On the Origin of Asymmetric Interactions between Permeant Anions and the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel Pore

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ABSTRACT Single channel and macroscopic current recording was used to investigate block of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel pore by the permeant anion Au(CN)₂⁻. Block was 1–2 orders of magnitude stronger when Au(CN)₂⁻ was added to the intracellular versus the extracellular solution, depending on membrane potential. A point mutation within the pore, T-338A, strongly decreased the asymmetry of block, by weakening block by intracellular Au(CN)₂⁻ and at the same time strengthening block by external Au(CN)₂⁻. Block of T-338A, but not wild-type, was strongest at the current reversal potential and weakened by either depolarization or hyperpolarization. In contrast to these effects, the T-338A mutation had no impact on block by the impermeant Pt(NO₂)²⁻ ion. We suggest that the CFTR pore has at least two anion binding sites at which Au(CN)₂⁻ and Pt(NO₂)²⁻ block Cl⁻ permeation. The T-338A mutation decreases a barrier for Au(CN)₂⁻ movement between different sites, leading to significant changes in its blocking action. Our finding that apparent blocker binding affinity can be altered by mutagenesis of a residue which does not contribute to a blocker binding site has important implications for interpreting the effects of mutagenesis on channel blocker effects.

INTRODUCTION

Understanding the mechanism of action of ion channels has been given a tremendous boost in recent years by the generation of direct structural information in the form of channel crystal structures (1,2). One of the most striking features of these structures has been the identification of multiple discrete permeant ion binding sites within the pores of both cation selective (3,4) and anion selective channels (5). Permeant ion binding within the pore is thought to be key to the function of ion channels, providing the link between ionic selectivity and rapid ion transport both in cation channels (1,6) and anion channels (7,8).

Although direct structural evidence for ion binding sites inside channel pores is a recent breakthrough, the existence of such sites has been postulated for decades based on biological evidence (9). Most useful in these studies have been high affinity probes of ion binding sites, such as Ba²⁺ in K⁺ channels (10,11), Cd²⁺ in Ca²⁺ channels (6), and SCN⁻ in Cl⁻ channels (12). For example, SCN⁻ has been used to probe the functional properties of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel pore (12,13) and also to identify key pore-forming amino acid residues that potentially contribute to anion binding sites inside the pore (14–17). More recently, pseudohalide anions (such as Au(CN)₂⁻ and Pt(NO₂)²⁻) that inhibit Cl⁻ permeation at relatively low concentrations have been used to probe the CFTR pore (8,18–21). The apparent affinity of Au(CN)₂⁻ binding is affected by point mutations throughout the putative pore region (21–23); however, since Au(CN)₂⁻ is highly permeate in CFTR (18) it is difficult to separate the effects of mutagenesis on Au(CN)₂⁻ binding and permeation. Au(CN)₂⁻ has also recently been used to modify irreversibly cysteine residues introduced into the CFTR pore by site-directed mutagenesis (24). The divalent pseudohalide Pt(NO₂)²⁻ has also been used effectively to probe the CFTR pore, with one important distinction from Au(CN)₂⁻ being that Pt(NO₂)²⁻ is impermeate (19). Since Pt(NO₂)²⁻ is able to enter the pore and interfere with Cl⁻ permeation when applied to either side of the membrane, it has been suggested that distinct anion binding sites must exist on either side of some barrier inside the pore that prevents Pt(NO₂)²⁻ movement (19,25).

One amino acid residue that has been implicated in Au(CN)₂⁻ binding in the CFTR pore is T-338 in the sixth transmembrane region (TM6). Thus, the point mutation T-338A significantly reduces the apparent affinity with which intracellular Au(CN)₂⁻ inhibits Cl⁻ current through the channel (21). A number of studies have implicated this threonine residue as being involved in determining the single channel conductance (26) and anion selectivity (26,27) of the pore, perhaps by contributing to the narrowest region of the pore (8,26). Initially it was suggested, based on substituted cysteine accessibility mutagenesis, that the amino acid side chain at this position was not in contact with the aqueous lumen of the pore (28); however, more recent studies have shown that the side chain does in fact line the pore (24,29,30). There is some evidence that mutagenesis of T-338 affects the binding of both permeant (17,21,27) and blocking anions.

