX, 3-isobutyl-methylxanthine; Isop, isoproterenol; MTSES[−], sodium [2-sulfonatoethyl]methanethiosulfonate; MTSET⁺, [2-(trimethylammonium)ethyl] methanethiosulfonate bromide; 2-ME, 2-mercaptoethanol; NEM, *N*-ethylmaleimide; PKA, protein kinase A; TM, transmembrane spanning segment.

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50–100 Hz and single channel records were analyzed using the event detection features of Clampfit 9 (Axon Instruments). Some experiments were done in TES buffer and were recorded at 10 kHz on Axopatch 200B onto DAT tape (model DTC-790, Sony, Tokyo, Japan) and subsequently filtered at 100 Hz (four-pole Bessel filter, Warner Instruments, Hamden, CT) and acquired by the computer at 400 Hz using the Fetchex program of pClamp (Axon Instruments) for analysis. In these experiments, pipette solution contained 200 mM NMDG-Cl, 5 mM MgCl₂, and 10 mM TES, adjusted to pH 7.4 with Tris). Channels were either activated on-cell with isoproterenol before excising into intracellular solution (200 mM NMDG-Cl, 1.1 mM MgCl₂, 2 mM Tris-EGTA, 1 Mg ATP, and 10 mM TES, pH 7.3, and 50 U/mL PKA (Promega), or were activated by PKA after excision. The single channel current for T338A CFTR was measured using 150 mM symmetrical [Cl[−]]. Single-channel i-V plots were obtained by determining the current amplitudes at V_m ranging from −100 mV to 100 mV at 20 mV intervals.

Open and closed current levels were first identified manually and then by transition analysis using a 50% cutoff between open and closed levels. We used an apparent open probability (P_o^*) as a measure of channel gating. We defined P_o^* as the ratio of NP_o -for-all-levels divided by N , where NP_o -for-all-levels was obtained after single-channel searching using the event detection features in Clampfit 9 and N is the apparent number of channels in a patch.

Recordings of single, T338C channels in experiments involving covalent modification posed additional challenges for two reasons. First, as indicated above, although variable oxidation states of the cysteine could be mitigated by treatment with reducing agents, we could not eliminate the possibility that in any particular patch there could be CFTR channels that would be insensitive to reducing agents and nonreactive toward MTS compounds. In addition, in some preliminary experiments, it was clear that after exposure to a reducing agent, the cysteine at 338 could spontaneously reoxidize to a variable extent over the time course of a typical experiment, 30–120 min. Finally, preliminary experiments indicated that, in contrast to results obtained previously with R334C CFTR, reaction of T338C channels with MTSET⁺ actually reduced single-channel conductance, rendering the resulting single-channel currents more difficult to discriminate from the small (0.1–0.3 pA) background Cl[−] channels sometimes seen in patches from oocytes. In view of these potential ambiguities, the single-channel data from experiments involving covalent modification were analyzed in the following way. Amplitude distributions were computed for each current level under each particular condition (reducing agent pretreated, MTSET⁺ modified, treated with reducing agent after MTSET⁺ modification) using the single-channel event detection feature of Clampfit 9 (Axon Instruments). Only opening amplitudes that appeared in all patches under a particular condition were counted as CFTR, and the data were analyzed so as to enable us to determine if changing the experimental condition resulted in the conversion of one amplitude type to another, i.e., an increase in prevalence of openings of one amplitude and a corresponding decrease in another. This procedure should result in the exclusion of background channels as well as CFTR channels that are in a *terminal oxidation state* that would be insensitive to either reducing agents or MTS reagents. All recordings involving MTSET⁺ modification were conducted at a pipette pH of 6.0 to maximize the conductance of unmodified channels. This acidic pH is expected to slow the rate of the thiol-disulfide exchange reaction, but not to prevent it (Roberts et al., 1986). Recording conditions were designed to maximize the likelihood of recording T338C single channels in each of three states: unmodified; MTSET⁺ modified; and MTSET⁺ modified, but subsequently exposed to 2-ME to reverse the modification (see text).

Several factors inherent in carrying out covalent modification experiments made it very difficult to accurately quantify channel gating. First, to accurately quantify single-channel amplitudes, the ATP concentration in the solution bathing detached patches was often manipulated to enhance the resolution of individual openings in multichannel recordings. Second, to reduce the disulfide bond between MTSET⁺ and the cysteine thiolate at 338 after modification, it was necessary to apply reducing agents to the cytoplasmic side of the patch, a maneuver that has been shown to alter CFTR gating (Harrington et al., 1999). Third, in some of the experiments, long

recordings were necessary (20–60 min) during which channel rundown may occur spontaneously. Therefore, the data presented focused on the change in channel amplitude brought about by covalent modification and its comparison to changes in macroscopic currents.

Reagents

Highly polar derivatives of methanethiosulfonate reagents, [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET⁺) and sodium [2-sulfonatoethyl]methanethiosulfonate (MTSES[−]) were obtained from Toronto Research Chemicals (Toronto, Canada). N-ethylmaleimide (NEM) was obtained from Sigma. Reducing agents included 2-mercaptoethanol (2-ME) and dithiothreitol (DTT), were obtained from Sigma. The molecular volumes of MTS reagents were determined using a molecular modeling program (Titan, Wavefunction, Irvine, CA).

Whole-cell data analysis

The apparent pK_a of the conductance was determined by titrating the pH of the bath. The value was estimated by fitting the conductance versus pH relation to a modified Henderson-Hasselbalch equation using the curve-fitting routines in SigmaPlot (SPSS, Chicago, IL) (see Eq. 2). The total conductance due to CFTR, g_{Cl} , was expressed as the sum of two components, one pH-dependent, $\Delta g(pH)$ and the other pH-independent, g_{min}^{exp} (Eq. 1),

$$g_{Cl} = g_{min}^{exp} + \Delta g(pH), \quad (1)$$

$$\Delta g(pH) = g_{Cl} - g_{min}^{exp} = \frac{\Delta g_{pH}^{max}}{1 + 10^{(pH - pK_a)}}, \quad (2)$$

where g_{min}^{exp} was operationally defined as the minimum conductance that was measured in the most basic solution in a particular experiment and Δg_{pH}^{max} was defined as the maximum change in conductance due to pH titration.

We interpreted the titration behavior of the conductance due to T338C CFTR as reflecting the pH-dependent change in the time-averaged, partial negative charge on any single thiolate anion. We used a modified Henderson-Hasselbalch equation (Eq. 3) to calculate this partial charge just as one would calculate the fraction of thiol in the ionized form

$$q = z \left\{ \frac{10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}} \right\}, \quad (3)$$

where $z = 1$ for cations and -1 for anions and q can range from either -1 to 0 or 0 to 1 , depending on the titratable group.

For ease of comparison, in T338C/R334X ($X = A$ or E) CFTRs and T338H/R334C CFTRs in which the cysteine was always blocked by reaction with MTS reagents or NEM, the titration curves were expressed in a normalized form. $\Delta g(pH)$ and Δg_{pH}^{max} were expressed as the fractional increase with respect to g_{min}^{exp} (Eq. 4),

$$\Delta g(pH)^{nor} = \frac{(\Delta g_{pH}^{max})^{nor}}{1 + 10^{(pH - pK_a)}}. \quad (4)$$

Modeling

The effects of charge changes on anion conduction at position 338 were interpreted using two types of models, similar to those employed by Smith et al. (2001). In both models, the pore was viewed as comprising a rate-

limiting central region flanked by two vestibules (Dani, 1986; MacKinnon et al., 1989; Lu and MacKinnon, 1994), where the electrical potential created by charged groups can influence local anion distribution. In one model the properties of the rate-limiting region were described using a constant-field, electrodiffusion model (Goldman, 1943) modified to incorporate charged inner and outer vestibules where ion concentrations are in equilibrium with those in the bulk solutions adjacent to the vestibules (Eq. 5). The electrostatic potentials of the inner and outer vestibules were represented by average values, Ψ_i and Ψ_o , respectively, which were positive for a positively charged vestibule and negative for a negatively charged vestibule. The model was fitted to the data using the curve-fitting routines contained in SigmaPlot (SPSS),

$$I_{Cl} = \frac{F^2}{RT} P_{Cl} (V_m + \Psi_i - \Psi_o) e^{\frac{F\Psi_o}{RT}} \left(\frac{[Cl]_b - [Cl]_c e^{\frac{-FV_m}{RT}}}{1 - e^{\frac{-F}{RT}(V_m + \Psi_i - \Psi_o)}} \right), \quad (5)$$

where V_m is the measured transmembrane potential and P_{Cl} is the chloride permeability of the narrow region of the pore, $[Cl]_b$ and $[Cl]_c$ are bath and cytoplasmic chloride concentrations, and F , R , and T have their usual meanings. In the second model the rate-limiting region was described using a four-barrier, three-well rate theory scheme incorporating a variable surface potential in the outer vestibule. I-V plots were simulated using a program developed by Dr. Ted Begenisich (Begenisich and Cahalan, 1980) and curves were fitted to the data by eye.

