

CFTR: What's It Like Inside the Pore?

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ABSTRACT The Cystic Fibrosis Conductance Regulator (CFTR) functions as a cAMP-activated, anion-selective channel, but the structural basis for anion permeation is not well understood. Here we summarize recent studies aimed at understanding how anions move through the CFTR channel, and the nature of the environment anions experience inside the pore. From these studies it is apparent that anion permeability selectivity and anion binding selectivity of the pore are consistent with a model based on a “dielectric tunnel.” The selectivity pattern for halides and pseudohalides can be predicted if it is assumed that permeant anions partition between bulk water and a polarizable space that is characterized by an effective dielectric constant of about 19. Covalent labeling of engineered cysteines and pH titration of engineered cysteines and histidines lead to the conclusion that the CFTR anion conduction path includes a positively charged outer vestibule. A residue in transmembrane segment 6 (TM6) (R334) appears to reside in the outer vestibule of the CFTR pore where it creates a positive electrostatic potential that enhances anion conduction. *J. Exp. Zool.* 300A:69–75, 2003. © 2003 Wiley-Liss, Inc.

WHAT DO WE KNOW ABOUT CFTR?

The cystic fibrosis conductance regulator (CFTR) is the product of the gene that is mutated in Cystic Fibrosis (CF), the most common, fatal genetic disorder in the Caucasian population. It is a 1480 amino acid protein consisting of five distinct domains (Collins, '92), including two membrane-spanning domains (MSD1, MSD2), each consisting of six transmembrane segments (TM); and three cytosolic domains, including two nucleotide-binding folds (NBF1, NBF2) and the regulatory domain (R domain) (Fig. 1). Numerous studies support the notion that CFTR functions as an anion-selective channel and that activation requires phosphorylation of the R-domain by protein kinase A (PKA) and hydrolysis of ATP at the two nucleotide binding folds (Anderson et al., '91; Rich et al., '91; Baukowitz et al., '94; Hwang et al., '94; Sheppard et al., '94; Carson et al., '95; Wilkinson et al., '96; Dawson et al., '99). However, the structural basis for anion permeation and conduction are not known.

CFTR is a critical element in the normal function of multiple organs including the lung, the pancreas, the intestine, the liver, the reproductive organs, and the sweat glands and ducts (Quinton, '99, and the references therein). CFTR also figures importantly in another devastating

disease, secretory diarrhea (e.g. Cholera), which is a worldwide health problem (Guggino, '94). While CF is the result of deficient Cl⁻ transport due to the disruption of CFTR function, secretory diarrhea is the result of chronic activation of CFTR and stimulation of Cl⁻ transport (Field and Semrad, '93; Gabriel et al., '94). Mutations causing CF can prevent the delivery of CFTR protein to the membrane or alter the gating behaviors or anion conduction (Drumm et al., '91; Mansoura et al., '98). Understanding the structural basis for anion permeation through the CFTR pore could aid the design of small ligands that compensate for inefficient Cl⁻ transport by the mutant protein or stabilize the protein conformation for efficient delivery.

ANION SELECTIVITY OF CFTR

A signal feature of an ion channel is its selectivity or its ability to discriminate one ion from another. The unique ion-selective pathways formed by different ion channels and the

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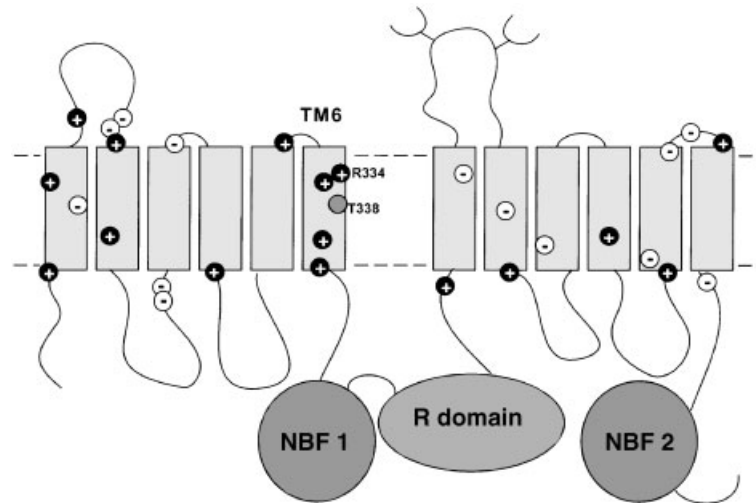