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within the pore. It has been suggested that T-338 contributes to the narrow, central region of the CFTR pore, which is the main determinant of selectivity between different permeant anions (reviewed by Linsdell (8)).

This work investigates the interaction between permeant and impermeant blocking ions and the CFTR pore. The complex effects of the T-338A mutant on channel block suggest that this important threonine residue forms a barrier to permeant anion movement inside the pore and that the existence of this barrier underlies asymmetric anion binding properties of the wild-type channel pore. In particular, our finding that mutagenesis of T-338 drastically alters the potency of Au(CN)₂⁻ block apparently without directly affecting an anion binding site has important implications for studies that seek to identify blocker binding sites within ion channel pores using mutagenesis.

**MATERIALS AND METHODS**

Experiments were carried out on baby hamster kidney cells stably transfected with human wild-type or T-338A-CFTR, prepared as described previously (17). Macroscopic and single channel patch clamp recordings were made from inside out patches excised from these cells, as described in detail previously (21–33). After patch excision and recording of background currents, CFTR channels were activated by exposure to protein kinase A (PKA) catalytic subunit plus MgATP (1 mM) in the cytoplasmic (bath) solution. As in a previous study (22), single channel currents were recorded after weak PKA stimulation (1–10 nM), whereas all macroscopic CFTR currents were recorded after maximal PKA stimulation (≈20 nM) and subsequent treatment with sodium pyrophosphate (PPi, 2 mM) to “lock” channels in the open state. Because single channel activity was recorded under conditions of weak PKA stimulation, patches contained a large number of CFTR channels with very low open probability, and as a result no information is contained in the relative open probability under different ionic conditions. Both intracellular (bath) and extracellular (pipette) solutions were based on one containing TES, 2 MgCl₂, pH 7.4, to which KAuCN (19,38) and subsequent treatment with protein kinase A (PKA) catalytic subunit plus MgATP (1 mM) in the cytoplasmic (bath) solution. As in a previous study (22), single channel currents were recorded after weak PKA stimulation (1–10 nM), whereas all macroscopic CFTR currents were recorded after maximal PKA stimulation (≈20 nM) and subsequent treatment with sodium pyrophosphate (PPi, 2 mM) to “lock” channels in the open state. Because single channel activity was recorded under conditions of weak PKA stimulation, patches contained a large number of CFTR channels with very low open probability, and as a result no information is contained in the relative open probability under different ionic conditions. Both intracellular (bath) and extracellular (pipette) solutions were based on one containing TES, 2 MgCl₂, pH 7.4, to which KAuCN (19,38) and subsequent treatment with protein kinase A (PKA) catalytic subunit plus MgATP (1 mM) in the cytoplasmic (bath) solution.

**RESULTS**

Asymmetric block of wild-type CFTR by Au(CN)₂⁻ ions

Au(CN)₂⁻ ions have previously been shown by our lab (19,38) and others (24) to cause a reduction in the amplitude of unitary CFTR Cl⁻ channel current, due to its ability to bind within the pore with high affinity and interrupt the flow of Cl⁻ ions. However, although Au(CN)₂⁻ is highly permeant in CFTR (18,21) and can inhibit CFTR Cl⁻ currents from either side of the membrane (24), its inhibitory effects have previously been studied at the single channel level only when present in the intracellular solution (19,24,38). Fig. 1 shows the block of unitary CFTR Cl⁻ currents recorded in inside out membrane patches by both intracellular and extracellular Au(CN)₂⁻. As reported previously (24,38), intracellular Au(CN)₂⁻ caused a voltage-dependent block of unitary Cl⁻ current at concentrations below 1 mM (Fig. 1, A–D). However, very much higher concentrations of Au(CN)₂⁻ were required in the extracellular solution to produce comparable levels of inhibition (Fig. 1, A–C and E). This asymmetry of blocking action is seen more clearly from single channel concentration-inhibition curves shown in Fig. 2 A. These curves further demonstrate that, unlike intracellular Au(CN)₂⁻, extracellular Au(CN)₂⁻ exhibits little or no voltage dependence of block. Fits to these curves by Eq. 1 were used to estimate the apparent affinity of Au(CN)₂⁻ block; results of these fits are shown in Fig. 2 B. Au(CN)₂⁻ inhibits unitary Cl⁻ current with significantly (~1–2 orders of magnitude) lower affinity when present in the extracellular versus the intracellular solution, depending on the membrane potential. Thus, despite its high permeability in the CFTR pore, Au(CN)₂⁻ shows highly asymmetric blocking effects on Cl⁻ movement through the pore.

