Side-Chain Accessibilities in the Pore of a K^+ Channel Probed by Sulfhydryl-Specific Reagents after Cysteine-Scanning Mutagenesis

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ABSTRACT To gain insight into the secondary structure of the ion conduction pathway of a voltage-gated K^+ channel, we used sulfhydryl-specific reagents of different diameters to probe amino acid side-chain accessibilities in the pore of the channel after cysteine-substitution mutagenesis. We identified five positions at which modified amino acid side chains are accessible from the aqueous lumen of the external channel vestibule. Covalent coupling of the 2-trimethylammonium-thioethyl group to cysteine thiols leads to position-dependent current reduction, suggesting a gradual narrowing of the pore. The fact that the modified side chains of two adjacent amino acids are reactive is not compatible with the ion conduction pathway forming a regular β-pleated sheet at these positions. The smaller thiol reagent Cd^{2+} reacts with modified side chains that are also accessible to the larger (*2-trimethylammoniumethyl)methanethiosulfonate (MTSET). Our results imply that the outer vestibule of a potassium-selective ion channel narrows over a short distance of three amino acids near a position where a regular β-structure is unlikely.

INTRODUCTION

Potassium (K^+) channels are ubiquitous membrane proteins that are important for the fine tuning of electrical properties in excitable cells. Each subunit of a tetrameric K^+ channel contains a conserved hydrophobic domain with six putative β-helical, membrane-spanning segments, S1–S6 (reviewed in Miller, 1991; Jan and Jan, 1992; Joho, 1992; Catterall, 1993). The region between S5 and S6 was shown to form a major part of the ion conduction pathway (MacKinnon and Yellen, 1990; Hartmann et al., 1991; Kavanaugh et al., 1991; Yellen et al., 1991; Yool and Schwarz, 1991; Kirsch et al., 1992). The side chains of several amino acids after S5 and before S6 are involved in K^+ channel block by tetraethylammonium (TEA) from the outside. Some residues located approximately in the middle of the S5–S6 linker region influence channel block by TEA from the inside and modify conductance properties. A synopsis of currently available data has led to several working models for the ion channel pore. Most models propose that residues after S5 and before S6 are located on the outside, and that part of the S5–S6 linker region penetrates the plane of the membrane, in some models by folding into two antiparallel β-strands (Fig. 1 *A*). In a tetrameric channel (MacKinnon, 1991), each subunit would contribute a short, β-pleated sheet to the ion conduction pathway, forming a structural motif reminiscent of an eight-stranded β-barrel (Bogusz and Busath, 1992; Durell and Guy, 1992). Recently, it has been shown that some residues in the cytoplasmic S4–S5 linker and part of the S6 segment influence conductance properties and channel block by intracellularly applied TEA, implying that parts of S4–S5 and S6 also contribute to the ion conduction pathway and to the inner mouth of the channel (Choi et al., 1993; Slesinger et al., 1993; Lopez et al., 1994). Although these studies have led to the identification of channel regions involved in ion permeation, there is no structural information demonstrating which amino acid side chains may directly line the ion conduction pathway and, perhaps, be involved in determining the channel's ion selectivity or in conformational changes associated with channel gating. To gain insight into secondary structure motifs of the pore-forming region, we used sulfhydryl-specific reagents to monitor side-chain accessibilities in the ion conduction pathway after cysteine-substitution mutagenesis.

MATERIALS AND METHODS

Reagents

Initial experiments were done with (2-trimethylammoniumethyl)methanethiosulfonate bromide (MTSET) kindly provided by Drs. A. Karlin and D. A. Stauffer (Columbia University). For subsequent experiments, MTSET was synthesized as described by Stauffer and Karlin (1994). Oocytes, clamped to −80 mV, were superfused with ND96 containing the appropriate concentration of MTSET. A fresh stock solution of 100 mM MTSET in ice-cold water was diluted into ND96 immediately before each experiment. Stock solutions of 100 mM $CdCl_2$ (Sigma Chemical Co., St. Louis, MO) in ND96 and 100 mM dithiothreitol (Boehringer Mannheim, Mannheim, Germany) in water were stored at 4 and −20°C, respectively, and freshly diluted into ND96 before each experiment. Currents were elicited at 20-s intervals by 400-ms test pulses to potentials between +10 and +40 mV, followed by 100 ms at −30 mV before returning to −80 mV.