Fig. 1. Predicted topology of CFTR protein including three intracellular domains (R domain, NBF1 and NBF2), transmembrane segments (TMs), and extracellular loops.

regulation of their activities by physiological factors make it possible for cells to carry out a variety of physiological tasks, from nerve and muscle function to salt and water secretion or absorption by epithelial cells. How does CFTR select or discriminate one anion from another and what are the determinants of channel conductance? Although Cl^- is the major permeant anion, the anion selectivity of CFTR is rather modest: the channel conducts a spectrum of anions, including NO_3^- , Br^- , formate, F^- , SCN^- , ClO_4^- , I^- and an array of pseudohalides (Anderson et al., '91; Tabcharani et al., '93; Linsdell and Hanrahan, '96; Linsdell et al., '97a, b; Tabcharani et al., '97; Linsdell et al., '98; Mansoura et al., '98; Smith et al., '99; Linsdell et al., 2000, 2001a; McCarty and Zhang, 2001). Nevertheless, Cl^- passes through the channel more easily than other anions because it either enters more readily or binds less tightly in the pore. To understand anion permeation through the CFTR pore, it is thus important to distinguish anion *permeability* selectivity from anion *binding* selectivity, each of which represents a different functional property of CFTR (Dawson, '96; Smith et al., '99). Permeability selectivity can be thought of as a measure of the relative ease of anion *entry* into the pore, whereas binding selectivity can be thought of as a measure of the relative tightness of anion *binding* in the pore (Dawson, '96; Smith et al., '99).

Anion permeation through a channel can be viewed as comprising three steps: (1) leaving the aqueous solution and entering the channel; (2) translocating within the channel; and (3) entering

the aqueous environment on the cell inside (Dawson et al., '99; Smith et al., '99) (Fig. 2). In order to enter the channel, anions must leave the aqueous solution where they are stabilized by water. As anions enter the channel, they experience some degree of dehydration and are stabilized by the channel. Smith et al. ('99) used a model for ion stabilization originally proposed by Born ('20) in which free energy of solvation of an anion was calculated, based on continuum electrostatics, and the solvent was viewed as a structureless dielectric continuum. The model focused on

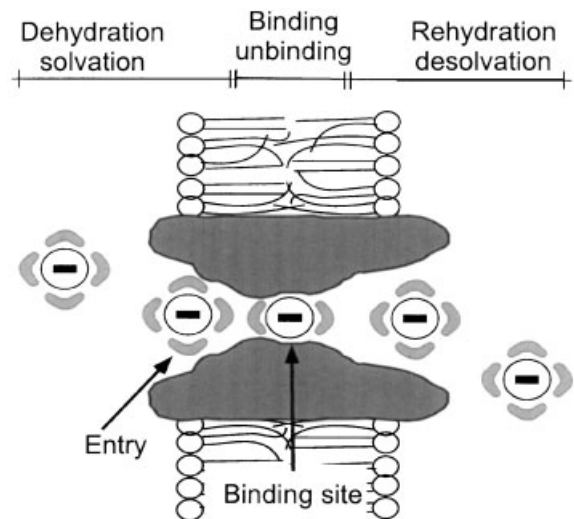


Fig. 2. A view of anion movement through the channel. This movement involves losing water, entering the channel (dehydration), traversing the channel (solvation), diffusing, and reentering the aqueous solution on the other end (rehydration).

the relative stabilization of anions by their surroundings, be it channel or water, and provided a simple, semi-quantitative approach to the investigation of the physical basis for anion translocation. The free energy required to stabilize an anion in the aqueous solution was represented by hydration energy (ΔG_{hyd}) that measures the strength of anion-water interaction and is determined by the dielectric constant of water and the radius of the anion (Born, '20; Dawson, '96; Smith et al., '99). The stabilization energy within a channel is represented by the solvation energy (ΔG_{solv}) that measures the strength of anion-channel interaction and is determined by the dielectric constant of the polarizable interior of the channel and the radius of the anion (Born, '20; Dawson, '96; Smith et al., '99). The magnitude of ΔG_{solv} does not reveal the physical nature of the reciprocal anionic radius, anion-channel interaction, but provides the basis for comparing the ability of different channels or other physical systems to stabilize anions.