RESULTS

The conductance of oocytes expressing T338C CFTR is highly sensitive to changes in bath pH

Testing the hypothesis that a cysteine at position 338 resides within the conduction pathway required that we alter the

charge at this locus. In previous experiments using R334C CFTR we found that charge change could be effected by means of covalent modification with thiol-directed reagents like MTSET⁺ or MTSES⁻, or by using changes in bath pH to alter the partial negative charge on the thiolate anion (Smith et al., 2001). Because the effect of amino acid substitutions at 338 on CFTR conduction had been interpreted as suggesting that T338 might reside near the narrow part of the pore (Linsdell et al., 1997, 1998, 2000; McCarty and Zhang, 2001), we used pH titration to evaluate the effects of charge changes at position 338, hoping to avoid potential obstruction of the pore by MTS reagents. Fig. 2 A contains a plot of g_{Cl} at $V_m = E_{rev}$ versus time from a representative experiment ($n = 5$) designed to test the pH-sensitivity of T338C CFTR. Channels were activated by exposure to a stimulatory cocktail containing 10 μ M Isop and 1 mM IBMX (Isop+IBMX). For reasons discussed in Materials and Methods and in a separate communication (Liu et al., 2002), oocytes were first exposed to a reducing agent (0.1–1 mM 2-ME or DTT) for 2–5 min after activation, to favor the simple thiolate form of the engineered cysteine. Afterwards, the bath pH was stepped to pH 6 and then pH 9. Acidifying the bath pH from 7.4 to 6 increased g_{Cl} at $V_m = E_{rev}$ from $71.5 \pm 10.1 \mu S$ to $134.3 \pm 12.2 \mu S$ ($n = 5$). Alkalinizing the bath pH from 6 to 9 decreased g_{Cl} at $V_m = E_{rev}$ from $134.3 \pm 12.2 \mu S$ to $21.4 \pm 5.2 \mu S$ ($n = 5$). Fig. 2 B contains the I-V plots obtained at pH 9, 7.4, and 6 from the same oocyte. It can be seen that as the bath pH became more acidic the I-V plot changed in two ways: g_{Cl} at $V_m = E_{rev}$

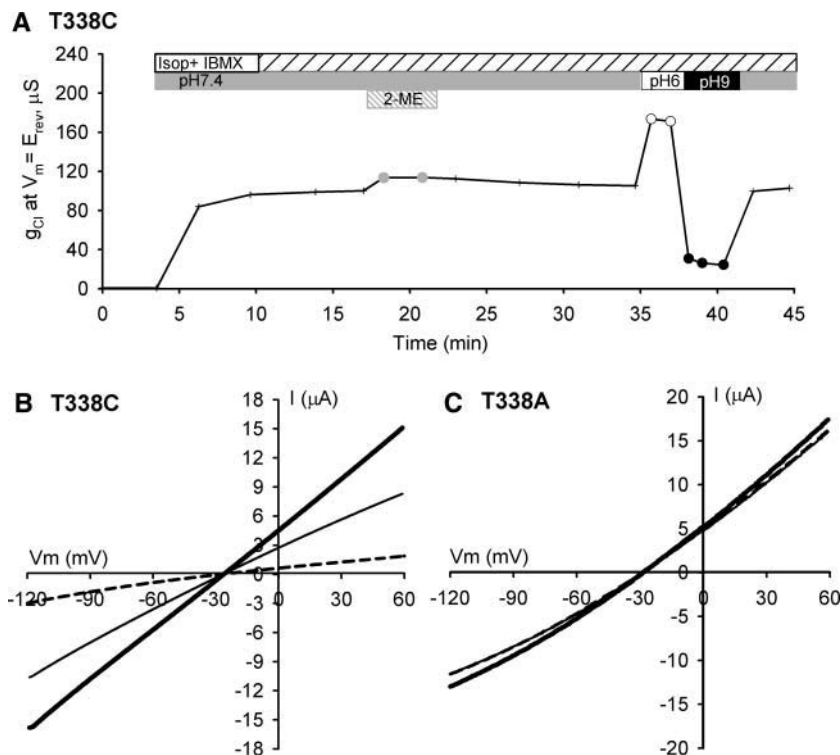


FIGURE 2 The pH-induced change in conductance of oocytes expressing T338C CFTR. (A) g_{Cl} at $V_m = E_{rev}$ versus time. The channels were activated by a stimulatory cocktail containing 10 μ M Isop and 1 mM IBMX (Isop+IBMX, hatched bar above). The crosshairs and circles depict times of measurements. At steady state, oocytes were exposed to 1 mM 2-ME (shaded circles) to ensure that the cysteine thiol was in a simple thiolate form. After a wash, the bath pH was changed sequentially to pH 6 (open circles) and pH 9 (solid circles), then to pH 7.4 again. These maneuvers induced changes in both conductance and the shape of the I-V curve. (B) The I-V plots obtained at pH 9 (dashed line), 7.4 (dotted line), and 6 (solid line) from an oocyte expressing T338C CFTR. (C) The I-V plots obtained at pH 9 (dashed line), 7.4 (dotted line), and 6 (solid line) from an oocyte expressing T338A CFTR.

increased and the shape changed from inward rectifying toward linearity. As documented in detail in Smith et al. (2001), this result is consistent with the expected influence of a negative charge in the conduction path that is increased at alkaline pH and reduced at a more acidic pH.

Changing the bath pH had essentially no effect on the conductances of oocytes expressing T338A CFTR (Fig. 2 C, $n = 3$), nor did the same maneuver alter the conductances of oocytes expressing T338S (Fig. 3) or wt CFTR (Smith et al., 2001), consistent with the idea that the pH-dependent change in conductance of T338C CFTR was due to the titration of the cysteine substituted at 338. As an additional test of the hypothesis that the pH-induced response seen in T338C CFTR was due to the titration of the engineered cysteine, we exposed oocytes expressing T338C CFTR to NEM, a reagent that forms a thioether bond with the cysteine, and thereby blocks titration of the thiol group. As summarized in Fig. 3, acidifying the bathing solution (pH 7.4–6) induced an $\sim 68\%$ (± 12) increase in conductance in oocytes expressing T338C CFTR whereas alkalinizing the bath (pH 7.4–9) decreased the conductance by $\sim 80\%$ (± 4). After exposure to 100 μM NEM, however, changing the bath pH had no effect on the conductances of these oocytes. Pre-exposure to 1 mM MTSET⁺ or 1 mM MTSES[−] also prevented the response of T338C CFTR conductance to a change in bath pH (X. Liu and D. C. Dawson, unpublished observation).

Changes in anion conduction brought about by titration of T338C CFTR are consistent with the predictions of a simplified charged-vestibule model

We compared the effect of charge changes at 338 to the predictions of simplified charged-vestibule models used previously to analyze the influence of charge at position 334 (Smith et al., 2001). As shown in Fig. 4, bath pH-induced changes in conductance and I-V shape could be described by models based either on the Goldman equation or rate theory, in which the *only* variable allowed to change with pH was the magnitude of the electrostatic potential of the outer vestibule, Ψ_o . Using the Goldman equation, the decrease in conductance (with respect to pH 6) observed at pH 7.4 and pH 9 could be attributed to changes in the outer vestibule potential of -27 mV and -124 mV, respectively. Using the rate theory model, the I-V curve at pH 7.4 was fitted by adding a Ψ_o of -35 mV and the I-V curve at pH 9 was fitted by adding a Ψ_o of -95 mV. The fit for pH 9 might have been improved if Ψ_o had been set to a more negative value, but -95 mV was the default limit of the program. The results of the modeling were consistent with the notion that the effects of charge changes at position 338 on anion conduction can be largely accounted for by changes in outer vestibule potential. This sort of model, although highly simplified, was also used by Lu and MacKinnon (1994) to describe the effects of a charged amino

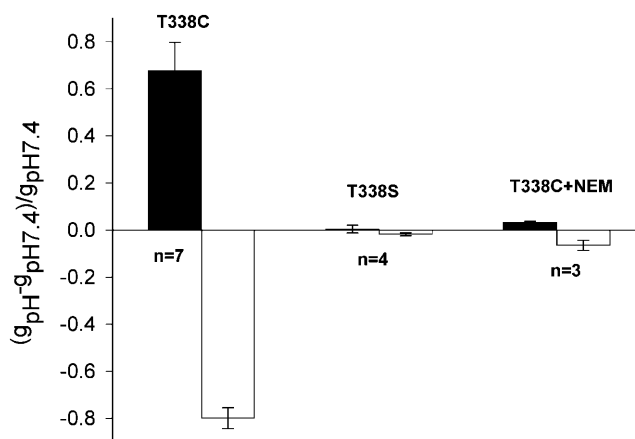


FIGURE 3 The pH-sensitivity of T338C CFTR conductance was due to the titration of the cysteine thiol. Oocytes expressing T338C or T338S CFTR were first exposed to a stimulatory cocktail containing 10 μM Isop and 1 mM IBMX at pH 7.4. After activation, oocytes were exposed to reducing agents followed by a wash. Bath pH was then changed to either pH 6 (solid bar) or pH 9 (open bar), then to pH 7.4. In some oocytes, after exposure to reducing agents, oocytes were first exposed to NEM after a wash, then to either pH 6 or pH 9. Premodification by NEM prevented the large pH-induced response seen in T338C CFTR conductance. T338S CFTR exhibited no pH-sensitivity.

acid side chain on Mg^{2+} blockade and K^{+} conduction in inwardly-rectifying potassium channels.