Site-directed mutagenesis, RNA synthesis, and electrophysiological recording

All mutants were generated, and RNA was synthesized as described (Zühlke et al., 1994, 1995). Approximately 150 pg in vitro-synthesized cRNA was injected into *Xenopus* oocytes, and 1–4 days after injection the cells were subjected to a standard two-electrode, voltage-clamp protocol (VanDongen et al., 1990). Experiments were done in ND96 at room temperature (22 ± 1°C). Both voltage-sensing and current-passing electrodes were filled with 3 M KCl and had resistances of 0.2–1.0 MΩ. The pClamp system was used to generate the voltage pulse protocol and for data acquisition. Signals were

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filtered at 500 Hz and digitized at 1–2 kHz. Linear capacitive and leakage currents were subtracted on line using a P/4 protocol. Membrane potentials were not corrected for series resistance errors.

Statistical analysis of results

Differences of current reduction were tested for statistical significance ($p < 0.05$) by single-factor ANOVA. The Tukey test was applied to determine which mutants were significantly different from wild type ($p < 0.05$).

RESULTS

Twenty-eight positions in the pore region between S5 and S6 of Kv2.1 (Frech et al., 1989), from K356 to T383, were each mutated to a cysteine (Fig. 1 *A*). After expression of mutant K^+ channels in *Xenopus* oocytes, accessibilities of cysteine side chains were probed by superfusion with the membrane-impermeant, sulfhydryl-specific reagent MTSET (Stauffer and Karlin, 1994). MTSET forms a mixed disulfide with a cysteine side chain provided the SH-group is accessible to the reagent. Covalent addition of the $-SCH_2CH_2N(CH_3)_3^+$ group may interfere with ion permeation if the modified side chain is in or near the ion conduction pathway as has been shown for the nicotinic acetylcholine receptor (Akabas et al., 1992). Current reduction, therefore, may be interpreted as structural evidence that the side chain of the modified amino acid faces the lumen of the pore in a region that is narrow enough to effect the passage of ions after covalent coupling of at least one positively charged molecule of approximately 6×8 Å.

After injection of *Xenopus* oocytes with similar amounts (~150 pg/oocyte) of cRNA, 14 of the 28 cysteine-substitution mutants expressed functional channels with macroscopic current levels similar to wild-type Kv2.1 (Fig. 1 *B*). Midpoints of activation and slope factors for most mutants, as well as K^+ versus Na^+ selectivities (data not shown), were similar to Kv2.1 with some possible changes detectable in I369C and T372C (Table 1). This suggests that the overall functional and structural integrity of the mutant channels was preserved and that the strategy of probing cysteine side-chain accessibilities may yield valuable structural information. Fig. 1 *A* shows a hypothetical arrangement of the amino acids between S5 and S6 of Kv2.1. Shaded positions indicate cysteine-substitution mutants expressing K^+ current levels of similar magnitude like wild-type Kv2.1. Many, but not all, of the functional substitutions are at positions at which amino acid variations are found in different members of the voltage-sensitive K^+ channel family. Most of the nonfunctional channel mutants are at conserved positions or in the deep pore thought to be closely involved in ion permeation (Yool and Schwarz, 1991; Heginbotham et al., 1992; Kirsch et al., 1992a, b).

We studied the effect of extracellularly applied MTSET on all 14 functional cysteine-substitution mutants. The Y380C mutation in Kv2.1 was a good candidate to test accessibility of the mutated side chain because the equivalent position in the *Shaker* B channel (threonine 449), when mutated to a tyrosine, had been shown to render the channel highly sensitive to TEA block from the outside (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992). Superfusion of oocytes expressing Y380C mutant channels with 3 mM MTSET led to a rapid decrease in peak current. At saturating concentrations, current amplitudes were reduced to approximately one-third of the initial values (Fig. 2). In agreement with the expectation that the cysteine side chain was covalently modified, washout of the reagent did not reverse the effect. Superfusion with 1 mM dithiothreitol (DTT), however, restored current amplitudes close to the level present at the beginning of the