Block of T-338A-CFTR by Au(CN)₂⁻

We also investigated Au(CN)₂⁻ block of unitary currents in T-338A-CFTR. As described in the introduction, this mutant...
has been shown to exhibit elevated unitary Cl\textsuperscript{−} conductance (26), increased Au(CN\textsubscript{2})\textsuperscript{−}/Cl\textsuperscript{−} permeability (21), and weakened block by intracellular Au(CN\textsubscript{2})\textsuperscript{−} at the macroscopic current level (21,22). The effects of intracellular and extracellular Au(CN\textsubscript{2})\textsuperscript{−} on wild-type and T-338A-CFTR single channel currents are compared in Fig. 3. These traces indicate that, whereas the inhibitory effects of 100 \textmu M intracellular Au(CN\textsubscript{2})\textsuperscript{−} are weakened in T-338A, the inhibitory effects of 1 mM extracellular Au(CN\textsubscript{2})\textsuperscript{−} are enhanced in this mutant.

Au(CN\textsubscript{2})\textsuperscript{−} block of T-338A-CFTR unitary currents is explored in more detail in Fig. 4. Unitary current-voltage relationships recorded in the presence of the same concentration of Au(CN\textsubscript{2})\textsuperscript{−} (1 mM) in either the intracellular or extracellular solution are shown in Fig. 4 A. The unitary current-voltage relationship recorded in the presence of extracellular Au(CN\textsubscript{2})\textsuperscript{−} shows an unusual “N”-shape, suggesting that block is stronger close to 0 mV than at either strongly hyperpolarized or strongly depolarized membrane potentials (Fig. 4 A). In the presence of intracellular Au(CN\textsubscript{2})\textsuperscript{−}, the current-voltage relationship appears linear at hyperpolarized voltages but outwardly rectified at depolarized voltages (Fig. 4 A). The concentration dependence of Au(CN\textsubscript{2})\textsuperscript{−} block

![FIGURE 1 Asymmetric block of wild-type CFTR by Au(CN\textsubscript{2})\textsuperscript{−} ions. (A) Example single channel currents recorded from inside out patches at membrane potentials of +50 mV and −50 mV, as indicated. In each case the closed state of the channel is indicated by the line on the far left. Currents were recorded in the absence of Au(CN\textsubscript{2})\textsuperscript{−} (control) or with Au(CN\textsubscript{2})\textsuperscript{−} present in the intracellular solution (100 \textmu M) or the extracellular solution (10 mM). In both cases Au(CN\textsubscript{2})\textsuperscript{−} causes a reduction in unitary current amplitude, as further demonstrated from amplitude histograms prepared from these current traces at +50 mV (B) and −50 mV (C) and also from mean unitary current-voltage relationships recorded under these conditions (D and E). Mean of data from 3–5 patches in D and E.](image-url)
of T-338A single channel currents is shown in Fig. 4, B and C. The apparent affinity of block is similar for both internal and external \(\text{Au(CN)}_2^-\), and in both cases the apparent \(K_d\) is minimal close to 0 mV and increases with either hyperpolarization or depolarization of the membrane potential (Fig. 4 C).

