Side Chain Orientation in the Selectivity Filter of a Voltage-gated Ca\(^{2+}\) Channel*

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Four glutamate residues (EEEE locus) are essential for ion selectivity in voltage-gated Ca\(^{2+}\) channels, with ion-specific differences in binding to the locus providing the basis of selectivity. Whether side chain carboxylates or alternatively main chain carbonyls of these glutamates project into the pore to form the ion-binding locus has been uncertain. We have addressed this question by examining effects of sulfhydryl-modifying agents (methanethiosulfonates) on 20 cysteine-substituted mutant forms of an L-type Ca\(^{2+}\) channel. Sulfhydryl modifiers partially blocked whole oocyte Ba\(^{2+}\) currents carried by wild type channels, but this block was largely reversed with washout. In contrast, each of the four EEEE locus glutamate → cysteine mutants (0 position) was persistently blocked by sulfhydryl modifiers, indicating covalent attachment of a modifying group to the side chain of the substituted cysteine. Cysteine substitutions at positions immediately adjacent to the EEEE locus glutamates (±1 positions) were also generally susceptible to sulfhydryl modification. Sulfhydryl modifiers had lesser effects on channels substituted one position further from the EEEE locus (±2 positions). These results indicate that the carboxylate-bearing side chains of the EEEE locus glutamates and their immediate neighbors project into the water-filled lumen of the pore to form an ion-binding locus. Thus the structure of the Ca\(^{2+}\) channel selectivity filter differs substantially from that of ancestral K\(^{+}\) channels.

Mechanistically, ion selectivity in Ca\(^{2+}\) channels relies upon differences between ions in affinity for the EEEE locus. For a solution containing both Ca\(^{2+}\) and Na\(^{+}\), for example, Ca\(^{2+}\) is permeant and Na\(^{+}\) is not because Ca\(^{2+}\) binds more tightly to the locus than does Na\(^{+}\) (4, 7–10). Upon identification of the EEEE locus as the essential structural feature underlying Ca\(^{2+}\) channel selectivity, it was generally presumed that the carboxylate-bearing side chains of the EEEE locus glutamates projected into the aqueous pore where they could form a cation-binding structure rather like that of organic chelators such as EGTA. Evidence supporting this idea has been provided by studies of proton block of Ca\(^{2+}\) channel currents. In these studies, amino acid substitutions in the EEEE locus were shown to disrupt proton block of Ca\(^{2+}\) channel currents, and the nature of the disruptions could be most readily described by a structural model requiring projection of carboxylate groups into the aqueous pore (11–13). For any single residue, steric constraints do not allow main chain carbonyls and side chain carboxylates to project in the same direction, so that the relative orientation of the amino acid chain lining the pore of a channel with a carboxylate-based EEEE locus would differ by ~180° axial rotation from the orientation of the pore-lining chain in a channel with a carbonyl-based EEEE locus.

In contrast to the proposed carboxylate-based ion-binding sites in the selectivity filter of Ca\(^{2+}\) channels, ion-binding sites in the selectivity filter of K\(^{+}\) channels are probably formed by main chain carbonyl groups. Evidence for carbonyl-based binding sites in K\(^{+}\) channels is provided by the x-ray crystallographic structure of a bacterial K\(^{+}\) channel, which has revealed that carbonyl groups of the main peptide chain project into the pore to form K\(^{+}\)−binding sites in this channel’s selectivity filter (14). The bacterial K\(^{+}\) channel has a signature sequence and pore structure that are closely related to those of eukaryotic K\(^{+}\) channels (15, 16), indicating that the selectivity filter of eukaryotic K\(^{+}\) channels is likely to be very similar in structure to that of the ancestral bacterial K\(^{+}\) channel. Indeed, before the bacterial K\(^{+}\) channel structure had been solved, eukaryotic K\(^{+}\) channels had been predicted to utilize main chain carbonyl groups to form selectivity filter K\(^{+}\)−binding sites (15).