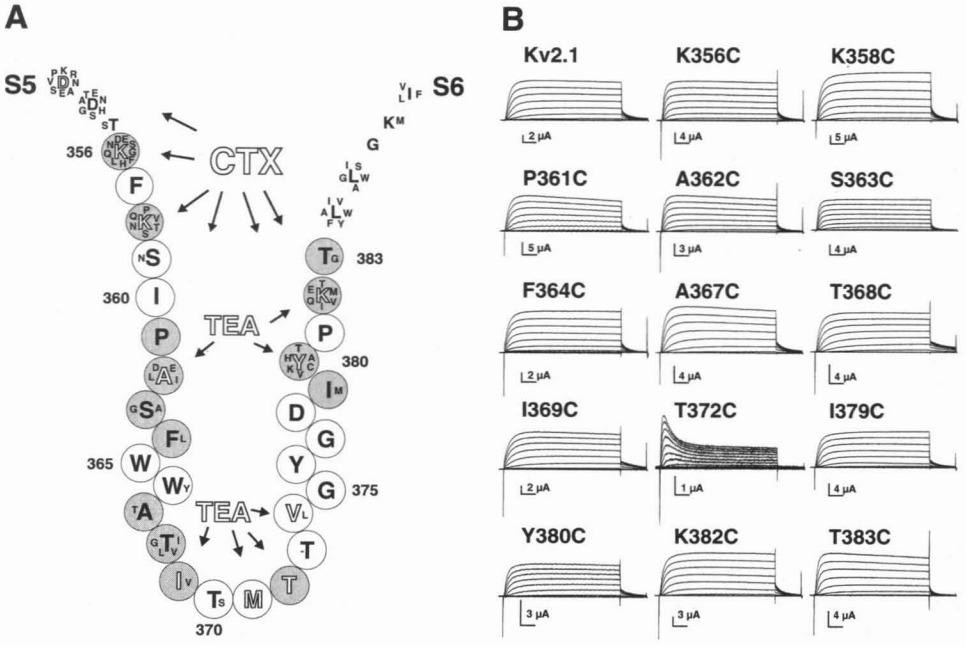

FIGURE 1 (*A*) Hypothetical pore structure of a voltage-gated K^+ channel. Large letters represent amino acids in the S5–S6 region of Kv2.1 (Frech et al., 1989). Small letters indicate residues found in other known voltage-gated K^+ channels. Positions involved in block by charybdotoxin (CTX), external TEA (*top*), and internal TEA (*bottom*) are highlighted (reviewed in Joho, 1992). Circles indicate positions that were mutated to cysteines; mutants identified by filled circles led to K^+ current amplitudes comparable with wild-type Kv2.1. (*B*) Current traces of Kv2.1 and 14 cysteine-substitution mutants. K^+ channel-expressing oocytes were held at −80 mV, and test pulses of 400-ms duration were applied in 10-mV increments up to +40 mV, followed by 80 ms at −30 mV before returning to the holding potential. The unlabeled scale bar indicates 50 ms.

TABLE 1 Midpoints of activation and slope factors

Mutant	$E_{0.5}$ (mV)	k (mV)	N
WT	1.8 ± 5.2	11.2 ± 1.8	6
K356C	−1.7 ± 5.0	10.5 ± 2.5	6
K358C	6.3 ± 4.2	13.4 ± 1.3	6
P361C	8.3 ± 3.9	13.7 ± 1.9	7
A362C	8.7 ± 4.0	15.0 ± 2.7	4
S363C	6.8 ± 4.2	13.5 ± 1.9	5
F364C	8.4 ± 4.4	13.7 ± 2.9	5
A367C	3.8 ± 2.9	11.0 ± 1.5	7
T368C	−0.4 ± 1.5	11.6 ± 1.2	4
I369C	13.6 ± 10.9	14.6 ± 5.1	4
T372C	17.5 ± 4.6	13.9 ± 2.0	6
I379C	0.4 ± 2.9	10.9 ± 0.9	6
Y380C	2.0 ± 2.2	11.5 ± 2.2	7
K382C	3.5 ± 3.6	11.0 ± 2.0	6
T383C	9.3 ± 6.5	12.2 ± 2.8	6