Single-channel conductance of T338C CFTR is pH-sensitive

Fig. 5 A contains examples of T338C CFTR single-channel currents recorded from detached inside-out patches with the pH of the pipette solution (containing 1 mM 2-ME) buffered to either pH 6 or pH 7.4. At pH 6 ($V_m = -100$ mV) the dominant single-channel current amplitude was 0.8–0.9 pA, whereas with a more alkaline (pH 7.4) pipette solution, the dominant current amplitude was a 0.6-pA current that was not seen at pH 6. The result is consistent with the notion that alkalinizing the pipette solution reduced single-channel conductance. We observed an increase in open channel noise at the more alkaline pH, 7.4, and—as can be seen in Fig. 5 A—this, in combination with the rectification of the single-channel current, made it more difficult to resolve single-channel events at $V_m = 100$ mV. Occasionally, in patches containing several channels, we observed events with smaller current amplitudes, 0.1–0.3 pA, with or without 2-ME in the pipette. These current amplitudes were comparable at pH 6 and pH 7.4. Because of the smaller amplitude and rare occurrence of these channels, their sensitivity to intracellular ATP could not be confidently determined. We speculate that these events might be attributed to the opening of some endogenous channels or alternatively, to T338C CFTR channels that were in oxidation

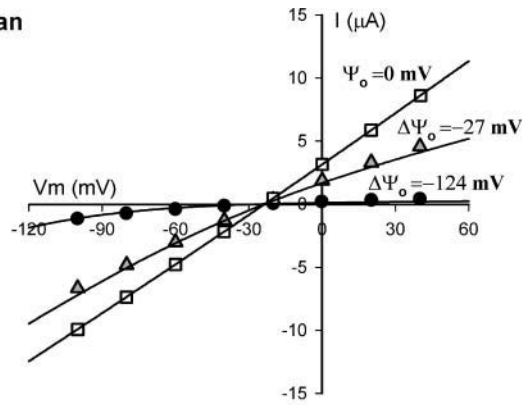
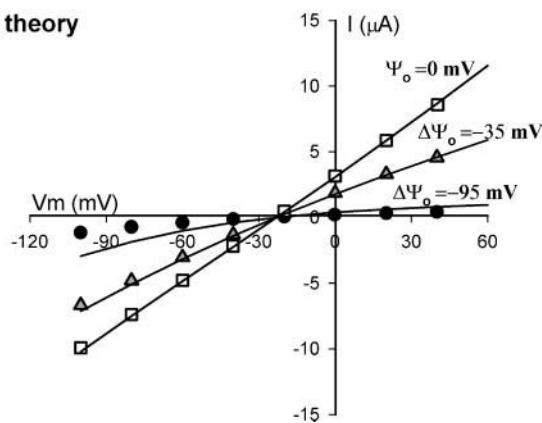
A Goldman**B Rate theory**

FIGURE 4 Modeling I-V data using continuum (Goldman) and rate theory models. (A) Using the continuum model, the I-V curve corresponding to pH 6 (open squares) obtained from an oocyte expressing T338C CFTR was first fitted by fixing Ψ_o at zero, resulting in a P_{Cl} of $3.51 \times 10^{-7} \text{ cm}^3/\text{s}$, a C_i of 42 mM, and a Ψ_i of 20.9 mV. All of the fitted curves are shown as solid lines. I-V curves corresponding to pH 7.4 (shaded triangles) and pH 9 (solid circles) were subsequently fitted with P_{Cl} , C_i , and Ψ_i held constant. The decreased conductances in pH 7.4 and pH 9 with respect to pH 6 were attributed to a shift of the outer vestibule potential by -27 mV and -124 mV , respectively. (B) When using the rate theory model, the predicted currents were scaled according to the macroscopic conductance (g_{Cl} at $V_m = E_{rev}$) at a pH of 6. (The rate theory fitting procedure differed in one detail from that reported previously. Smith et al., 2001, scaled the currents predicted by the model in each condition to the macroscopic I-V plot for that condition. We have found, however, that more satisfactory fits were obtained if a single scaling factor was used for all three plots. This procedure attributed the effects of MTSET⁺ and MTSES⁻ on the conductance of R334C CFTR to a change in Ψ_o of 45 mV and -30 mV , respectively, a prediction somewhat different from that reported by Smith et al., 2001, in which the effects of MTSET⁺ and MTSES⁻ were attributed to a change in Ψ_o of 50 mV and -10 mV , respectively.) The I-V data obtained at pH 6 (open squares) were fitted using barrier heights (in units of RT) of 4.3, 5, 3, and 5, and well depths (RT) of -1.8 , -2 , and -1 , whereas Ψ_i and Ψ_o were fixed at zero. With the same barrier heights and well depths, the I-V data at pH 7.4 (shaded triangles) were fitted by adding a Ψ_o of -35 mV and the I-V data pH 9 (solid circles) were fitted by adding a Ψ_o of -95 mV , the default limit of the program.

states that could not be reduced by 2-ME, i.e., sulfinic or sulfonic acids (Giles et al., 2003; Jacob et al., 2003).

Fig. 5 B contains single-channel i-V plots demonstrating that alkalinizing the pipette solution not only reduced the

conductance, but also altered the shape of the i-V plot so as to produce significant inward rectification similar to that seen in macroscopic recordings. Like those seen in macroscopic recordings, pH-induced changes in single-channel conductance and i-V shape could be predicted using Goldman or rate theory models in which only Ψ_o was permitted to vary with pH. The Goldman model attributed the effect of changing bath pH from pH 6 to pH 7.4 to a change in Ψ_o of -35 mV . Fitting the same i-V curves using the rate theory model (Fig. 5 C) predicted a change in Ψ_o of -50 mV . Single-channel recording also indicated that the conductance of T338A CFTR channels was not sensitive to changes in bath pH (Fig. 5 C, inset).

It is also of interest to note that the ratio of the single channel conductance at pH 6 and pH 7.4 for T338C CFTR was ~ 1.8 , a value comparable to the ratio observed for the macroscopic conductances (1.7). This result is consistent with the idea that the pH-induced change in the macroscopic conductance was primarily due to a change in single-channel conductance, rather than an effect on channel gating. Because we employed different ionic conditions for single-channel and macroscopic measurements of the conductance ratio (see Materials and Methods), we used the fitted parameters for the single-channel results to determine how this ratio would be affected by changing the ionic conditions. Changing the concentration from 200 mM symmetrical Cl^- (used for single-channel recordings) to that obtained from fitting the macroscopic conductance ($[\text{Cl}^-]_o = 105.6 \text{ mM}$ and $[\text{Cl}^-]_i = 42 \text{ mM}$) predicted a change in the conductance ratio, $\gamma_{\text{pH}6}:\gamma_{\text{pH}7.4}$ of, at most, 9%.

Alteration of the charge on the engineered cysteine thiolate did not affect channel gating. As summarized in Fig. 6 we could discern no relationship between external (pipette) pH and the apparent open probability, P_o^* , determined as NP_o/N . At $[\text{ATP}] = 1 \text{ mM}$, P_o^* averaged 0.22 ± 0.06 at pH 6 and 0.21 ± 0.04 at pH 7.4. At $[\text{ATP}] = 2 \text{ mM}$, P_o^* averaged 0.27 ± 0.06 at pH 6 and 0.27 ± 0.02 at pH 7.4. No pH-induced change in P_o^* was observed in T338A CFTR, which averaged 0.85 ± 1.2 at pH 6 and averaged 0.74 ± 0.15 at pH 7.4 ($[\text{ATP}] = 1 \text{ mM}$). We did not study in detail the gating difference between these two mutants, but the result is not surprising in light of previous studies in which amino acid substitutions in the TMs were found to alter CFTR gating (Sheppard et al., 1993; Cotten and Welsh, 1999; Zhang et al., 2000, 2002).

The macroscopic conductance due to T338C CFTR exhibits a pK_a that is more acidic than expected for a simple thiol in free solution

Shown in Fig. 7 is a representative titration curve obtained by measuring the conductance of an oocyte expressing T338C CFTR at different values of bath pH ($n = 5$). To favor the simple thiolate form of the cysteine, titration was conducted in the presence of either 2-ME or DTT. The apparent pK_a was