The K\(^{+}\) channel images are so compelling that the idea that Ca\(^{2+}\) channels, like K\(^{+}\) channels, might utilize main chain carbonyl groups to bind and select permeant ions has become more plausible. Furthermore, the fact that Ba\(^{2+}\) block of K\(^{+}\) channels results from the binding of Ba\(^{2+}\) to selectivity filter carbonyls (17) resonates with this contrarian view of selectivity filter structure in Ca\(^{2+}\) channels because Ba\(^{2+}\) is a divalent cation that binds with high affinity in both K\(^{+}\) and Ca\(^{2+}\) channels. Hence, it is possible that over the course of evolution Ca\(^{2+}\) channels may have preserved features of the selectivity filter structure first evolved in K\(^{+}\) channels and, in particular, the reliance upon carbonyls to form high affinity binding sites for permeant ions.

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To test these competing descriptions of selectivity filter structure in Ca$^{2+}$ channels, we have used the substituted cysteine accessibility method to examine whether pore-lining amino acids project side chains into the pore lumen or whether they instead project side chains away from the lumen and into the bulk of the protein (18, 19). This method has been used to determine side chain orientation of pore lining residues in many kinds of ion channels, including nicotinic receptors (18, 20), $\gamma$-amino butyric acid type A receptors (21, 22), N-methyl-D-aspartate receptors (23, 24), P2X$_{2}$ receptors (25), K$^{+}$ channels (26–33), Na$^{+}$ channels (34–37), cyclic nucleotide gated channels (38), chloride channels (39), the cystic fibrosis transmembrane conductance regulator (40, 41), and an excitatory amino acid transporter channel (42). We report here results of the application of this method to voltage-gated Ca$^{2+}$ channels.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Cysteine substitutions were introduced into the $\alpha_{1c}$ subunit of an L-type Ca$^{2+}$ channel using a megaprimer polymerase chain reaction method, as described previously (5, 6). All cysteine substitution mutants were confirmed by sequencing both polymerase chain reaction method, as described previously (5, 6). All experiments were carried out at room temperature (22–23 °C). Data are reported as means ± S.E., with the number of measurements (n) in parentheses.

**RESULTS**

**Effects of Cysteine Substitution on Channel Function**—Fig. 1 indicates the locations of each of the single cysteine substitutions introduced into the Ca$^{2+}$ channel selectivity filter (substituted positions enclosed in diamonds). In total, 20 single substitution mutants were made: for each of the four EEEE locus glutamates (position 0) and for all 16 sequence neighbors at their nearest two amino- and carboxyl-terminal positions (±2, ±1, and ±2 positions). Before testing the action of sulphydryl-modifying agents, the effects of cysteine substitution on channel function were examined.

Voltage-clamp currents were collected for each of the cysteine substitution mutants, and their large amplitudes showed that the mutants expressed well in oocytes. In Fig. 2A, examples of superimposed families of voltage-clamp currents are Ca$^{2+}$ channels were elicited every 15 s by 150-ms depolarizing test pulses from a holding potential of −80 mV. Currents were filtered at 1kHz (−3dB; 4-pole Bessel filter), sampled at 2 kHz, and leak- and capacitance-subtracted. Oocytes were perfused continuously (~1 ml/min) with a solution containing 40 mM Ba(OH)$_{2}$, 52 mM tetraethylammonium hydroxide, 5 mM HEPES, pH adjusted to 7.4 using methane sulfonic acid. Methanethiolosulfonate (MTS)$^{3}$ reagents were dissolved in the 40 mM Ba$^{2+}$ solution immediately prior to their application (<2 min) via the bath perfusion system, at approximately equi-effective concentrations: 2 mM MTSEA$^{-}$, 1 mM MTSET$^{-}$, or 10 mM MTSES$^{-}$ (Toronto Research Chemicals, Toronto, Canada). The effects of a higher concentration of MTSEA$^{-}$ (10 mM) on WT and the 20 cysteine substitution mutants were indistinguishable from the effects of 2 mM MTSEA$^{-}$. All experiments were carried out at room temperature (22–23 °C). Data are reported as means ± S.E., with the number of measurements (n) in parentheses.