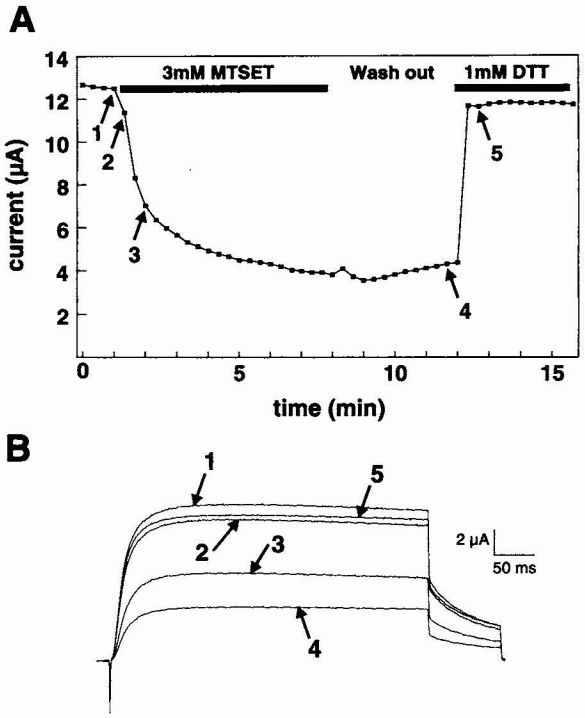

FIGURE 2 Current reduction after superfusion with MTSET. (*A*) An oocyte expressing the mutant Y380C was held at −80 mV and pulsed every 20 s to +40 mV for 400 ms (*arrow* 1). The oocyte was superfused with 3 mM MTSET (*arrows* 2 and 3) for several minutes. The current amplitude is reduced within seconds and remains low after washout of the reagent (*arrow* 4). Superfusion with 1 mM DDT reverses the effect rapidly (*arrow* 5). (*B*) Current traces recorded at the times indicated by the arrows in *A*.

experiment (Fig. 2). Current traces did not change in shape, but current amplitudes decreased between test pulses, suggesting that MTSET reacts with the modified side chain during the 20-s interpulse intervals when the channel is in the closed state.

To compare maximal reduction of K^+ currents for different cysteine-substitution mutants, it was important to determine a dose-response relationship. Micromolar concentrations of MTSET led to significant current reduction, and 1 mM MTSET provided saturating conditions (Fig. 3). To test

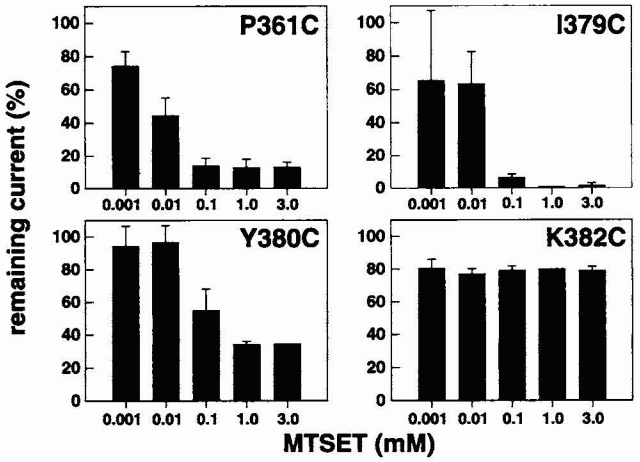

FIGURE 3 Dose-response relationship for four MTSET-reactive Kv2.1 mutants. Oocytes expressing Kv2.1 mutants were held at −80 mV and pulsed every 20 s for 400 ms to potentials between +10 mV and +40 mV. Oocytes were superfused for 5 min with the indicated concentrations of MTSET in ND96. The fraction of remaining peak current is shown (mean ± SD; n = 2–6 oocytes, except where error bar is not present).

side-chain accessibilities in the 14 functional cysteine-substituted channels, all subsequent experiments were performed with saturating concentrations of 1–3 mM MTSET. Only four mutants showed obvious current reduction (Fig. 4). K^+ currents in the mutants P361C, I379C, Y380C, and K382C are significantly reduced by 88 ± 2, 99 ± 1, 67 ± 6, and 22 ± 3%, respectively (mean ± SD; $n \geq 3$ oocytes). Although mutant K356C showed only 11 ± 3% current reduction (not statistically significant), the effect was reproducible (n = 5 oocytes) and could only but consistently be reversed by addition of 1 mM DTT. Moreover, this mutant