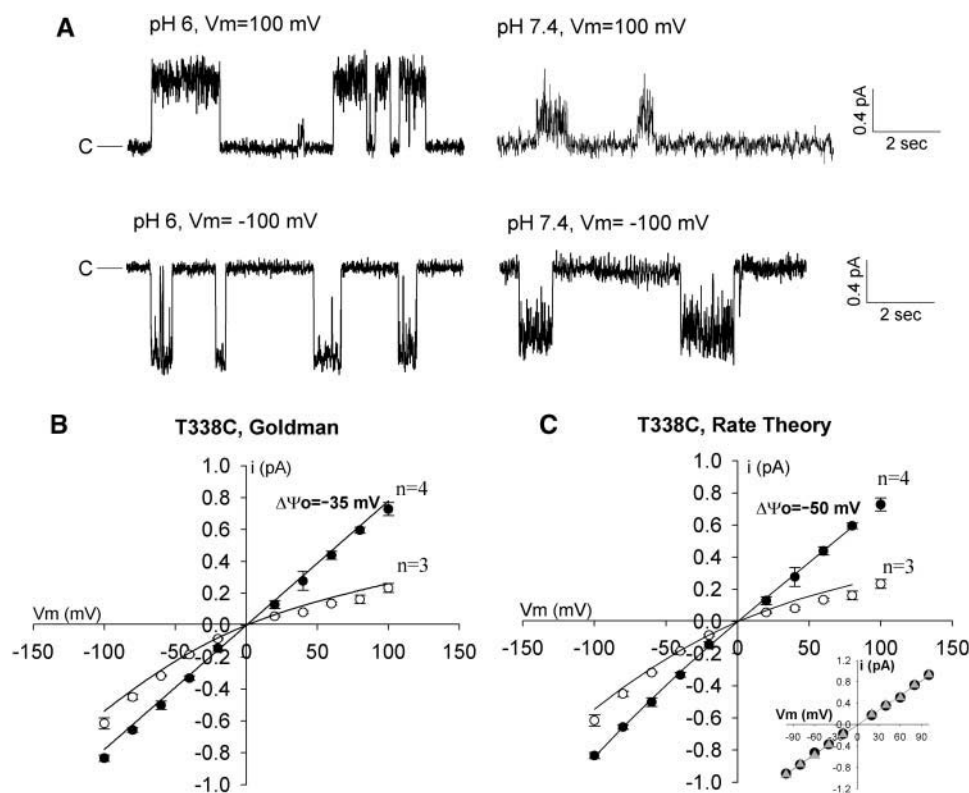


FIGURE 5 The pH-dependence of single-channel amplitudes. (A) Examples of single-channel current records obtained from inside-out patches detached from oocytes expressing T338C CFTR. (B) The *i*-*V* plot for T338C CFTR. The fitted curves (solid lines, Goldman equation) were obtained by fixing Ψ_o and Ψ_i at zero and C_i at 200 mM, then first fitting the *i*-*V* plot for pH 6 (solid circles) to Eq. 4, resulting in a P_{Cl} of 1.04×10^{-14} cm³/s. The *i*-*V* curve corresponding to pH 7.4 (open circles) was then subsequently fitted while holding P_{Cl} constant, resulting in a Ψ_o of -41 mV. (C) Fitting with the rate theory model. The fitted curves are shown as solid lines. The *i*-*V* data at pH 6 were first fit by holding Ψ_i and Ψ_o at zero and using barrier heights (*RT*) of 10.9, 11.5, 9.5, and 12, and well depths (*RT*) of -1.8, -2, and -1. With the same barrier heights and well depths, the *i*-*V* curve at pH 7.4 was fit by adding a Ψ_o of -50 mV. (Inset) T338A CFTR conductance was insensitive to changes in bath pH. Shown are examples of single-channel *i*-*V* plots obtained from inside-out patches detached from oocytes expressing T338A CFTR at pH 6 (solid circles) and pH 7.4 (shaded triangles). All the recordings for T338A CFTR were done in the presence of 150 mM symmetrical $[Cl^-]$.

independent of the concentration of the reducing agents over a range of 50 μ M to 1 mM, indicating that these species did not contribute to the value of the apparent pK_a . For this particular oocyte, the resulting g_{Cl} at $V_m = E_{rev}$ versus pH curve was described by a Henderson-Hasselbalch relation with an apparent pK_a of 7.3 (average 7.41 ± 0.05 , $n = 10$). Shown for comparison (dashed lines) are two curves, one predicted for a pK_a of 8.3, a value more like that expected for a cysteine in aqueous solution (Lindley, 1960), and another predicted for a pK_a of 10.3 as seen in simple thiolates such as methylthiol and ethylthiol (Irving et al., 1964; Kreevoy et al., 1964; Stewart, 1985). This result is consistent with the presence of a nearby positive charge, like R334, that could stabilize the thiolate anion (see Discussion and Supplementary Material for details).

To investigate the effect of charge at position 334 on the titration behavior of T338C CFTR, we examined the conductance of oocytes expressing double mutants, T338C/R334A, T338C/R334E, and T338C/R334D CFTR. Shown in Fig. 8 A are representative titration curves for the conductance for T338C CFTR and two of these double mutants ($n = 5$ each). Neutralizing the charge at 334 (R334A) resulted in a pK_a that was more than one pH unit more basic (8.78 ± 0.03 , $n = 4$) than that determined for T338C CFTR. The substitution of acidic residues, however,

did not result in a large additional shift of the apparent pK_a to more alkaline values (8.84 ± 0.05 for T338C/R334D CFTR, $n = 4$ and 8.96 ± 0.08 for T338C/R334E CFTR, $n = 5$). We speculate that this result may reflect, at least in part, the effect of amino acid substitutions on the orientation and spatial relations of the charges (see Discussion). In the range of pH used, glutamic and aspartic acid should exist primarily in the deprotonated form.

To test the generality of titration results obtained with T338C CFTR, we compared the titration behavior of T338H CFTR with that of a double mutant, T338H/R334C, in which it was possible to change the charge at position 334 by means of chemical modification. Summarized in Fig. 8 B are the results obtained from oocytes expressing either T338H or chemically modified T338H/R334C CFTR ($n = 3-4$ for each mutant). Shown are sample titration curves for T338H and T338H/R334C CFTRs in which the cysteine was modified by MTSET⁺, MTSES⁻, and NEM (neutral). The solid lines are the fitted curves and the dashed lines are extrapolations to lower pH values that were not well tolerated by oocytes. The conductance of oocytes expressing T338H CFTR was essentially not titratable, as if the pK_a of the histidine was more acidic than 4.0. Similarly, the conductance due to MTSET⁺-modified T338H/R334C CFTR was not very sensitive to pH titration. However, the conductances of

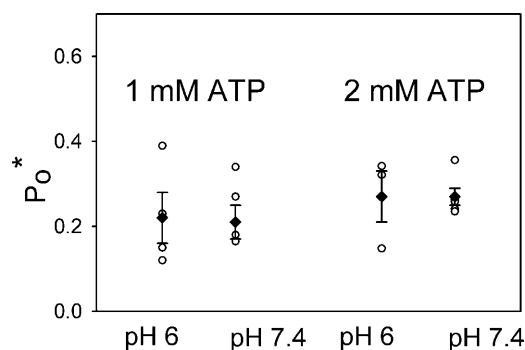


FIGURE 6 Alteration of charge at the engineered cysteine at position 338 did not affect channel gating. No relationship exists between external (pipette) pH and the apparent open probability, P_o^* . \circ , Individual experiments, with either 1 or 2 mM ATP in the intracellular bath solution in which values of P_o^* were computed (see Methods) over periods ~ 1 min (1 mM ATP) or from 2.5 to 25 min (2 mM ATP). \blacklozenge , Mean \pm SE for each condition ($n = 3-5$).

NEM- or MTSES⁻-modified T338H/R334C CFTR were more titratable and the apparent pK_a values were shifted toward more basic values. Because the range of pH values tolerated by oocytes was limited, the apparent pK_a values could not be estimated accurately for T338H and T338H/R334C variants. Nevertheless, the results were in qualitative agreement with the hypothesis that the charge at position 334 creates an electrostatic potential that alters the pK_a of a titratable residue at 338.

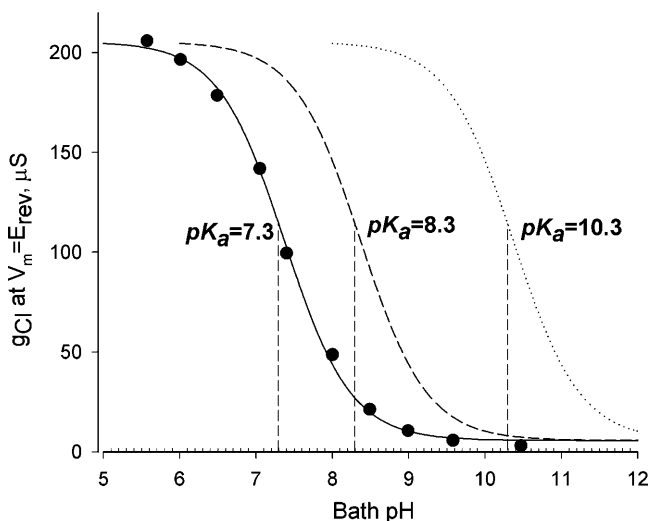


FIGURE 7 Titration of conductance due to T338C CFTR. A representative titration curve obtained from an oocyte expressing T338C CFTR. The oocytes used in this group of experiments were treated with reducing agents (2-ME or DTT) at various concentrations (50 μM to 1 mM), followed by pH titration (pH 5.3–10.5) in the presence of the respective reducing agent. The actual data are shown in solid circles. The solid line is the fitted curve. The dashed line is the predicted titration curve for a thiol group having a pK_a of 8.3. The dotted line is the predicted titration curve for a thiol group having a pK_a of 10.3.