1 The abbreviations used are: MTS, methane thiosulfonate; MTSEA$^{-}$, (2-aminomethyl) methane thiosulfonate; MTSET$^{-}$, (2-trimethylammonium)ethyl methanethiosulfonate; MTSES$^{-}$, 2-sulfonatoethyl) methanethiosulfonate; DTT, 1,4-dithiothreitol; WT, wild type; DTT, dithiothreitol.

**FIG. 1. Location of Cys substitutions in pore-lining regions of the L-type Ca$^{2+}$ channel and structure of thiol modifying reagents. A, membrane topology of the $\alpha_{1c}$ L-type Ca$^{2+}$ channel. B, amino acid sequences of the four pore lining (P) loops. Cysteine substitutions were introduced at each of the positions (indicated as diamonds). The residue numbers for each of the four selectivity filter glutamates are marked to the left of each glutamate. For ease of reference, the positions of pore lining residues are referred to relative to the selectivity filter glutamate in each loop, and these are numbered as 0 in each pore lining loop. C, structures of methanethiolosulfonate reagents. The MTS reagents tested all fit into a cylinder of length 10 Å and diameter of 5.8 Å or less. The methanethiosulfonate chain common to all three MTS reagents has a diameter of 4.8 Å, and the diameters of the different headgroup are indicated.
illustrated for WT and all five of the motif I substitution mutants. For the other 15 mutants, maximum inward currents were comparable in size with those shown in Fig. 2A.

Current-voltage relationships constructed from these data showed that both gating and selectivity were altered in some of the mutants. Regarding gating changes, it can be seen in Fig. 2B that mutant channel activation between −20 and 0 mV was dispersed over a range of −10 mV, with the WT data falling in the middle of the range. In addition to the effects on steady-state gating, the kinetics of gating were also affected in some cases; compare, for example, the inactivation kinetics of the M392C mutant with those of WT (Fig. 2A). Overall, however, the effects of cysteine substitution on gating were small in size, and they therefore had little consequence for the study of sulfhydryl modifier action.

More significant than altered gating were, for some of the substitutions, the relatively large changes in reversal potential. In motif I, the −1 (M392C) and +1 (G394C) substitutions were virtually identical to WT in reversal potential, the −2 (T391C) and +2 (W395C) substitutions differed from WT by 10−15 mV, and the position 0 substitution (E393C) differed by −30 mV from WT (Fig. 2B). The effects of cysteine substitution on reversal potential are summarized for all of the mutants in Fig. 2C; the Glu → Cys (position 0) substitutions exhibited the largest reductions in reversal potential, corresponding to the largest reductions in selectivity for Ba2+ over K+. Substitutions at other positions (e.g., T391C) clearly differed from WT in reversal potential, but about half of the substitutions were little different from WT.

Selectivity can also be examined by measuring the ability of one permeant ion to block the flux of another permeant ion species, which provides the relative binding affinity for the higher affinity ion. A pairing of Cd2+ and Ba2+ is useful for this purpose because Cd2+ permeates Ca2+ channels but at such a low rate that it blocks the flux of other ions, including Ba2+ (46). For WT and the motif IV substitutions, examples of Cd2+ block of Ba2+ currents and corresponding dose inhibition relationships are illustrated in Fig. 3 (A and B). Relative to WT, some motif IV mutants were more potently blocked by Cd2+ (A1447C, +1 position), and others were less potently blocked (W1448C, +2 position). Considering all 20 mutants, half-block (IC50) values ranged from 0.15 to 1.5 μM, as compared with an IC50 of 0.6 μM for WT (Fig. 3C). Thus mutant IC50 values differed only modestly from the WT value.