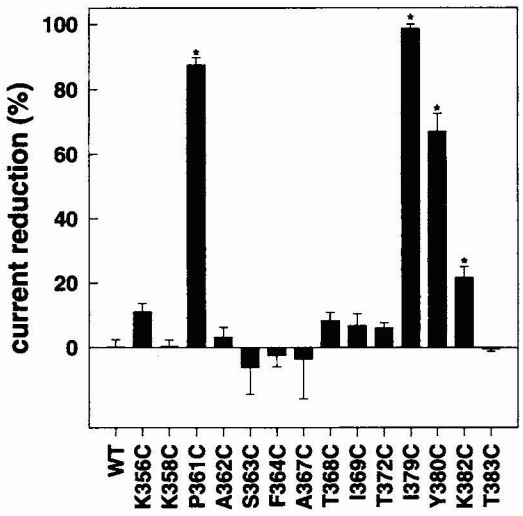

FIGURE 4 Effect of MTSET on 14 cysteine-substitution mutants in the pore of Kv2.1. Oocytes were superfused for 5 min with saturating concentrations of MTSET (1–3 mM) in ND96. MTSET effects were measured as described in Fig. 3. Percent reduction of K^+ current (in %) is shown for each mutant (mean ± SD; n = 2–7 oocytes; *significantly different from wild type at $p < 0.05$).

was clearly sensitive to Cd^{2+} (see below) and Zn^{2+} (data not shown). We believe, therefore, that the side chain at position 356 is accessible. The remaining mutants and wild-type Kv2.1 did not show any significant effects (Fig. 4). The thiol groups at these positions may not face the lumen of the channel pore because they are buried in the interior of the protein. Alternatively, the ion conduction pathway may be too narrow for the membrane-impermeant MTSET molecule to penetrate and reach a reactive thiol group when applied from the outside, or some side chains may have reacted with MTSET, yet we were unable to detect any K^+ current reduction because the mutated position is not close enough to the pore to interfere with ion permeation. Finally, an exposed thiol group may not generate a reactive thiolate anion and participate in disulfide formation because its pK_a is too high.

To study whether the reactive cysteine side chains show state-dependent accessibility, we applied 1 mM MTSET for 5 min in the absence of depolarizing test pulses. After complete washout of the reagent, we observed the same reduction of current amplitudes for K356C, P361C, I379C, Y380C, and K382C as observed in the presence of test pulses, suggesting that the mutated side chains at these positions were indeed accessible while the channel was in the closed state (Fig. 5; data not shown for K356C and K382C). We tested reversibility of the MTSET effect on the three mutants with the highest degree of inhibition. After superfusion of 1 mM DTT, we saw clear, position-dependent differences. For the Y380C mutant, 50% recovery was achieved in less than 20 s. In marked contrast, the mutants P361C and I379C recovered to 50% of the initial current levels after approximately 6 and 2 min, respectively (Fig. 5).

To probe the narrow region of the pore that may not be reached by MTSET, we used a smaller thiol reagent. Extracellular application of Cd^{2+} led to results that were in agreement with the ones obtained with MTSET. Mutants affected by MTSET showed also significant current reduction after extracellular application of 0.1 and 1.0 mM Cd^{2+} (Table 2). Particularly, mutant K356C, which showed only a marginal effect with MTSET, was significantly affected by as little as 0.1 mM Cd^{2+} (34% current reduction). Mutant T372C is inhibited by 35% in presence of 1 mM Cd^{2+}; however, unlike the other Cd^{2+}-sensitive mutants that are rapidly blocked and unblocked by addition and removal of 0.1 mM Cd^{2+}, the block of T372C takes several minutes to develop, requires 1 mM Cd^{2+} and cannot be reversed by washout of Cd^{2+} (data not shown). The crystal radius of Cd^{2+} is approximately 1 Å (Cotton and Wilkinson, 1980). Although the diameter of the unhydrated Cd^{2+} ion is less than one-third that of MTSET, we were unable to uncover additional positions in the deeper pore region, suggesting that no modified side chains are easily accessible to the smaller reagent from the outside.