The unusual ‘‘U’’-shaped voltage dependence of apparent blocker affinity for external \(\text{Au(CN)}_2^-\) is a consequence of the ‘‘N’’-shaped unitary current-voltage relationship (Fig. 4 A), and the slight ‘‘U’’-shape also seen with internal \(\text{Au(CN)}_2^-\) (Fig. 4 C) hints at a similar, perhaps less pronounced, bi-phasic voltage dependence of block. To confirm this unusual current-voltage relationship shape, we recorded macroscopic T-338A current-voltage relationships over extended voltage ranges (−100 to +100 mV) in the absence of \(\text{Au(CN)}_2^-\) or with \(\text{Au(CN)}_2^-\) in the internal (2 mM) or external (1 mM) solutions, after maximal channel activation with PKA and ATP and subsequent channel ‘‘locking open’’ with PPi. As shown in Fig. 5 A, under these conditions T-338A-CFTR exhibits an almost completely linear macroscopic current-voltage relationship over the voltage range −100 to +100 mV, which, due to the locking of channels in the open state, should reflect the open channel unitary current-voltage relationship. In the presence of \(\text{Au(CN)}_2^-\) in either the intracellular or extracellular solution; however, the macroscopic current-voltage relationship shows an ‘‘N’’-shape reminiscent of the shape of the unitary current-voltage relationships in the presence of \(\text{Au(CN)}_2^-\) (Fig. 4 A). Quantification of the relative chord conductance at different membrane potentials indicates that, under control conditions, conductance in T-338A is maximal around 0 mV and decreases very slightly at strongly hyperpolarized and strongly depolarized membrane potentials (Fig. 5 B). In contrast, in the presence of both intracellular and extracellular \(\text{Au(CN)}_2^-\) conductance is minimal close to 0 mV and increases greatly when the membrane potential is hyperpolarized or depolarized (Fig. 5 B). By analogy with the single channel current-voltage relationships and voltage dependence of \(K_d\) shown in Fig. 4, this reflects strong block by both intracellular and extracellular \(\text{Au(CN)}_2^-\) at 0 mV and relief of block by both hyperpolarization and depolarization of the membrane potential.

The inhibitory effects of intracellular and extracellular \(\text{Au(CN)}_2^-\) on wild-type and T-338A-CFTR are compared directly in Fig. 6. These data show that the T-338A mutation significantly decreases the apparent affinity of block by intracellular \(\text{Au(CN)}_2^-\) (especially at hyperpolarized membrane potentials where the block of wild-type is strongest) while significantly increasing the apparent affinity of block by extracellular \(\text{Au(CN)}_2^-\).

**Block of T-338A-CFTR by other small anions**

We hypothesized that the dramatic weakening of the asymmetry of \(\text{Au(CN)}_2^-\) block that we observed in T-338A-CFTR was related to the fact that \(\text{Au(CN)}_2^-\) is a permeant blocker of the channel pore (18,21). We therefore wondered if the T-338A mutation would affect the apparent affinity of block by small anions that act as open channel blockers but that are impermeant. One such anion is the divalent pseudohalide ion \(\text{Pt(NO}_2\text{)}_2\text{I}^-\), which is impermeant but can block the channel from either the intracellular and extracellular side of the membrane (19,25). Indeed, we have previously shown by macroscopic current recording that the T-338A mutation does not significantly affect block by intracellular \(\text{Pt(NO}_2\text{)}_2\text{I}^-\) (19). To investigate the blocking effects of extracellular \(\text{Pt(NO}_2\text{)}_2\text{I}^-\) we used single channel recording from inside out membrane patches in the absence or presence of \(\text{Pt(NO}_2\text{)}_2\text{I}^-\) in the extracellular solution (Fig. 7). Unitary current-voltage relationships recorded under these conditions (Fig. 7 B),
as well as the fractional current observed in the presence of 1 mM Pt(NO₂)₄²⁻ (Fig. 7 C), indicate that Pt(NO₂)₄²⁻ causes a strongly voltage-dependent inhibition of unitary current amplitude that appears similar in both wild-type and T-338A-CFTR.

Unitary Cl⁻ current in wild-type CFTR is also blocked by another permeant anion, SCN⁻ (13), albeit with a considerably lower affinity than that observed for Au(CN)₂⁻. However, although SCN⁻ shows a fairly similar permeability to Au(CN)₂⁻ (12,18,21), its blocking effects are quite symmetrical, with an ~2-fold higher $k_a$ having been reported for extracellular SCN⁻ block of unitary Cl⁻ currents (14.2 mM at 0 mV membrane potential) compared to intracellular SCN⁻ (6.2 mM at 0 mV) (13). Since one of the most striking effects of the T-338A mutation was the weakening of the asymmetry of Au(CN)₂⁻ block, we also wondered how this mutation would affect the already quite symmetrical blocking effects of SCN⁻. As shown in Fig. 8, A and B, 10 mM SCN⁻ had broadly similar blocking effects on T-338A unitary Cl⁻ currents when present in either the intracellular or extracellular solution. As with Au(CN)₂⁻ block, the unitary current-voltage relationship in the presence of SCN⁻ was noticeably ‘‘N’’-shaped (Fig. 8 B). As a result, the voltage dependence of block, like that of Au(CN)₂⁻ showed a ‘‘U’’-shape, with strongest inhibition close to 0 mV (Fig. 8, C and D). Whereas SCN⁻ caused a similar degree of inhibition of wild-type CFTR (13) (Fig. 8, C and D), the voltage dependence of block was more conventional, with intracellular SCN⁻ blocking most strongly at hyperpolarized membrane potentials (Fig. 8 C) and extracellular SCN⁻ blocking most strongly at depolarized potentials (Fig. 8 D).