Cysteine substitution of EEEE locus glutamates did not reduce Cd2+ binding affinity, unlike what has been described previously for aspartate, glutamine, and alanine substitution in the EEEE locus (5). Some cysteine substitution mutants (E1446C and A1447C) even bound Cd2+ more tightly than did WT. The unique behavior of the cysteine substitutions was not surprising, though, because Cd2+ avidly binds to thiol groups of cysteines.

Taken together, the effects of cysteine substitution on Ca2+ channel expression, gating, and selectivity were, with the exception of reversal potential for Glu → Cys mutants, rather modest in size. In addition, the increased Cd2+ binding affinity exhibited by some of the cysteine mutants hints that at least some of the tested positions project side chains into the pore and particularly that E1446 projects its side chain into the pore. A more telling examination of side chain orientation was carried out using methanethiosulfonate reagents, which, unlike Cd2+, do not have significant affinity for carboxylate groups.

Effects of Methanethiosulfonates on Cysteine-substituted Channels—Methanethiosulfonates are sulfhydryl-specific reagents that can covalently modify exposed cysteine residues. The MTS reagents MTSEA, MTSET, and MTSES covalently attach ethylamine, ethyltrimethylammonium, or ethylsulfonate moieties, respectively, to the thiol group on the cysteine side chain. For exposed cysteine thiols in the narrow region of a channel pore, attachment of these moieties can obstruct the flow of ions, so that testing for persistent MTS block provides an indication of cysteine side chain accessibility and, by extension, of side chain orientation in the WT channel.

We first examined the action of MTS reagents on WT channels to test for effects via endogenous thiols. Application of the cationic methanethiosulfonate reagent MTSEA+ to oocytes expressing WT channels produced partial block of Ba2+ current (Fig. 4A). WT currents were blocked by 11 ± 1% (n = 9; Fig. 5A), but this block was reversed when MTSEA+ was washed out of the recording chamber (Fig. 4B, top). Two other MTS reagents, MTSET+ and MTSES+, had similarly small effects.
that reversed with wash when tested on WT channels (block was 11 ± 3%, n = 8 and 13 ± 1%, n = 8 respectively; Fig. 5, B and C).

Examples of the action of MTSEA on each of the four Glu → Cys substitution mutants are also presented in Fig. 4. MTSEA blocked nearly all of the Ba²⁺ current in each of these mutants, but unlike for WT, current did not fully recover subsequent to MTSEA washout. Modification by MTSEA could not be reversed by application of the disulfide reducing agent DTT in three of the four Glu → Cys mutants, but DTT was able to slowly reverse the action of MTSEA on the motif IV Glu → Cys mutant (E1446C, bottom panel in Fig. 4B). It may be that DTT cannot fit into the pore of these latter three MTSEA-modified channels. Fractional block by MTSEA is summarized for all 20 cysteine substitution mutants in Fig. 5A. The results were similar, but not identical, across the four motifs, with the general pattern within motifs as follows. MTSEA block was greatest in size for the mutants at position 0 (Glu → Cys; 94–97% block), followed by that for the mutants at the ±1 positions (45–89% block), and block at the ±2 positions was in most cases smaller yet nonexistent. The only significant deviations from this pattern were in motif II (+2 position) and motif III (±2 positions). For these three ±2 position mutants, block by MTSEA was as large as that observed for the ±1 positions.

We tested two other MTS reagents on the cysteine substitution mutants. MTSET differs from MTSEA in two ways: (i) MTSET is larger in molecular diameter (Fig. 1); and (ii) it cannot cross the cell membrane owing to its permanent charge, whereas MTSEA, a primary amine, spends a fraction of the time in its neutral form and is thus able to cross the cell membrane (47). Fig. 5B shows the pattern for MTSET block of the substitution mutants. The MTSET pattern was similar to that for MTSEA in that the Glu → Cys mutants exhibited large fractional block, and the +1 position mutants were sensitive to MTSET. Also, the +2 position of motifs II and III was sensitive to MTSET, whereas the +2 position of motifs I and IV was not, just as was observed for MTSEA. Two aspects of the MTSET block pattern differed from that for MTSEA: no −2 position mutant was sensitive to MTSET block, and only one of the four −1 position mutants (motif IV) was sensitive to MTSET, perhaps because MTSET is too bulky to reach and modify these deeper positions or perhaps because MTSET cannot cross the membrane to reach these positions.