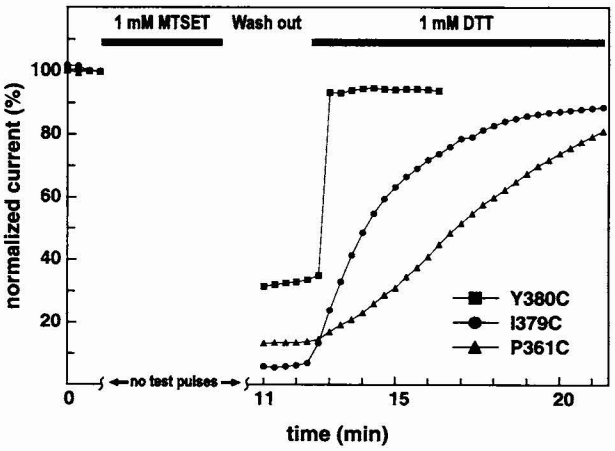

FIGURE 5 Amino acid side chains are accessible in the closed state. Oocytes were superfused for 5 min with 1 mM MTSET in ND96, followed by complete washout in ND96 before applying regular test pulses at 20-s intervals. After several pulses, 1 mM DTT in ND96 was applied, and the time course of recovery was monitored. All current amplitudes are normalized to the initial current values before application of MTSET.

DISCUSSION

The results presented here lead us to refine the secondary structure model for the external vestibule and part of the pore of the voltage-gated K^+ channel Kv2.1. The new working model provides some clues about atomic dimensions (Fig. 6). The tyrosines at position 380 in the four subunits are thought to provide a high affinity TEA binding site by forming an aromatic bracelet accommodating a TEA molecule 8 Å in diameter (Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992). Therefore, because of charge repulsion and this space constraint, we assume that only a single charged MTSET molecule (diameter 6 Å) may approach close to and covalently couple with one of the cysteines substituted for tyrosine at position 380. The effects of MTSET progressively increase from K382C to Y380C to I379C, suggesting that the ion conduction pathway becomes gradually narrower in this region. The fact that DTT reverses the effect of covalently modified cysteines at positions 361, 379, and 380 implies that there is still enough space for an uncharged cylindric

TABLE 2 Current reduction in percent of initial current amplitude after external application of Cd^{2+}

	0.1 mM $CdCl_2$		1.0 mM $CdCl_2$	
Mutants	(%)	N	(%)	N
WT	0.7 ± 3.0	5	8.1 ± 3.1	5
K356C	34.3 ± 5.7*	3	59.4 ± 4.9*	3
K358C	5.1 ± 5.0	2	3.0 ± 9.6	2
P361C	21.5 ± 8.5*	2	54.9 ± 24.1*	4
A362C	3.2 ± 5.0	7	9.9 ± 6.8	7
S363C	−0.2 ± 1.2	7	7.6 ± 3.1	6
F364C	2.6 ± 4.1	5	11.7 ± 6.1	6
A367C	1.9 ± 0.9	3	9.7 ± 2.3	3
T368C	0.4 ± 1.2	4	10.4 ± 4.3	4
I369C	−10.6 ± 6.6	3	−11.3 ± 13.5	3
T372C	6.8 ± 3.0	6	35.5 ± 10.5*	7
I379C	76.2 ± 15.4*	5	95.0 ± 5.2*	3
Y380C	27.8 ± 8.2*	4	94.9 ± 1.1*	3
K382C	22.1 ± 1.1*	2	39.6 ± 3.5*	2
T383C	7.5 ± 2.9	4	6.1 ± 1.8	2