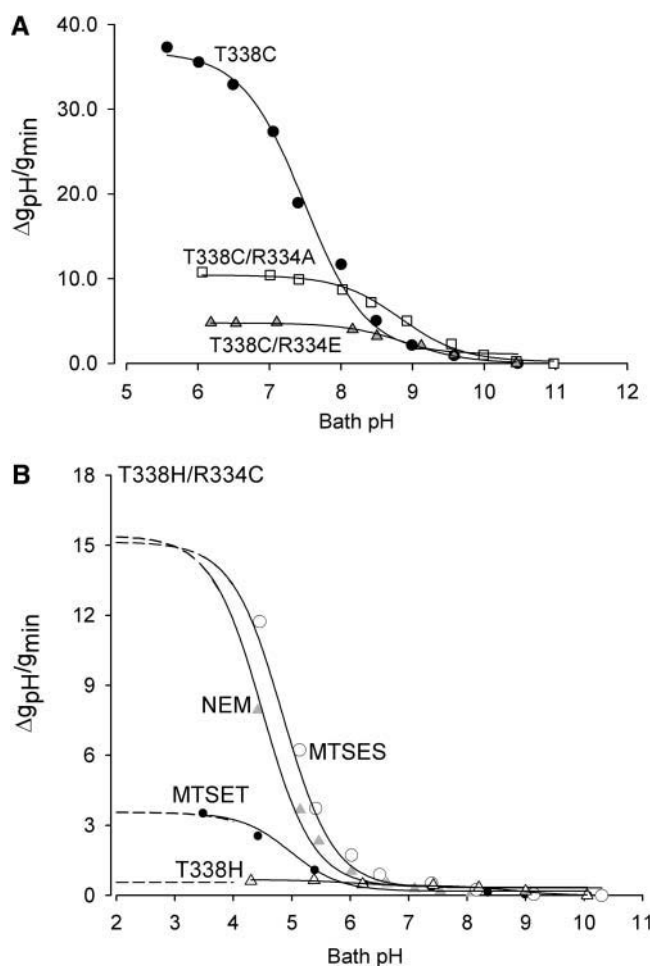


FIGURE 8 The pH-induced changes in the conductances of oocytes expressing T338C/R334A, T338C/R334E, T338H, or T338H/R334C CFTR. (A) Sample titration curves of conductances of oocytes expressing T338C CFTR (solid circles), T338C/R334A (open squares), or T338C/R334E CFTRs (shaded triangles). The titration was performed in the presence of reducing agents as described in Fig. 7 ($n = 5$ for each mutant). The solid lines are the fitted curves. (B) Sample titration curves for T338H (open triangle) and T338H/R334C CFTR modified by MTSET⁺ (solid circles), MTSES⁻ (open circles), and NEM (shaded triangles). The solid lines are fitted curves. The dashed lines are extended curves at low pH values that were calculated based on the fitting results.

Rates of covalent modification of T338C CFTR by MTSET⁺ and MTSES⁻ are consistent with a model featuring a positive vestibule potential

If, as suggested by the shift in the apparent pK_a of a cysteine or a histidine at 338, this portion of the CFTR pore is characterized by a positive electrostatic potential, the potential should also affect the relative rates of covalent modification by MTSET⁺ and MTSES⁻ (Stauffer and Karlin, 1994; Pascual and Karlin, 1998; Karlin and Akabas, 1998). Fig. 9 contains the results of experiments in which the time course of modification was measured at pH 7.4 for oocytes expressing either T338C CFTR or a double mutant

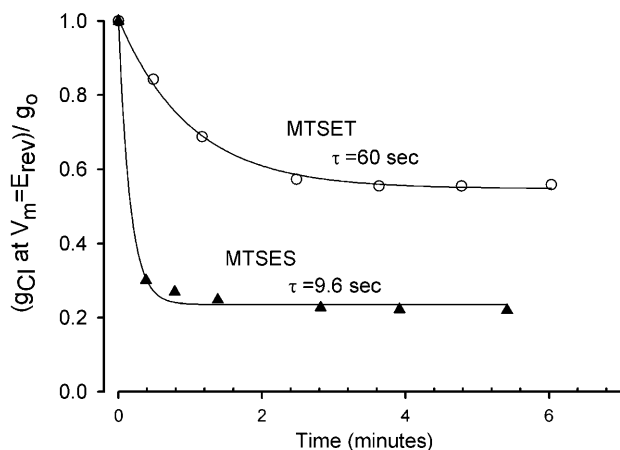
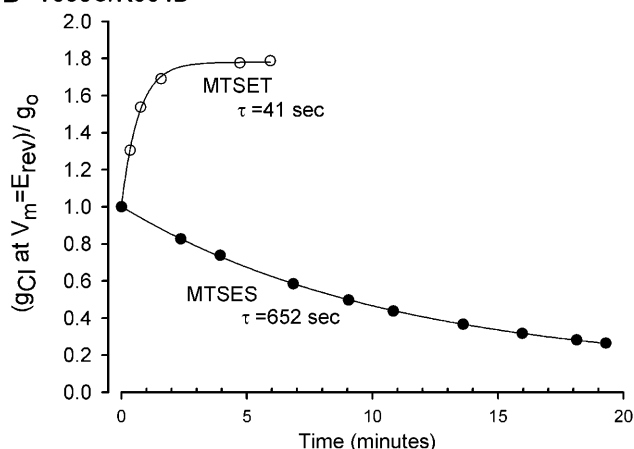
A T338C**B T338C/R334D**

FIGURE 9 Representative time course of modification of T338C CFTR and T338C/R334D CFTR conductance by MTSET⁺ ($n = 3$) and MTSES⁻ ($n = 3$). Data were normalized according to g_{Cl} at $V_m = E_{rev}$ at time zero (g_0). (A) After activation, oocytes expressing T338C CFTR were first exposed to reducing agents (2-ME) followed by a wash and were then exposed to 25 μ M MTSET⁺ (○) or MTSES⁻ (▲). (B) After activation, oocytes expressing T338C/R334D CFTR were first exposed to reducing agents (2-ME) followed by a wash and were then exposed to 25 μ M MTSET⁺ (○) or MTSES⁻ (●).

in which the arginine at 334 was replaced by aspartic acid (T338C/R334D CFTR). Note that covalent modification of T338C CFTR with either reagent decreased the macroscopic conductance. In the case of MTSES⁻ this decrease seems likely to be due, at least in part, to the deposition of a negative charge in the conduction path, as seen with titration of the cysteine thiolate. The fact that modification by MTSET⁺ also reduced the conductance suggests an additional effect, however, such as a change in gating, partial obstruction of the pore, or both. Single-channel recordings (see below) showed that modification of T338C CFTR by MTSET⁺ reduced single-channel conductance, as expected if the ethyl(trimethylammonium) moiety partially obstructs anion flow through the pore.

The rate of modification of T338C CFTR by MTSES⁻ exceeded that for MTSET⁺, a result that is even more remarkable given the fact that the intrinsic rate of reaction of MTSES⁻ with simple thiols is ~ 12 -fold less than that for MTSET⁺, due to the electrostatic interaction of the thiolate anion and the MTSES⁻ during thiol-disulfide exchange (Karlin and Akabas, 1998). Introduction of negative charge at 334 (in T338C/R334D) reversed the relative reaction rates so that modification by MTSET⁺ was more rapid. Note that in this double-mutant modification by MTSET⁺ produced an increase in conductance, as expected if the effect of the negative charge on the aspartic acid at 334 was partially compensated by the deposition of a positive charge at 338. The time constants for MTSET⁺ and MTSES⁻ modification of T338C CFTR ($[MTS] = 25 \mu$ M) averaged 64.5 ± 2.2 s ($n = 3$) and 11.3 ± 1.9 s ($n = 3$), respectively. The time constants for MTSET⁺ and MTSES⁻ modification of T338C/R334D CFTR ($[MTS] = 25 \mu$ M) averaged 39.8 ± 15.8 s ($n = 3$) and 641 ± 27.7 s ($n = 3$), respectively. Calculations based on a simple kinetic model for the ratio of the rates of reaction of MTSET⁺ and MTSES⁻, including a correction for the difference in the intrinsic rates of the MTS-thiolate reactions (see Discussion), suggested that charged reagents modifying T338C CFTR sensed an electrostatic potential that was ~ 54 mV positive with respect to the bath. When the arginine at 334 was replaced with aspartic acid the change in the relative rates suggested a vestibule potential of ~ -4 mV, a negative shift of 58 mV.

Covalent modification of T338C CFTR by MTSET⁺ adds a positive charge, but also partially obstructs the pore

The reduction of T338C CFTR macroscopic conductance by MTSET⁺ suggested that at this locus, the deposition of the ethyl(trimethylammonium) moiety might partially block the pore. Direct evidence for pore obstruction by MTSET⁺ was obtained by recording T338C CFTR single-channel currents before and after modification. To favor the unmodified condition, oocytes were preincubated in 1 mM 2-ME or DTT for ~ 1 h before recording, and 2-ME (1 mM) was also included in the pipette solution. The first current trace depicted in Fig. 10 A contains a segment of a representative single-channel current trace from an oocyte pretreated with 1 mM DTT for ~ 1 h. 2-ME (1 mM) was also included in the pipette. The majority of current amplitudes observed in this condition were ~ 0.9 pA ($V_m = -100$ mV), similar to those shown in Fig. 5 A (pH 6, $V_m = -100$ mV).

To achieve maximum MTSET⁺ modification, after incubation in 1 mM DTT or 2-ME for ~ 5 min, oocytes were exposed to 1–5 mM MTSET⁺ for ~ 10 min. In some experiments, MTSET⁺ (50–250 μ M) was also included in the pipette solution. The effective concentration of MTSET⁺ in the pipette likely varied because the half-life of MTSET⁺ is only ~ 10 min at room temperature according to Stauffer