The anionic MTS reagent MTSES was also tested on the cysteine substitution mutants. The pattern of block by MTSES was even more restricted than that for MTSET, but nevertheless, all of the Glu → Cys mutants were blocked by this reagent (Fig. 5C). The only one of the eight −2 or −1 position mutants that may have been sensitive to MTSES was the −1 position mutant in motif IV, and the percentage of block of current carried by this mutant was small. Among the eight +1 and +2 position mutants, only those in motif II (+2) and motif III (+1 and +2) exhibited significant sensitivity to MTSES. Thus MTSES was able to modify at least some superficial pore entrance positions and all of the EEEE locus positions, but deeper positions were apparently inaccessible to MTSES. That anionic MTSES could enter the selectivity filter of a strongly cation-selective Ca²⁺ channel was not anticipated and is considered under “Discussion.”

Reversal of MTS Block by a Disulfide Reducing Agent—Block that persists after removal of an MTS reagent can in principle be reversed by agents that reduce the disulfide bond that attaches MTS reagent headgroups to exposed cysteine thiois. Within the narrow confines of an ion channel pore, however, disulfide reducing agents may not physically have access to the disulfide bond formed between an MTS reagent and a substituted cysteine. In such cases, persistent block by MTS reagents would not be reversible by a disulfide reducing agent. For Ca²⁺ channels, this seems to be the case for many of selectivity filter cysteine substitution mutants: 11 of the 20 substitution mutants were blocked persistently by MTSET, but the disulfide reducing agent DTT was able to clearly reverse modification in only six of these.

Of the six mutants whose block could be reversed by DTT, four bore cysteine substitutions at the more superficial +1 or +2 positions in motifs II, III, and IV, and the other two bore cysteine substitutions at positions 0 and −1 of motif IV. Examples of reversibility with 2 mM DTT for four +1 and +2 position substitution mutants are shown in Fig. 6. In these mutants,
DTT was able to largely and relatively rapidly reverse persistent block by MTSET\(^1\).

In addition to the +1 position mutant in motif IV, the 0 and −1 position mutants in this motif were also susceptible to DTT reversal of MTS block. The bottom panels of Fig. 4 showed that persistent MTSEA\(^1\) block of E1446C (motif IV) could be slowly reversed by DTT, and similar results were obtained for MTSET\(^1\) and MTSES\(^2\). MTSEA\(^1\) and MTSET\(^1\) modification of the adjacent −1 position, G1445C, could also be reversed by DTT, as evidenced by the reversal of block in this mutant during DTT application (not shown). MTSES\(^2\) did not modify this position and therefore showed no reversal with DTT. It is unclear why DTT has a greater ability to reverse MTS block in motif IV, although one possibility is that this motif is somewhat more externally disposed than are the others.

**DISCUSSION**

The main finding in this work is that the side chains of the EEEE locus glutamate residues are likely to project into the aqueous pore lumen. Side chains of all residues at the +1 and −1 neighboring positions also project into the pore lumen, as do at least some −2 and +2 position side chains. Luminal orientation of the glutamate side chains is congruent with the idea that EEEE locus carboxylate groups form the ion-binding locus of the \(\text{Ca}^{2+}\) channel selectivity filter. These results and those of other workers (11–13) indicate that the protein fold of the selectivity filter in voltage-gated \(\text{Ca}^{2+}\) channels is distinctly different from that of a bacterial \(\text{K}^+\) channel, the only member of the superfamily of voltage-gated ion channels for which a crystal structure has yet been obtained. The strength of these findings and their interpretation are considered below.