**Origin of the voltage dependence of Au(CN)₂⁻ block of T-338A-CFTR**

A striking feature of T-338A-CFTR block by both Au(CN)₂⁻ and SCN⁻ is the ‘‘N’’-shaped current-voltage relationship in the presence of blocker (Figs. 4 A, 5 A, and 8 B), reflecting a blocking event that is strongest close to 0 mV and is weakened by both hyperpolarization and depolarization. To investigate whether this reflected dependence of block on voltage itself or on the rate of Cl⁻ movement through the pore, we investigated block of T-338A-CFTR macroscopic currents by intracellular Au(CN)₂⁻ in the presence of different transmembrane Cl⁻ concentration gradients (Fig. 9). Whether Cl⁻ was reduced to 20 mM in either the intracellular or extracellular solution, current inhibition by addition of 1 mM Au(CN)₂⁻ was still associated with an ‘‘N’’-shaped current-voltage relationship (Fig. 9 A). As a result, maximal current inhibition occurred close to the current reversal potential (Fig. 9 B) under all ionic conditions studied. This suggests that voltage-dependent Cl⁻ movement through the pore—rather than membrane potential per se—is responsible for the weakening of Au(CN)₂⁻ inhibition at extreme membrane potentials that results in the ‘‘N’’-shaped current-voltage relationships observed.

**Rate theory models of Au(CN)₂⁻ interaction with wild-type and T-338A-CFTR**

To illustrate how the T-338A mutation might decrease the apparent affinity of block by internal Au(CN)₂⁻ and yet increase the apparent affinity of block by external Au(CN)₂⁻, we used the single channel I/V relationships shown in Figs. 1 and 4 to develop simple rate theory models of the CFTR pore. As described in the Discussion, our results could reflect either one or two Au(CN)₂⁻ binding sites in the pore, and to reflect this we have developed two models, with either one or two anion binding sites (energy wells) within the pore. Best fit energy profiles for both Cl⁻ and Au(CN)₂⁻ were simultaneously optimized by the fitting procedure, and the effects of the T-338A mutation were modeled by altering a single parameter—i.e., by lowering one energy barrier for both Cl⁻ and Au(CN)₂⁻ movement in the pore (see Discussion). Relative energies for the two different anions are shown graphically as energy barrier profiles in Fig. 10, A–D. The predictions

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of the models (Fig. 10, E–H) indicate that both the single binding site model (Fig. 10, A and B) and the two binding site model (Fig. 10, C and D) are able to replicate the most salient features observed experimentally—strong block of wild-type by internal Au(CN)_{2}^{−}, weak block of wild-type by external Au(CN)_{2}^{−}, weakened block of T-338A by internal Au(CN)_{2}^{−}, and yet strengthened block of this mutant by external Au(CN)_{2}^{−}.