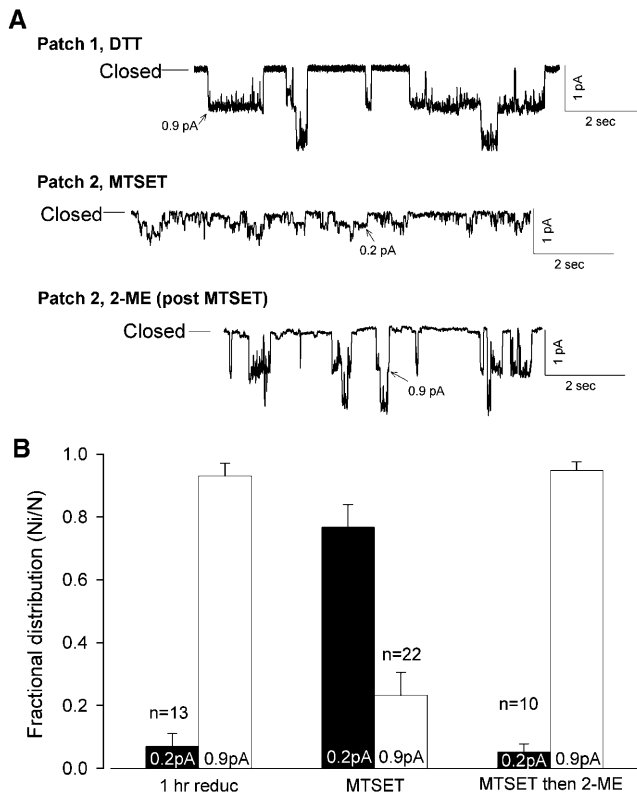


FIGURE 10 MTSET⁺ modification of T338C CFTR single-channel conductance. (A) Segments of representative single-channel current traces obtained from an oocyte pretreated with 1 mM DTT for ~1 h. 2-ME (1 mM) was also included in the pipette (*Patch 1, DTT*). An oocyte that was first treated with 1 mM DTT for ~5 min, then with 2 mM MTSET⁺ for ~10 min (*Patch 2, MTSET⁺*), and from the same patch as in *Patch 2, MTSET⁺*, but recorded after 45-min exposure to 20 mM intracellular 2-ME (*Patch 2, 2-ME, post-MTSET⁺*). The bath concentration of ATP was manipulated to facilitate the resolution of single-channel events. (B) MTSET⁺-induced changes in two major current levels observed in three cases: patches obtained from oocytes pretreated with 1 mM ME or DTT for ~1 h before patch formation; patches obtained from oocytes pretreated with 2 mM MTSET⁺ before patch formation with or without MTSET⁺ included in the pipette solution; and patches containing MTSET⁺-modified channels that were exposed to 20 mM intracellular 2-ME for >20 min. Although we observed multilevel openings in most of the patches, under reducing conditions the dominant current level was 0.9 pA. MTSET⁺-treatment caused a marked increase in the frequency of events at the 0.2-pA level and a decrease in the frequency of events at the 0.9-pA level, whereas treatment with 2-ME reduced the frequency of events at the 0.2-pA level and increased the frequency of events at the 0.9-pA level. The results suggested a 75% decrease in single channel current at $V_m = -100$ mV at pH 6 due to MTSET⁺-induced modification of T338C CFTR. N_i is the number of the number of single-channel events of each current amplitude, 0.2 pA or 0.9 pA, recorded at $V_m = -100$ mV, and N is the total number of such events.

and Karlin (1994). The second trace in Fig. 10 A is a segment of a representative trace obtained from an oocyte that was first treated with 1 mM DTT for ~5 min, then with 2 mM MTSET⁺ for ~10 min. In this condition, the majority of current amplitudes were ~0.2 pA.

In a few experiments, it was possible to record the activity of modified channels and then reverse the modification by

perfusing the bath (cytoplasmic side) with membrane permeant reducing agent, 2-ME (20 mM) (Rojas et al., 1991). The third trace depicted in Fig. 10 A was from the same patch as in *Patch 2, MTSET⁺*, but was recorded after 45-min exposure to 20 mM intracellular 2-ME. The majority of current amplitudes were ~0.9 pA after 2-ME exposure, consistent with the notion that modified channels (0.2 pA) were converted to unmodified channels (0.9 pA) by 2-ME. The design of these experiments precluded a comparison of single-channel gating in the different conditions as the bath concentration of ATP was manipulated to facilitate the resolution of single-channel events, and incubation of detached-patches with 2-ME might be expected to influence gating (Harrington et al., 1999).

The fractional distribution of single-channel current amplitudes for the three conditions obtained from multiple patches is shown in Fig. 10 B. After extensive incubation in 2-ME, the single-channel current amplitudes were dominated by a current of ~0.9 pA, identical to the current amplitude at pH 6 shown in Fig. 5 A. There were a small number (<10%) of openings at a level of ~0.2 pA. These currents cannot represent the MTSET⁺-modified channels, as these oocytes had never been exposed to the compound. After prolonged exposure to MTSET⁺ the dominant single-channel current amplitude was 0.2 pA and the overall occurrence of 0.9 pA current was reduced to ~20%. Approximately one-third of the patches had no 0.9 pA currents in this condition. Exposure of modified patches to 20 mM 2-ME resulted in a reappearance of the 0.9-pA openings together with a small number of 0.2-pA events. This result is consistent with the notion that the 0.2-pA event represented MTSET⁺-modified T338C CFTR channels. Based on the single-channel amplitude observed under reducing conditions, it seems likely that the 0.2-pA events included a small contribution from a background channel or a terminal oxidation state of T338C CFTR; see Materials and Methods. The fact that the modification of the T338C CFTR channel was not always complete may reflect the acidic pH as well as some degradation of the MTSET⁺. The reduction in single-channel conductance of ~75% was comparable to that seen in macroscopic recordings (see below) in which the reduction in conductance varied from 20 to 80% (Fig. 12), suggesting that the attenuation of macroscopic conductance is largely attributable to a reduction in single-channel conductance. The variability in the impact of MTSET⁺ on macroscopic conductance may indicate that not all the cysteines were in the simple thiolate form, which is required for MTSET⁺ reaction. This variability persisted even after overnight treatment of oocytes with 1 mM DTT (X. Liu and D. C. Dawson, unpublished observations), suggesting that in some cases the cysteine at 338 was oxidized to either a sulfinic acid or sulfonic acid that was insensitive to reducing agents.

As an additional test of the effect of MTSET⁺ we examined changes in single-channel current amplitude in experiments in which the recording pipette was backfilled with MTSET⁺ to

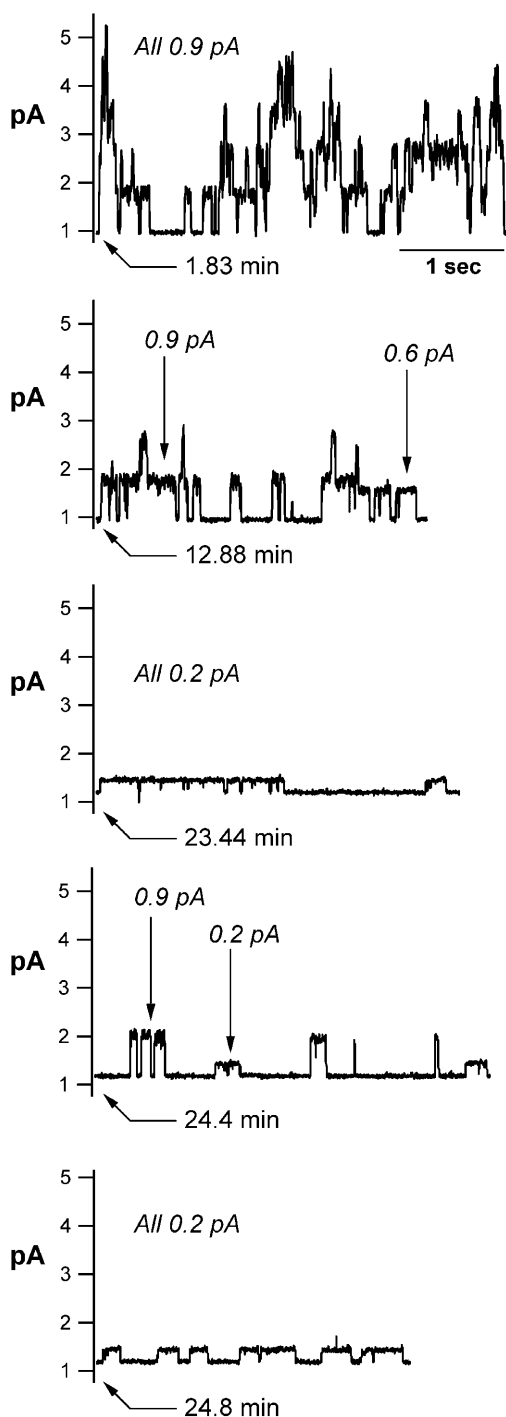


FIGURE 11 Real-time modification of T338C-CFTR channels activated by PKA and ATP in an excised, inside-out patch. The pipette was backfilled with pipette solution containing 100 μ M MTSET⁺. During the course of the recording, MTSET⁺ diffused to the pipette tip, and modified the T338C-CFTR channels there. Shown are several segments of record from an experiment lasting >25 min; the elapsed time from forming the seal to the beginning of the segment is shown below the trace. Current amplitudes of representative openings are listed. Pipette solution was pH 6.0 to enhance channel amplitudes before modification. As is often the case in long experiments of wt CFTR expressed in oocytes, channel activity ran down during the course of the record, so the effect of MTSET⁺ on gating could not be readily quantified.

monitor modification in real time (Smith et al., 2001). Fig. 11 shows segments of a record from a long experiment of this type; similar results were seen in seven patches. At the beginning of the experiment, all channel openings exhibited the amplitude associated with unmodified channels (0.9 pA). As the recording progressed, channel openings transitioned to the lower amplitude associated with the modified state (0.2 pA). Partway through the recording, a small number of openings showed an intermediate conductance level (0.6 pA). This 0.6-pA conductance level was rare, and may represent a subconductance state, such as those seen occasionally in wt-CFTR and several pore-domain mutants (Z.-R. Zhang, G. Cui, X. Liu, B. Song, D. C. Dawson, and N. A. McCarty, unpublished), given the fact that this state also was seen occasionally in patches that were treated only with 2-ME or DTT (X. Liu and D. C. Dawson, unpublished observation). The data shown in Fig. 10 indicate that prolonged exposure to 20 mM 2-ME led to a prevalence of openings (0.9 pA) characterized by the full (unmodified) single-channel conductance, whereas prolonged exposure to MTSET⁺ led to a prevalence of openings (0.2 pA) characterized by the lower (modified) single-channel conductance, with the occasional openings characterized by the unmodified conductance amplitude. Consistent with these observations, openings at the end of the long, real-time modification experiments exhibited predominantly, but not exclusively, the lower single-channel conductance characteristic of the MTSET⁺-modified state (Fig. 11). The fact that large conductance channels remained at the end of the experiment could reflect the reduced rate of the thiol-disulfide exchange reaction at pH 6 and the expected degradation of the reagent over the time course of the recording.