**Sensitivity of WT to MTS Reagents—**

MTS reagent block of the WT channel was reversible with washout, indicating that this block did not result from covalent modification of endogenous cysteine residues. The mechanism of reversible MTS block of WT channels described here is unknown. Possibilities include block by protons released as MTS reagents hydrolyze in solution, physical block of the pore by noncovalent MTS binding, or noncovalent MTS action elsewhere on the channel resulting in modified gating. In any case, the fact that MTS block of the WT channel was reversible, along with the small magnitude of this block (−11%), combined to make identification of covalent action of MTS reagents on cysteine substitution mutants straightforward.

In contrast to the findings reported here, it has been previously reported that sulfhydryl-modifying agents inhibit WT
Fig. 6. MTSET\(^+\) block was reversed by DTT in some mutants. A, superimposed Ba\(^{2+}\) current records illustrating the reversibility by DTT of MTSET\(^+\) block in D737C, W738C, W1147C, and A1447C. Control currents (con) were acquired first, and then MTSET\(^+\) was applied at 1 ms for 2 min, and wash records were acquired 5 min after MTSET was washed from the bath. DTT (2 ms for 5–10 min) was applied last, after the 5-min wash period. B, examples of the time courses of MTSET\(^+\) and DTT action. Peak inward Ba\(^{2+}\) currents were acquired every 15 s and are plotted as circles. Filled circles correspond to the records in A. Bars mark the periods during which MTSET\(^+\) or DTT were applied. Holding potential, ~80 mV; test potential, +20 mV.

\(\alpha_{1C}\)-based channels. In transfected HEK 293 cells, it was found that treatment with sulfhydryl-oxidizing agents reduced WT \(\alpha_{1C}\) current (48) and that MTSEA\(^-\), but not MTSET\(^+\) or MTSSES\(^-\), blocked ~30–45% (49, 50) of WT \(\alpha_{1C}\) current. Testing our WT combination of channel subunits (\(\alpha_{1C}\beta_{2A}\alpha_{2A}\alpha_{3}\)) in HEK 293 cells, we too have found that MTSEA\(^-\) but not MTSET\(^+\) persists persistently and effectively (~90%) blocks WT \(\alpha_{1C}\) channels (data not shown). In our experiments, block by extracellularly applied MTSEA\(^-\) was prevented by inclusion of a scavenging thiol, 15 mM cysteine (47), in the whole cell patch pipette, which indicates that MTSEA\(^-\) modifies WT \(\alpha_{1C}\) channels in HEK 293 cells from the intracellular side of the membrane. In comparing HEK 293 cells with Xenopus oocytes, perhaps the much smaller surface-to-volume ratio of the oocytes allows for a much more effective action of endogenous scavenger thiols so that \(\alpha_{1C}\) channels are little affected by MTSEA\(^-\) that crosses the oocyte membrane.

**MTS Access to the Narrow Region of the Pore**—Whether MTS reagents will block current through the pore of a cysteine substitution mutant is dependent upon several factors in addition to the accessibility of cysteine thiols. For example, in narrow regions of the pore, MTS reagents may be too large in diameter to reach and modify exposed cysteines, whereas in wider pore regions, attachment of MTS reagent headgroups to exposed cysteines may not obstruct ion flux. Based on the cut-off size for permeability of small organic cations, the minimum pore diameter has been estimated as ~6 Å for the skeletal muscle Ca\(^{2+}\) channel (51). The largest diameter organic cation that passes through the skeletal muscle channel is tetramethylammonium, and this cation has also been found to permeate the WT \(\alpha_{1C}\) channel studied here (not shown). MTS reagents are cylindrical molecules that in some instances can pass lengthwise through ion channel pores. For the MTS reagents, the sulfonyl group common to MTS reagents is the widest part of MTSEA\(^-\) and MTSSES\(^-\), at 4.8 Å diameter, and the trimethylammonium headgroup is the widest part of MTSET\(^+\), at 5.8 Å. Based on size considerations alone, MTSEA\(^-\) and MTSSES\(^-\) would be expected to access the narrowest region of the pore, and MTSET\(^+\) would also just fit into the narrow region.