Permeability Selectivity

The work required for anion entry into the channel is the *difference* between the hydration energy (ΔG_{hyd}) and the solvation energy (ΔG_{solv}); it is this difference that determines the partition of an anion between water and the channel. It represents an energy barrier ($\Delta G_{\text{eq}} = \Delta G_{\text{solv}} - \Delta G_{\text{hyd}}$) that an anion must overcome to enter the channel. The analysis of the impact of this energy barrier can be derived by one of two different approaches, electrodiffusion or rate theory (Dawson, '96; Smith et al., '99). In the electrodiffusion approach based on the Nernst-Planck equation, the energy barrier is related to the partition coefficient (β) for an anion (Dawson, '96). This coefficient describes the near equilibrium distribution of the permeant anion between the aqueous solution and the channel,

$$\beta = \exp(-\Delta G_{\text{eq}}/RT) \quad (1)$$

The partition coefficient, β , together with the diffusion coefficient, D , the effective cross-sectional area A and the length, l , defines the permeability ($P = \beta DA/l$). By assuming that the variation in D is negligible, the *difference* in the work required to move ions i and j from the solution into the channel, $[\Delta \Delta G_{\text{eq}}]_{ij}$, can be related to the permeability ratio (P_i/P_j) (Dawson, '96; Smith et al., '99),

$$P_i/P_j = \beta_i/\beta_j = \exp([\Delta \Delta G_{\text{eq}}]_{ij}/RT) \quad (2)$$

where P_i/P_j can be determined experimentally by measuring the shift in the reversal potential when a test anion is substituted for the reference anion. This approach has the advantage of minimizing the effects of anion binding, because the ionic throughput at the reversal potential is zero.

The rate theory approach, in which the permeation path is viewed as a landscape of energy barriers and wells, gives rise to a similar relationship between permeability selectivity and the energetics of permeation (Dawson, '96; Mansoura and Dawson, '98; Hille, 2001),

$$P_i/P_j = \exp([\Delta \Delta G_{\text{peak}}]_{ij}/RT) \quad (3)$$

where $\Delta \Delta G_{\text{peak}}$ is equivalent to $\Delta \Delta G_{\text{eq}}$ in the Nernst-Planck model (Dawson, '96; Smith et al., '99), thus,

$$\Delta \Delta G_{\text{peak}} = \Delta \Delta G_{\text{eq}} = \Delta \Delta G_{\text{solv}} - \Delta \Delta G_{\text{hyd}} \quad (4)$$

In order to compare anion selectivity between CFTR and other anion channels or physical systems, Smith et al. ('99) determined $\Delta \Delta G_{\text{solv}}$ for a panel of halides and pseudohalides by measuring the permeability ratio of a test anion and a reference anion in the wild type CFTR which was expressed in *Xenopus* oocytes. ΔG_{hyd} of an anion can be calculated from the reciprocal radius of the anion ($1/r$) and the dielectric constant of water (ϵ_w), using the Born equation [$\Delta G_{\text{hyd}} = -(K/2)(1/r)(1 - 1/\epsilon_w)$ where $K = (N_e^2/4\pi\epsilon_0) = (1386 \text{ kJ/mole})$ where N is Avogadro's number, e is the electrical charge, and ϵ_0 is the permittivity of free space.] (Born, '20), so that $\Delta \Delta G_{\text{solv}}$ of the channel can be estimated as the difference between $\Delta \Delta G_{\text{hyd}}$ and $\Delta \Delta G_{\text{peak}}$. $\Delta \Delta G_{\text{solv}}$, derived from the permeability ratio is a measure of the free energy change that is associated with anion entry.

Smith et al. ('99) found that ΔG_{hyd} and $\Delta \Delta G_{\text{peak}}$ for halides and pseudohalides varied linearly with the reciprocal anionic radius, $1/r$. Hence ΔG_{solv} was also linearly related to $1/r$, following the so-called "lyotropic" selectivity sequence. This selectivity pattern was simulated with a Born-type analysis in which the polarizable interior of wild type CFTR was characterized by an effective dielectric constant of 19. The effective dielectric constant (ξ_m) was estimated from ΔG_{solv} by using the Born equation ($\Delta G_{\text{solv}} = -(K/2)(1/r)(1 - 1/\xi_m)$), (Born, '20). It provides an empirical description of the polarizability within the channel.