If, as suggested by single-channel recordings, MTSET⁺ partially obstructs the pore of T338C CFTR, we reasoned that the net effect of MTSET⁺ on macroscopic conductance might consist of two components: one due to obstruction and another due to the change in charge. In previous experiments using R334C CFTR (Smith et al., 2001) we showed that, in general, the total charge change due to covalent modification was the sum of the charge added by the deposited group and the pH-dependent charge on the thiolate that is neutralized in the formation of a mixed disulfide bond. The results shown in Fig. 12 compare the effects of covalent modification of T338C CFTR at pH 6 and pH 9, values chosen such that the partial charge on the cysteine thiolate would vary from near zero (pH 6) to near -1 (pH 9). At pH 9, MTSET⁺ increased T338C CFTR conductance by 50–110% (84 ± 15 , $n = 3$) whereas at pH 6, the same treatment reduced the conductance due to T338C CFTR by 20–80% ($n = 16$). At pH 6, the blocking efficacy of MTSET⁺ seemed to fall roughly into two groups. If we define the two groups using 50% block as the cutoff, in one group (>50%) the reduction in the macroscopic conductance averaged 68% (± 2.6 , $n = 10$), whereas in the second group (<50%), the reduction averaged only 34% (± 3.5 , $n = 6$). This apparent twofold difference in efficacy is

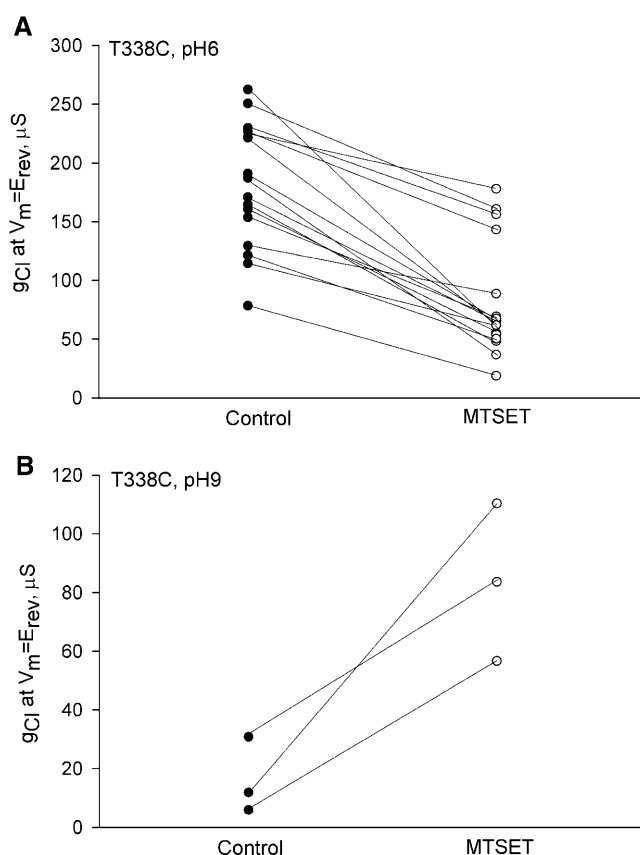


FIGURE 12 The pH-dependent effect of MTSET⁺ on whole-cell T338C CFTR conductance. After activation, oocytes were first exposed to 1 mM 2-ME or DTT, then to either pH 6 or pH 9, followed by exposure to MTSET⁺ (1 mM) at the same pH. (A) At pH 6, MTSET⁺ reduced the conductance due to T338C CFTR by variable amount (20–80%). (B) At pH 9, the conductance was increased by MTSET⁺.

consistent with the hypothesis that in any single oocyte a variable portion of the cysteines may exist in an oxidation state that prevents modification by MTSET⁺. The blocking effect of MTSET⁺ at position 338 contrasts to the effect of the same reagent at position 334, where MTSET⁺ increased the conductance of R334C CFTR by $\sim 45\%$ (± 16) at pH 6 and by $\sim 92\%$ (± 12) at pH 9 (Smith et al., 2001). Taken together with the results of single-channel recordings, the present result is compatible with the hypothesis that the impact of modification by MSTET⁺ at 338 is a result of the combined effects of a change in charge and partial obstruction of the pore, but we cannot eliminate the possibility that covalent modification at this position also results in some change in gating.

DISCUSSION

T338 lies within the CFTR pore

The results presented here are consistent with the hypothesis that T338 lies within the anion conduction pore of CFTR, but the location of this residue has been a matter of some con-

troversy. Cheung and Akabas (1996) reported that T338C CFTR did not react with externally applied MTSET⁺ or MTSES⁻ and concluded that the residue did not lie on the outward-facing, water-accessible surface of the protein. The effects of amino acid substitution at this locus, however, led Linsdell et al. (1997, 1998, 2000) and McCarty and Zhang (2001) to identify this residue as critical for anion permeation and binding. Although the reason for the discrepancy between our findings and those of Cheung and Akabas (1996) is not completely clear at present, one contributing factor may be the increased reactivity (reduced pK_a) of the cysteine thiol at position 338 which renders it more susceptible to reactions that could change its oxidation state and effectively block reactions with MTS reagents.

Two results presented here argue that T338 lies within the pore. First, a cysteine substituted at 338 was clearly accessible to polar thiol-directed reagents as well as protons and the polar reducing agent, dithiothreitol. Second, charge changes at 338, effected by pH titration of the cysteine thiolate, altered anion conduction in the manner predicted by a model featuring a charge located in the outer vestibule of the pore. Thus this locus meets the criterion proposed in a previous study of residue 334 (Smith et al., 2001) for a pore-lining residue, namely that altering the charge in the vestibule of the pore should change anion conduction in a charge-dependent manner, the key criterion being a simultaneous alteration of conductance and the shape of the I-V (or i-V) plot. Although this criterion may not apply to all pore-lining residues, it provides one test for residues that lie in the outer vestibule of the pore, defined generally as a wider region that precedes a narrow, rate-limiting portion of the pore where anions bind (Cai and Jordan, 1990; Dani, 1986; MacKinnon et al., 1989). The observation that modification of T338C CFTR by MTSET⁺ produced a net reduction in single-channel conductance, whereas similar modification of R334C CFTR increased single-channel conductance, is compatible with the notion that the pore narrows over the length of the helical turn.

The pK_a of T338C CFTR is consistent with a model in which T338C resides in the pore where it senses a positive electrostatic potential

The pH titration of the conductances of oocytes expressing T338C CFTR was consistent with the stabilizing effect of a nearby positive charge on the thiolate anion, and the behavior of double mutants suggested that one source of this charge could be R334. The effects of substitution for R334 were not completely consistent, however. Whereas the alanine substitution rendered the apparent pK_a more than one pH unit more alkaline, neither the glutamic acid nor aspartic acid substitution produced a significant additional shift. This apparent asymmetry was evident in both of the independent methods used to estimate Ψ_o , i.e., the ΔpK_a and the ratio of reaction rates for MTSET⁺ and MTSES⁻.

Although the underlining cause of the asymmetry is unclear at present, the similarity of the results of the two independent methods for determining Ψ_o nevertheless provides strong evidence that a positive electrostatic potential exists in the vestibule. The asymmetry suggests that the insertion of the negative charge (Glu or Asp) might change the conformation of the protein in such a way as to diminish the impact of the charge on Ψ_o , or increase the impact of other, as yet unidentified, positive charges.

The influence of the local protein environment on the pK_a of a titratable group has been of interest to investigators for many years and has been modeled for proteins of known structure (Antosiewicz et al., 1994, 1996; Bashford and Karplus, 1990; Bashford and Gerwert, 1992; Dillet et al., 1998; Fitch et al., 2002; Gilson and Honig, 1988; Oberoi and Allewell, 1993; Peters et al., 1998; Yang et al., 1993). In the case of T338C CFTR, although we lack the information as to a three-dimensional structure, we can nevertheless make use of some of the general principles that have emerged from the analysis of other proteins. As shown in Supplementary Material, the change in pK_a of a particular titratable group in the putative vestibule can be approximated by Eq. 6,

$$\Psi_o^q = -\frac{2.3 RT}{F} \Delta pK_a, \quad (6)$$

where Ψ_o^q is the component of the time-averaged electrostatic potential sensed by the titratable group that is attributable to local charge other than that of the titratable group itself; in this case, the cysteine thiolate anion. Ideally, Ψ_o^q would represent the potential that pertains, in our model, to the vestibule of the wt CFTR pore lacking the engineered cysteine. We cannot expect this to be exactly the case, however, due to the inevitable effects of amino acid substitution on protein conformation. The total potential, Ψ_o^T , for T338C CFTR, would also contain a pH-dependent component due to the cysteine thiolate at 338. As indicated above, at alkaline pH, the charge at 338 would be likely to make a large contribution to Ψ_o^T in T338C CFTR.