**Differing Accessibilities of MTS Reagents**—For each of the three MTS reagents tested, block of position 0 (Glu \(\rightarrow\) Cys) substitution mutants was greater than for other pore positions. As detailed under “Results,” additional similarities in patterns of fractional block are also evident among the three MTS reagents (Fig. 5). Equally clear are the differences among the block patterns of the various MTS reagents. These differences in patterns of block raise two questions: (i) For a given MTS reagent, why does fractional block differ among the mutants? (ii) For a given mutant, why does fractional block differ among the three MTS reagents?

The simplest interpretation of the differences in degree of block among the mutants is that some residues are more accessible to MTS reagents than are others. Absence of persistent block, for example, could indicate an inaccessible side chain. Among mutants that are persistently blocked by MTS reagents, their differing degrees of MTS block might be interpreted as indicating differences in diameter along the pore axis; the 0 position glutamates exhibit nearly full block because position 0 is the narrowest part of the ~2 to +2 region, and neighboring ±1 positions exhibit only partial block because ion flux is slowed but not fully blocked by MTS headgroup attachment in these hypothetically wider segments of the pore. This is an attractive interpretation in that it fits with the idea that no region could be narrower than that formed by the 0 position glutamates because this part of the pore (the EEEE locus) strips permeant ions down to a dehydrated diameter. However, this architectural interpretation is probably only part of the explanation because steady-state block does not necessarily report accurately on side chain accessibility; as outlined above, absence of block does not prove that the particular side chain thiol was inaccessible nor that it was unmodified by MTS reagent. Further, partial block may variously result from full modification of an exposed thiol that produces only partial obstruction of ion flux or from incomplete modification of a poorly accessible thiol. However, focusing on those mutants with large fractional block values, we conclude that at least the 0 and ±1 positions project side chains into pore.

What are the origins of the large differences among MTS reagents in fractional block of a given cysteine substitution mutant? Concerning the two cationic reagents MTSEA\(^-\) and MTSET\(^+\), MTSEA\(^-\) is smaller in diameter and membrane-permeant, properties that may allow this latter reagent to penetrate deeper into the pore or alternatively to enter the pore from the cytoplasmic side of the membrane (47). Either of these differences could be responsible for the unique ability of MTSEA\(^-\) to block all −1 mutants and one −2 mutant. In regard to differences with MTSSES\(^-\), this reagent is an anion and so was not expected to easily penetrate a highly cation-selective channel possessing a cluster of negatively charged carboxylates in its selectivity filter; presumably, the anionic nature of MTSSES\(^-\) accounts for the fact that block was restricted to the more external cysteine substitution mutants (0 to +2). The EEEE
locus is very close to the extracellular pore mouth (9, 10, 46), so it may be that MTSES' -backs into the pore, leaving its sulfonate moiety in the extracellular solution. The sulphhydril-reactive sulfur of MTSES' is in the middle of the molecule, 5 Å distant from the anionic sulfonate of the headgroup.

A final question of accessibility concerns the inability of DTT to reverse persistent block produced by MTS action at deeper pore positions (−2, −1, and 0 positions). This molecule fits into a cylinder of 4 Å diameter and is therefore expected to be able to reach any part of the pore. DTT was able to reverse MTS block in some cases when modification occurred at the external entrance positions (+1, +2, and one 0 position). The inability of DTT to reverse persistent block by MTS action at deeper pore positions may reflect its limited accessibility in mutant pores bearing a covalently attached MTS reagent headgroup. It is also possible that DTT, a neutral molecule, cannot enter the deeper pore whether the pore is MTS-modified or not.