The $1/r$ relationships of ΔG_{hyd} and ΔG_{solv} indicate that the interactions of larger anions with both water and channel are weaker than those of smaller anions. This is because the

distance between the charge center of larger anions and their ligands is greater. This is true whether the ligands are water molecules in solution or protein ligands within a channel. Larger anions are more permeant than smaller anions - i.e. they have a larger permeability ratio - because the *difference* between ΔG_{hyd} and ΔG_{solv} is smaller for larger anions than for smaller anions. The difference between ΔG_{hyd} and ΔG_{solv} determines the relative ease of entry into the channel by permeant anions. Even though larger anions experience weaker interactions with water and with the channel, they partition into the channel more readily because they see a smaller *difference* between ΔG_{hyd} and ΔG_{solv} , thus facing a lower energy barrier to entry into the channel (Fig. 3). Thus, though both ΔG_{hyd} and ΔG_{solv} were large (>100 kJ/mol), ΔG_{peak} for halides and pseudo-halides is relatively small (<10 kJ/mol), a condition necessary to achieve a significant anion throughput.

A linear relationship between $1/r$ and the solvation energies of permeant anions was also found to pertain to other anion channels like the GABA (gamma amino butyric acid) receptor, glycine receptor and the outwardly rectifying chloride channel in T84 cells; but each was characterized by a different effective dielectric constant (Smith et al., '99). Remarkably, the PVC-TDMAC [poly(vinyl chloride)-tridodecylmethylammonium chloride] membrane used in the

manufacturing of anion-selective electrodes exhibited a similar relationship between ΔG_{solv} and $1/r$. PVC membrane is a simple physical system that shares no structural similarity with ion channels. Its relative anion selectivity is determined solely by the relative anion partition coefficients between water and PVC. These results suggest that anion channels like CFTR may not require a specialized structural component to determine anion permeability selectivity, unlike the selectivity filter that "recognizes" K^+ in a K^+ channel (Smith et al., '99; Doyle et al., '98; Zhou et al., 2001).

Binding Selectivity

Anion binding selectivity of wild type CFTR also follows a "lyotropic" selectivity sequence, i.e. larger anions bind more tightly in the CFTR pore. This is exemplified by the fact that a small quantity of a larger anion such as SCN^- blocks Cl^- conductance (Tabcharani et al., '93; Overholt et al., '95; Linsdell et al., '97b; Tabcharani et al., '97; Smith et al., '99; Linsdell, 2001a, b; Gong et al., 2002). To understand the physical basis for anion binding selectivity, Smith et al. ('99) analyzed the solvation energy associated with anion binding in the pore of CFTR. In this case, the relative free energy of transfer ($\Delta\Delta G_{\text{eq}}$) or the well depth ($\Delta\Delta G_{\text{well}}$) for anion binding was derived from the ratio of the half-maximal inhibition constants of the test anions with respect to the reference anion ($\text{C}(\text{CN})_3$).

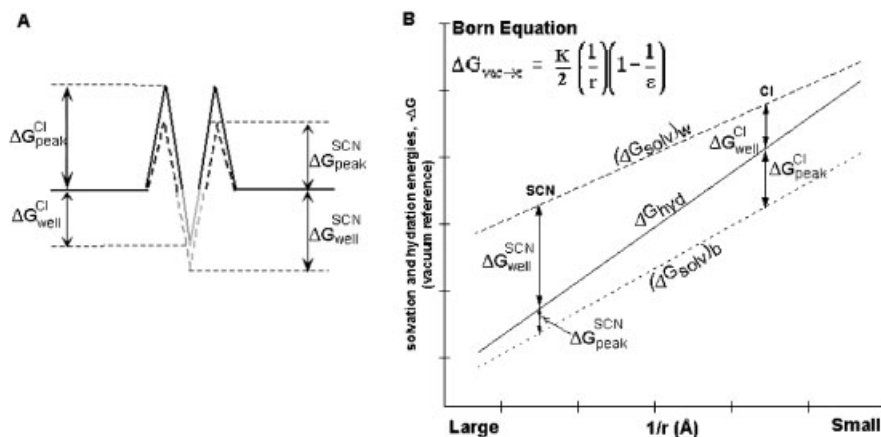


Fig. 3. (A) A cartoon illustrating energy barriers and wells as experienced by permeant anions within the CFTR pore. (B) $1/r$ plots for ΔG_{solv} and ΔG_{hyd} based on the results of Smith et al. ('99). The slopes of the lines for the solvation energies were exaggerated so that the relative changes can be seen more clearly while the patterns of the changes were maintained. Cl^- and SCN^- are shown as examples for smaller and larger anions, respectively. For anion entry, the difference