Eq. 6 is based on two simplifying assumptions (see Supplementary Material). First we assumed that the change in pK_a due to differential solvation of the protonated form of the titratable group is negligible (Sitkoff et al., 1994; Florian and Warshel, 1997); and second, we assumed that the change in pK_a due to differential solvation of the thiolate anion is zero, as if the anion is solvated in the vestibule as in free solution. By setting the solvation penalty to zero we ensure that Eq. 6 provides an estimate of the minimum value of Ψ_o^q . Any solvation effect, as it would shift the pK_a of the thiolate in alkaline direction, would increase the positive value of Ψ_o^q required to achieve the observed pK_a of the cysteine at 338.

There are at least two estimates of Ψ_o^q , the vestibule potential seen by a cysteine at 338, which are of interest. One is the absolute value of Ψ_o^q with respect to the bath that would

be derived from the shift in pK_a with respect to that for a simple thiol in aqueous solution. A second calculation would be the component of Ψ_o^q due to R334, Ψ_o^{R334} . A calculation of the absolute value of Ψ_o^q relative to the bath is complicated by some ambiguity as to what to take as the pK_a of the cysteine thiolate in the aqueous solution. Choices would include free cysteine, or simple compounds such as methylthiol or ethylthiol or linear helical peptides containing a cysteine (Irving et al., 1964; Kreevoy et al., 1964; Stewart, 1985; Kortemme and Creighton, 1995). The pK_a values range from 8.3 for free cysteine to ~ 10.3 for methylthiol or ethylthiol, and would translate into values for Ψ_o^q ranging from 53 mV to 171 mV positive with respect to the bath using a pK_a of 7.4 for T338C CFTR. It is important to note in this regard that conduction models—from Goldman and rate theory—used to analyze the change in I-V plots induced by change in bath pH yield only relative values of Ψ_o^q . Although arguments might be advanced in favor of choosing one of these pK_a values over another, we chose to focus on the electrostatic effect of R334.

A comparison of the apparent pK_a of T338C CFTR with that of the double mutant, T338C/R334A, suggests that the amino acid substitution at position 334 shifted the pK_a from ~ 7.4 to 8.8 or ~ 1.4 units. This ΔpK_a implies a change in Ψ_o^q (Eq. 6) of 83 mV. A comparison of T338C/R334A ($pK_a = 8.8$) with T338C/R334E ($pK_a = 8.9$), would suggest a further change in Ψ_o^q of ~ -6 mV.

The relative rates of modification of T338C CFTR by MTSET⁺ and MTSES[−] provide another estimate of Ψ_o^q (Stauffer and Karlin, 1994; Pascual and Karlin, 1998; Karlin and Akabas, 1998; Wilson et al., 2000; Elinder et al., 2001). To analyze the rates of modification we assumed that the concentration of the MTS reagents in the vestibule is determined by Ψ_o^q , as if the reagents equilibrate with Ψ_o^q , the component of Ψ_o^T , due to local charge other than that of the cysteine thiol. The ratio of the rates of modification is given by Eq. 7 (see Supplementary Material),

$$\frac{k_i^{\text{MTSES}}}{k_i^{\text{MTSET}}} = \frac{k_i^{\text{MTSES}}}{k_i^{\text{MTSET}}} \exp\left(2 \frac{F}{RT} \Psi_o^q\right). \quad (7)$$

Using the ratio for $k_i^{\text{MTSES}}/k_i^{\text{MTSET}}$ of 1/12 measured for 2-ME (Karlin and Akabas, 1998), the relative rates of modification for these two compounds yields a value for Ψ_o^q of 54 mV for T338C CFTR and -4 mV for T338C/R334D CFTR, comparable to the values estimated from the shift in cysteine pK_a resulting from the replacement of arginine by alanine or aspartic acid. The absolute values of Ψ_o^q must be interpreted with caution since we do not know to what extent structural differences between these two mutants might have resulted from the amino acid substitution (see below), but both of the measurements used to estimate the electrostatic potential of the vestibule indicated an asymmetry between the impact of basic and acidic residues at 334.

Prediction of the electrostatic effects of R334

If we ignore the possible effects of structural changes in the CFTR protein produced by amino acid substitution, then the change in Ψ_o^q calculated from the difference in the pK_a of T338C/R334 and T338C/R334A can be taken to be a crude measure of Ψ_o^{R334} , the component of Ψ_o^q due to the native arginine, and we can compare the value derived experimentally with that predicted on the basis of first principles. In the absence of detailed structural information we adopted a macroscopic approach based on Coulomb's law which yields for the electrostatic potential, Ψ , at a radial distance, r , from a charge, q (Eq. 8),

$$\Psi = \frac{1}{4\pi\epsilon_o\epsilon_{\text{eff}}} \left\{ \frac{q}{r} \right\}, \quad (8)$$

where ϵ_o is the permittivity of free space and ϵ_{eff} is the effective dielectric constant. The value ϵ_{eff} is best viewed as an empirical parameter that takes into account permanent and induced dipoles, mobile charges and protein relaxation that serve to diminish the field produced by q at a distance r (Mehler and Eichele, 1984; Pickersgill, 1988; Mehler, 1990; Mehler and Guarnieri, 1999; Schutz and Warshel, 2001). If we assume a helical conformation for TM6 in the region between 334 and 338, we can estimate the radial distance, r , to be ~ 5.4 Å. Using values of $\Psi_o^{R334} = 83$ mV, $(4\pi\epsilon_o)^{-1} = 8.99 \times 10^9$ V m/Coulomb, and $q = 1.6 \times 10^{-19}$ Coulomb, Eq. 8 predicts a value for ϵ_{eff} of ~ 35 for the interaction of R334 with a cysteine at 338. This value is comparable to that derived by a number of investigators using Eq. 8 (Mehler and Eichele, 1984; Pickersgill, 1988; Mehler, 1990; Sham et al., 1998; Mehler and Guarnieri, 1999; Schutz and Warshel, 2001).

A rather different approach to estimating the electrostatic potential was used by Elinder et al. (2001) to evaluate the impact of charge on a cysteine engineered into the S5 segment of the *Shaker* K^+ channel. These investigators envisioned the charge as being located on the boundary between a region of high dielectric constant, like a water-filled vestibule, and a region of relatively low dielectric constant, like the protein interior. In such a situation, the potential on the aqueous side can be approximated (McLaughlin, 1989) by Eq. 9,

$$\Psi(r) = \frac{2q \exp(-r/L_D)}{(4\pi\epsilon_o\epsilon_w)r}, \quad (9)$$

where $\exp(-r/L_D)$ is the Debye-Hückel screening factor, L_D is the Debye length in the aqueous solution, r is the radial distance, and ϵ_w is the dielectric constant of water. If we take L_D to be 10 Å, ϵ_w to be 80, r to be 5.4 Å, and q and $(4\pi\epsilon_o)^{-1}$ as above, then Ψ_o^{R334} is predicted to be ~ 42 mV.

The results obtained using two rather different approaches are both consistent with the hypothesis that the observed shift in the apparent pK_a of T338C CFTR could be the result of the

electrostatic effect of R334. Both calculations, however, are subject to important qualifications. For example, the actual geometrical relation of the two charges is unknown. The use of 5.4 Å as the distance between the cysteine thiolate and the arginine ignores the size and orientation of the side chains as well as the fact that the actual distance may be charge-dependent. It would not be surprising to find that the thiolate-arginine ion pair of T338C/R334 was closer together than the thiolate-glutamic acid pair of T338C/R334E, even if there were no major change in structure that accompanied the mutations. It is also possible that amino acid substitutions at 334 altered the relation of the cysteine at 338 to other, yet unidentified, charges or dipoles. The asymmetry in the impact of a positive versus a negative charged residue at 334, that was apparent in both the shift in thiol pK_a and the relative reaction rates for MTSET⁺ and MTSES[−], also suggests that the amino acid substitutions lead to local changes in the geometry of the interacting residues.

A working electrostatic model for the CFTR pore

The results and analysis presented here are consistent with a working model for the CFTR pore that features an outer vestibule that couples a narrow, rate-limiting region to the external bathing solution. We propose that this vestibule contains water sufficient to solvate anions about as well as in free solution, and that it contains at least one fixed positive charge (R334). The charge enhances the partition of anions into the vestibule by creating a positive electrostatic potential which, at position 338, is of the order of 40–80 mV positive with respect to the bath, a value comparable to that predicted by the singly-charged vestibule model of Dani (1986). The total electrostatic potential may be somewhat greater due to the influence of other, as yet unidentified residues.

We propose that the positive electrostatic potential in the outer vestibule of the CFTR pore enhances channel conductance by raising the anion concentration adjacent to the narrow rate-limiting region. The titration behavior of cysteine residues placed within the vestibule of the CFTR pore is a useful tool for probing this electrostatic potential. In the absence of a three-dimensional structure the difference in the pK_a of an engineered cysteine could, in principle, be used to construct a crude map of the electrical potential due to residues such as R334. When a structure is determined this approach can be used to test directly the predictions of electrostatic models based on the three-dimensional coordinates. If it is assumed for the sake of simplicity that the physical obstruction produced by MTSET⁺ and MTSES[−] (molecular volumes 206 Å³ and 176 Å³, respectively) are approximately the same, then the difference in the impact of the two reagents is consistent with the expected opposite effect of the two charges. However, it is not possible at this time to rule out an additional effect on single-channel gating.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at <http://www.biophysj.org>.

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