**Structure of the Ca2⁺ Channel Selectivity Filter**—When the EEEE locus was first identified as the core of the Ca2⁺ channel selectivity filter, it seemed likely that the side chain carboxylates of the glutamates, and not their main chain carbonyls, projected into the pore lumen to form an oxygen-based anionic binding site for divalent metal cations. This thinking was influenced by the known structures of divalent metal ion-binding sites in organic chelators like EGTA and in protein motifs like EF-hands. In EGTA-like chelators, four carboxylates combine to form the divalent metal ion-binding site. In EF-hand sites, side chain carboxylate groups provide many of the essential coordinating oxygen atoms. In both of these cases, Ca2⁺ is coordinated within a pocket of seven (EF-hand) or eight (EGTA) coordinating oxygen atoms (52, 53). The examples of the tetracarboxylate chelators and EF-hand structures conform to the general principle that charged binding sites preferentially bind multivalent metal ions (52). Neutral binding sites such as those formed by crown ethers exhibit little discrimination among metal ions based on ion valence. The charge selectivity of tetracarboxylate sites is thought to derive from the ability of the high positive charge density of divalent cations to overcome electrostatic repulsion between the anionic carboxylate oxygens, thereby allowing close-packed, 8-fold coordination of Ca2⁺. In contrast, monovalent cations lack the charge density needed to stabilize close packing of coordinating carboxylate oxygen atoms and thus have low binding affinity in such charged sites. Perhaps a similar effect is involved in the selectivity of Ca2⁺ channels for Ca2⁺ ions (radius 0.99 Å) over similar-sized Na⁺ ions (0.95 Å), which is the crucial accomplishment of Ca2⁺ channel pores in a physiological setting.

**Selectivity Filter Structure in the Superfamily of Voltage-gated Ion Channels**—Voltage-gated K⁺ channels and cyclic nucleotide-gated channels appear to have evolved from a K⁺-selective antecedent (54, 55). In turn, the voltage-gated K⁺ channels are surmised to have given rise to voltage-gated Na⁺ channels and from these evolved the voltage-gated Na⁺ channels. How have the forces that drove this evolution affected selectivity filter structure in this ion channel superfamily?

Ca2⁺ channels alone share the exceptionally high ion selectivity of K⁺ channels. Despite this similarity in performance, their selectivity filter structures differ in a key way, so that K⁺ channels use main chain carbonyl oxygen atoms to coordinate K⁺ ions, whereas Ca2⁺ channels use side chain carboxylate oxygen atoms to coordinate Ca2⁺ ions. Apparently, evolutionarily pressure has driven Ca2⁺ channel pore structure to diverge from that of K⁺ channels such that selectivity filter residues in Ca2⁺ channels have become rotated by 180° around the axis of the peptide chain.

In comparison with Ca2⁺ and K⁺ channels, Na⁺ channels are less selective, exhibiting, for example, a slight permeability to Ca2⁺ (55). The side chains in the Na⁺ channel selectivity filter project into the aqueous pore (34, 36), a pattern that may be derived from the pore structure of an ancestral Ca2⁺ channel. The selectivity filter of Na⁺ channels bears fewer carboxylates than does the corresponding structure in Ca2⁺ channels, which may be important in allowing monovalent Na⁺ ions to stabilize close-packed Na⁺-coordinating oxygen atoms.

Cyclic nucleotide-gated channels possess an intermediate kind of selectivity: they are permeated by monovalent cations (Na⁺ and K⁺) and, albeit more slowly, by divalent cations (Ca2⁺ and Mg2⁺). At pore-lining positions homologous to the EEEE locus of Ca2⁺ channels, cyclic nucleotide-gated channels also bear glutamate residues, and as in Ca2⁺ channels, the side chains of these residues are likely to project into the pore lumen (56). This structure is in accord with the strong preference of tetracarboxylate-based binding sites for divalent over monovalent cations; owing to tighter binding, Ca2⁺ and Mg2⁺ transiently block monovalent current through cyclic nucleotide-gated channels. In sum, K⁺ channels seem to be the only members of the voltage-gated ion channel superfamily that use main chain carboxyl oxygen atoms to select for permeant ions in their selectivity filters, with all other superfamily members using side chain carboxylates or other side chain moieties for this purpose.

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