between $(\Delta G_{\text{solv}})_{\text{b}}$ and ΔG_{hyd} was smaller for SCN^- than for Cl^- ($\Delta G_{\text{peak}}^{\text{SCN}} < \Delta G_{\text{peak}}^{\text{Cl}}$); thus SCN^- entered the channel more readily than Cl^- . For anion binding on the other hand, the difference between $(\Delta G_{\text{solv}})_{\text{w}}$ and ΔG_{hyd} was larger for SCN^- than Cl^- ($\Delta G_{\text{well}}^{\text{SCN}} > \Delta G_{\text{well}}^{\text{Cl}}$); thus SCN^- bound more tightly in the channel than Cl^- . The net result is that the channel conducts Cl^- better than SCN^- .

In order to arrive at a model for permeation, Smith et al. ('99) considered the total energy of an anion at any point in the channel to be the sum of two components. One component reflected the solvation that an anion would experience within a simple dielectric tunnel that would not bind anions. An "inner sphere" component of the solvation energy was used to simulate a narrow portion of the channel where elements of the channel lining stabilize the anion. As expected, ΔG_{solv} for anion binding followed a linear relationship with $1/r$, as predicted for a "lyotropic" selectivity sequence. ΔG_{solv} was slightly larger than ΔG_{hyd} , because anion binding inside the pore gave rise to an energy-well (Fig. 3). The linear relationship between ΔG_{solv} and $1/r$ indicated that larger anions interact with the channel more weakly than do smaller anions. However, it is the *difference* between ΔG_{solv} and ΔG_{hyd} (or ΔG_{well}) that determines the depth of the well. The observation that larger anions "bind more tightly" in the pore than smaller anions is explained on the basis that larger anions are more easily dehydrated and experience a deeper well depth (Fig. 3).

EXISTENCE FOR A CHARGED OUTER VESTIBULE

Smith et al. (2001) employed highly polar, thiol reactive reagents and *Xenopus* oocytes expressing cysteine-substituted CFTR constructs to identify the CFTR pore. No functional impact was observed in wild type CFTR upon exposure to these reagents, but covalent charge changes at position 334 (R334C CFTR) produced charge-dependent effects on macroscopic conductance and the shape of the I-V plot. The charge changes brought about by pH titration of engineered cysteine and histidine residues or amino acid substitutions at the same position produced similar charge-dependent responses. The results were consistent with the notion that R334 in wild type CFTR resides in the portion of the pore where its charge gives rise to a positive, outer vestibule potential.

Summarized in Figure 4 are charge-induced changes in R334C or R334H CFTR conductance that result from alteration of external pH or exposure of oocytes expressing R334C CFTR to charged methanethiosulfonate (MTS) reagents. The data have been replotted from Smith et al. (2001). Acidification of the bath solution of oocytes expressing R334C or R334H CFTR, or modification of R334C CFTR by a positively charged MTS