**DISCUSSION**

**Au(CN)_{2}^{−} is an asymmetric blocker of the wild-type CFTR channel pore**

Inhibition of wild-type CFTR by Au(CN)_{2}^{−} has been investigated in many previous studies (18,21,24,33,38). When applied to the cytoplasmic face of inside out membrane patches, Au(CN)_{2}^{−} has two inhibitory effects on CFTR Cl− currents—a high affinity reduction in channel open probability, and a lower affinity, voltage-dependent reduction in unitary current amplitude (38). This second effect appears to reflect binding of Au(CN)_{2}^{−} within the open channel pore, where it impedes Cl− movement through the pore. External Au(CN)_{2}^{−} has been reported to inhibit wild-type CFTR with a broadly similar potency and probably by the same mechanism (18,24); however, these effects have been observed only at the whole cell current level. It is somewhat surprising, therefore, that the effects of Au(CN)_{2}^{−} on CFTR single channel current amplitude show such strong asymmetry—with external Au(CN)_{2}^{−} being 1–2 orders of magnitude lower affinity than intracellular Au(CN)_{2}^{−} (Fig. 2). Thus the inhibitory effects of extracellular Au(CN)_{2}^{−} that we observe at the single channel level (Fig. 1)—which suggest a K_d of ~10 mM (Fig. 2)—appear to be much weaker than those reported using whole cell recording (18,24). This discrepancy may reflect multiple inhibitory effects of extracellular Au(CN)_{2}^{−} that contribute to the overall inhibition observed at the whole cell level—a situation analogous to the multiple inhibitory effects of intracellular Au(CN)_{2}^{−} that contribute to its blocking actions on macroscopic Cl− currents (38). Thus it is possible (but not investigated) that external Au(CN)_{2}^{−} could affect the open probability of the channel; it has been shown previously that CFTR gating is sensitive to extracellular anions including Cl− itself (39). It is less likely that Au(CN)_{2}^{−} is permeating the channel to inhibit channel open probability at an intracellular site of action, since the inhibitory effects of external Au(CN)_{2}^{−} on whole cell currents have been shown to be readily reversible (24). Irrespective of the reasons for these discrepancies, the strikingly different effects of Au(CN)_{2}^{−} on single channel current amplitude suggest that Au(CN)_{2}^{−} ions bind much more tightly inside the pore when added to the intracellular versus the extracellular solution.

Why is Au(CN)_{2}^{−} block of Cl− permeation through the pore so strongly asymmetric? It is well known that large organic anions usually block the CFTR channel pore only from the inside, due primarily to their interaction with a positively charged amino acid side chain (K-95 in TM1) in the wide inner vestibule of the pore (40–42). These large substances are thought to be excluded from the extracellular mouth of the pore for reasons related to their size (43). In
contrast, Au(CN)$_2^-$ is highly permeant in CFTR (in fact, with a higher permeability than Cl$^{-}$ itself) (18,21), such that it is expected to have access to binding sites throughout the pore when present in either the intracellular or the extracellular solution. Nevertheless, its interactions with binding sites within the pore appear to be very different depending on which side of the membrane it is applied from. This could reflect either a single Au(CN)$_2^-$ binding site in the pore that is more accessible from the intracellular side of the membrane or separate binding sites that underlie block by intracellular and extracellular Au(CN)$_2^-$, with the intracellular site showing a greater affinity for Au(CN)$_2^-$.

Currently we do not have enough information to be able to discriminate between these two models, and as a result we have developed rate theory models having either one or two binding sites (energy wells). Indeed, the energy profiles generated for wild-type CFTR reflect the general features described above; the single site model (Fig. 10 A) shows a lower energy barrier (resulting in easier access) for Au(CN)$_2^-$ entry from the intracellular side of the membrane, whereas the two-site model (Fig. 10 C) shows a deeper energy well (resulting in tighter binding) for the more cytoplasmically located binding site. Although we find that the single binding site model is the simplest that can reproduce our single channel data (Fig. 10, D and E), there are several factors that we consider as favoring the second model with two binding sites. First, we believe that there are blocker binding sites in both the outer and inner mouths of the pore, since the impermeant anion Pt(NO$_2$)$_2$ can block Cl$^{-}$ permeation when added to either side of the membrane, apparently by binding to different sites (19,25). This is also consistent with the presence of a blocker binding site in the outer pore vestibule proposed by Muanprasat et al. (44).

Second, point mutations in both the extracellular and intracellular mouths of the pore affect Au(CN)$_2^-$ block, and this may reflect disruption of separate binding sites. Thus, Au(CN)$_2^-$ block is greatly weakened by mutagenesis of R-334 (in TM6) (22,23), a positively charged residue which is involved in attracting extracellular Cl$^{-}$ ions into the outer mouth of the pore (45), and of K-95 (in TM1) (22), the positive charge of which attracts intracellular Cl$^{-}$ ions into the wide inner pore vestibule (40). Finally, block by extracellular Au(CN)$_2^-$ is apparently voltage independent (Fig. 2 B); although the voltage dependence of intracellular Au(CN)$_2^-$ block is complex in origin (33), this lack of voltage dependence would at first glance appear inconsistent with extracellular Au(CN)$_2^-$ ions moving deeply into the pore to block at a binding site located in the inner pore vestibule (Fig. 10 A).
Au(CN)$_2^-$ block of the T-338A-CFTR channel pore is much more symmetrical

Previously we showed, using macroscopic current recording, that block by intracellular Au(CN)$_2^-$ was greatly weakened in T-338A-CFTR and suggested that T-338 may contribute to a lyotropic anion binding site in the pore (21,22). However, the results here at the single channel level indicate that whereas this mutation does weaken block by intracellular Au(CN)$_2^-$, it actually strengthens block by extracellular Au(CN)$_2^-$ (Fig. 6). This apparently contradictory effect of a single mutation is difficult to reconcile with disruption of an Au(CN)$_2^-$ binding site in the pore. Instead, we now propose that the T-338A mutation alters the movement of Au(CN)$_2^-$ within the pore, changing its ability to access one or more binding sites the properties of which are not directly affected by the mutation. Consistent with this idea, block by impermeant Pt(NO$_2$)$_4^{2-}$ ions is not affected by the T-338A mutation, whether Pt(NO$_2$)$_4^{2-}$ is added to the intracellular (19) or extracellular solution (Fig. 7). This suggests that Pt(NO$_2$)$_4^{2-}$ ions are restricted to binding sites on the same side of the membrane to which they are applied and that the T-338A mutation has no impact either on the Pt(NO$_2$)$_4^{2-}$ binding affinity of these sites or on Pt(NO$_2$)$_4^{2-}$ movement between these sites.

The ways in which the T-338A mutation affects Au(CN)$_2^-$ inhibition can be interpreted in terms of either the single or
double binding site models shown in Fig. 10. If Au(CN)$_2^-$ is considered as acting at a single site, then the T-338A mutation could increase the rate of Au(CN)$_2^-$ movement between this site and the extracellular solution. This would have the effect of weakening Au(CN)$_2^-$ block from the inside (since Au(CN)$_2^-$ would now better be able to escape its binding site to the extracellular solution, relieving block) and at the same time strengthening block from the outside (since Au(CN)$_2^-$ would now better be able to reach its binding site). In the two binding site model, the effects of the T-338A mutation could result from an increase in the rate of Au(CN)$_2^-$ movement between the two sites. The effect on Au(CN)$_2^-$ block is very similar to that described above for the single site model; intracellular Au(CN)$_2^-$ block is weakened because of enhanced unblock by Au(CN)$_2^-$ permeation to the outside, whereas extracellular Au(CN)$_2^-$ block is strengthened because of increased access to the higher affinity, internal binding site. Note that in both of these suggested models, the effect of the T-338A mutation is to lower a barrier to Au(CN)$_2^-$ movement within the pore, rather than to affect an Au(CN)$_2^-$ binding site. Indeed, for the energy barrier profiles developed (Fig. 10), the effects of the T-338A mutation...
are reproduced by lowering the height of a single energy barrier—either the barrier between the binding site and the extracellular solution in the one-site model (Fig. 11 A) or the barrier between the two binding sites in the two-site model (Fig. 11 B).

The effects of the T-338A mutation are consistent with either the one-site or two-site model (Fig. 11). Indeed, a single binding site model is the simplest that can reproduce the effects of Au(CN)₂⁻ observed at the single channel level (Fig. 10 F). Nevertheless, we would interpret the lack of effect of this mutation on block by either intracellular or extracellular Pt(NO₃)₂⁻ block as supporting the two-site model, since these data are consistent with the idea that there are blocker binding sites located both external and internal to the location of T-338 within the pore, as illustrated in the two-site model (Fig. 11 B).