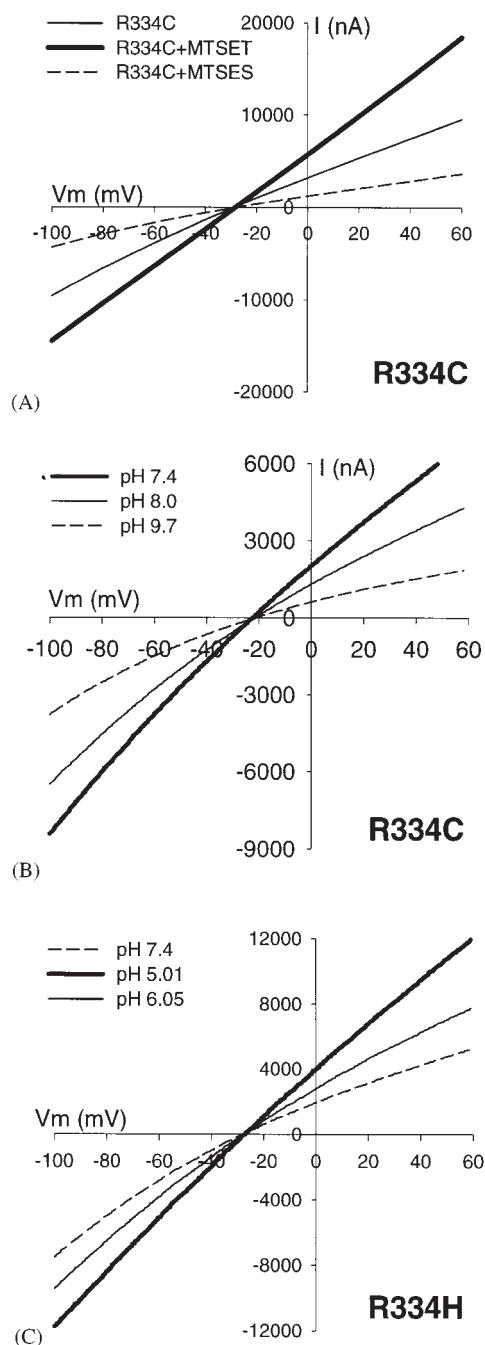


Fig. 4. Effects of charge modification at position 334. (A) Chemical modification. Increasing the positive charge (MTSET) increased conductance and altered the I-V shape toward linearity. Increasing the negative charge (MTSES) decreased conductance and rendered the I-V shape more inwardly rectified. (B) pH titration of R334C. (C) pH titration of R334H. Acidification produced an effect similar to that produced by MTSET, whereas alkalization produced an effect similar to that produced by MTSES. The data are replotted from Smith et al. (2001).

reagent, MTSET ([2-(trimethylammonium) ethyl] methanethiosulfonate bromide), increased the whole cell conductance and changed the shape of the I-V plots from inward rectification to linearity. On the other hand, alkalization of the bath solution of oocytes expressing R334C or R334H CFTR or modification of R334C CFTR by a negatively charged MTS reagent, MTSES (sodium [2-sulfonatoethyl]methanethiosulfonate), decreased the whole cell conductance and enhanced the inward rectification of the shape of the I-V plots. The effects of pH- or MTS-induced charge changes at position 334 on the conductance and the shape of the I-V plots were attributed to a change in the outer vestibule potential, as predicted by either an electrodiffusion or rate-theory model. MTSET modification of R334C CFTR conductance caused an appropriate doubling in single channel conductance and had no marked effect on the open probability of the channel (Smith et al., 2001). As a result it may be concluded that the arginine at position 334 in wild type CFTR resides in the external vestibule of the pore, where its charge causes the local Cl^- concentration to increase at a site adjacent to the rate-limiting step of the conduction path.

THE OUTER VESTIBULE POTENTIAL IS SENSED BY A PORE-LINING RESIDUE

The electrostatic potential exerted by R334 may be sensed by a titratable side chain inserted in place of a nearby pore-lining residue, T338. The latter is one helical turn cytoplasmic to R334 (Liu et al., 2001). T338C CFTR undergoes pH-dependent changes in g_{Cl} and I-V shape that are not seen in wild type, T338A or T338S CFTR. Preliminary data indicate that the pH induced changes in T338C CFTR conductance are due to a change in the single channel conductance without a concurrent change in the open probability. Changes in conductance and shape of the I-V plot are consistent with the notion that T338 resides in the outer vestibule of the CFTR pore and is located on cytoplasmic to R334. Titration of the macroscopic conductance due to T338C and T338H CFTR indicates that positive charges at R334, perhaps at K335, and perhaps elsewhere may cause the pKa of T338C CFTR to become more acidic than in free solution (≈ 7.4) and to render T338H CFTR non-titratable. Changing the charge at position 334 either by modification of R334C/T338H CFTR with polar thiol reactive reagents or by amino acid substitution (R334A/T338C) shifts the titration

curve in a direction that was predicted on the basis of a nearby positive charge being able to stabilize a titratable group (Liu et al., 2001).

CONCLUDING REMARKS

Functional studies have provided a limited view of the nature of the CFTR pore. The pore is envisioned as comprising an outer, positively charged vestibule, which couples the external bath to a narrow region where anions bind. A similar feature is found in crystallized bacterial CLC channels (Dutzler et al., 2002). Each conducting pore of the bacterial clc channel contains an ion-binding site where Cl^- is coordinated by four ligands from four separate regions. This binding site has been referred to as the selectivity filter. The conducting pore also has an inner and an outer vestibule that direct anions to the selectivity filter. Interestingly, the wider, water-filled vestibules contain basic amino acid residues (Arg 147 and Arg 451). These positive charges are likely to play a role similar to R334 in the CFTR channel. Their location in the pore would allow them to create electrostatic potentials that enhance conductance by increasing the local concentration of permeant anions adjacent to the selectivity filter.

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