### Voltage and chloride dependence of Au(CN)₂⁻ block

The voltage dependence of Au(CN)₂⁻ block is also strongly altered in T-338A-CFTR. In wild-type CFTR, block by intracellular Au(CN)₂⁻ shows a conventional voltage dependence, with block by this negatively charged substance being strengthened by hyperpolarization of the membrane potential (Figs. 1 D and 2 B). Block by external Au(CN)₂⁻ appears to be voltage insensitive (Figs. 1 E and 2 B), suggesting that the blocker binding site may be outside of the transmembrane electric field. Although some similarities are seen in T-338A (for example, block by internal Au(CN)₂⁻ is stronger at hyperpolarized than at depolarized voltages; Fig. 4 C), the most striking aspect of the voltage dependence of block in this mutant is the “U”-shaped apparent affinity-voltage
Au(CN)$_2^-$ block in this mutant is, in fact, more sensitive to the voltage-dependent flow of Cl$^-$ ions than to the transmembrane voltage itself. Again, this is consistent with the idea that blocking Au(CN)$_2^-$ ions can exit the pore in either direction; at hyperpolarized voltages, rapid Cl$^-$ efflux will sweep blocking Au(CN)$_2^-$ ions from the pore into the extracellular solution, whereas at depolarized potentials Cl$^-$ ions will flow into the intracellular solution and carry blocking Au(CN)$_2^-$ ions along with them.

**Role of T-338 as a barrier to anion movement in the pore**

Previously we suggested that T-338 contributes to an important lyotropic anion binding site in the pore, based on the finding that the T-338A mutation weakened open channel block by intracellular Au(CN)$_2^-$ ions (21,22). However, this work suggests that this mutation does not in fact affect an Au(CN)$_2^-$ binding site, but instead alters the ability of Au(CN)$_2^-$ to move between different sites within the channel pore. The finding that a point mutation in the pore can have such a large effect on the apparent affinity of a blocker (either increase or decrease) depending on the exact experimental protocol and our suggestion that this occurs without an actual modification of blocker binding sites, therefore, offers an important caveat for site-directed mutagenesis studies that seek to identify blocker binding sites within ion channel pores. Given the different effects of the T-338A mutation on the blocking effects of Au(CN)$_2^-$ and Pt(NO$_2$)$_2^{2-}$, this caveat might be particularly germane when investigating permeant blockers.

We suggest that the mutation T-338A removes or reduces a barrier to permeant anion movement inside the CFTR channel pore (Fig. 11). The existence of this barrier within the wild-type channel pore appears to play an important role in underlying asymmetric interactions between permeant ions and the pore that result in the strongly asymmetric blocking effects of Au(CN)$_2^-$ (Fig. 2). Removal of this barrier also allows Cl$^-$ ions to effectively sweep blocking Au(CN)$_2^-$ ions from the pore, resulting in a blocking action that is more dependent on voltage-dependent Cl$^-$ flux than on transmembrane voltage itself (Fig. 9). We propose that the barrier associated with T-338 effectively separates the outer and inner pore vestibules, both of which contain permeant anion binding sites with different properties.

It is tempting to speculate that the lyotropic anion binding sites situated external and internal to the T-338 “barrier” involve the pore-lining positive charges of R-334 and K-95, respectively. Indeed, of those amino acids that have been studied by site-directed mutagenesis, these two residues have the greatest impact on apparent Au(CN)$_2^-$ binding affinity (22). Furthermore, current models of the pore place these two residues on the extracellular and intracellular side of T-338, respectively (8). Unfortunately, mutagenesis of either of these two positively charged residues causes a considerable

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**FIGURE 11** Modeling of the effects of the T-338A mutation. Energy profiles shown in Fig. 10 are redrawn to show the effect of the T-338A mutation on Cl$^-$ and Au(CN)$_2^-$ movement in the pore, both for the one-site model (top) and the two-site model (bottom). In each case, solid lines represent the energy profile for wild-type, and dashed lines the changes made to model T-338A. The effects of the mutation are modeled as (top) lowering the energy barrier that exists between the single anion binding site and the extracellular solution, or (bottom) lowering the energy barrier that exists between the two anion binding sites. As illustrated in Fig. 10, these localized changes in energy profile were sufficient to model the salient differences in Au(CN)$_2^-$ interactions between wild-type and T-338A.
CONCLUSIONS

Our results suggest that T-338 contributes to a barrier to Cl\(^-\) channel conductance (26). Substitutions of T-338 also lead to a large reduction in single channel conductance (26).

REFERENCES