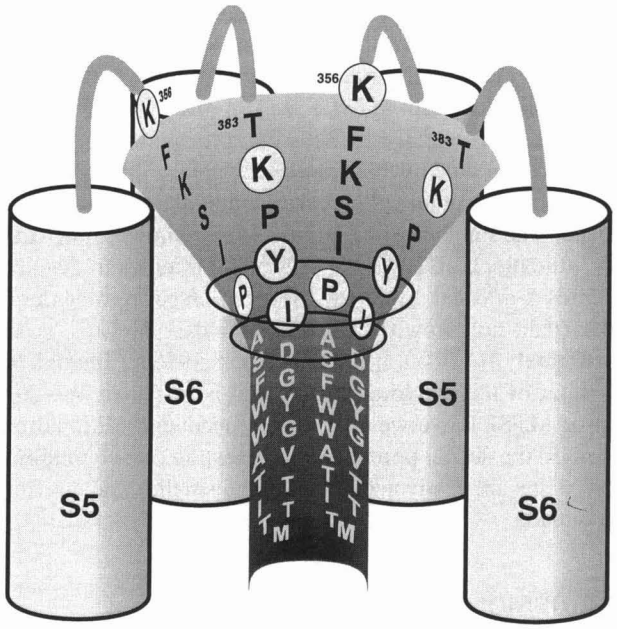

FIGURE 6 Side-chain accessibilities in the pore region of Kv2.1. Two of the four S5–S6 linker regions are shown together with the flanking S5 and S6 helices. Letters represent amino acids that have been mutated to cysteine. Circles indicate positions affected by MTSET. The pore gradually narrows from K382 to I379. The aromatic groups at the Y380 position have been postulated to form a TEA-binding bracelet to accommodate a molecule of 8-Å diameter (*top circle*) (Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992). At the I379 position, when mutated to a cysteine, the pore still allows access of at least one MTSET molecule with a diameter of 6 Å (*bottom circle*). None of the mutated positions located in the deep pore (*gray letters*) were found to react with MTSET in a way that affected ion permeation.

molecule with 4-Å diameter to have access to the bulky, covalently bound trimethylammonium-thioethyl group. It appears that attack on the disulfide end and removal of this group by DTT occur rapidly at the modified Y380C position but proceed approximately 20 and 6 times more slowly at the modified P361C and I379C positions, respectively. Although it is difficult to compare directly reaction rates of DTT with different disulfides in unknown chemical microenvironments, these findings support the results with MTSET, implying that the side chain at position 380 is more easily accessible from the aqueous environment of the outer channel vestibule than are the side chains at positions 361 or 379. We did not see similar kinetic differences during the onset of current reduction after application of MTSET.

Both side chains of the adjacent amino acids I379C and Y380C are accessible to MTSET and Cd^{2+}. This is not easily compatible with a model in which the pore is formed by a regular β-strand at these positions. The P361C mutant shows an MTSET effect intermediate between the effects seen for the I379C and Y380C mutants. Proline 361 is next to alanine 362, a position in *Shaker* that influences TEA block from the outside (MacKinnon and Yellen, 1990). Again, because of space constraints and charge repulsion, reaction with a single subunit may lead to current reduction. This suggests that the proline 361 is juxtaposed to isoleucine 379 and tyrosine 380 on the descending limb of the hairpin structure. It is remarkable that at three positions after proline 361 none of the mutated side chains show an effect with MTSET or Cd^{2+}. If the ion conduction pathway were in a β-strand conformation in this region and wide enough for the reagents to have access, one would expect at least one of the cysteine-substituted mutants to react. If it were in an α-helical conformation, reaction with the first or third residue after proline 361 might occur. The fact that none of the residues appear to be affected, either by MTSET or by Cd^{2+}, is in agreement with a model of a funnel-shaped outer vestibule that becomes too narrow in this region for these cations to penetrate, unless the reactive sites were only accessible in the open state but could not be reached because of an unfavorable voltage gradient or opposing K^+ ion flux. Our data, however, do not exclude the possibility that these residues are buried in the interior of the protein and that they do not form part of the ion conduction pathway. A mutant channel affected by Cd^{2+} but not by MTSET is T372C. Significant current reduction, however, is only apparent at 1 mM Cd^{2+}, takes minutes to develop, and is irreversible even in the presence of 1 mM DTT (data not shown). Currently, we do not know whether this slow effect is different in nature from the rapid block seen with the other Cd^{2+}-sensitive mutants, or whether Cd^{2+} ions have slow access to the deep pore and bind extremely tightly to the thiols at position 372. Although Cd^{2+} may not penetrate more deeply into the pore, the larger K^+ ion is still permeant. Because of the large enthalpy of hydration, the hydrated diameter of Cd^{2+} is substantially larger than that of K^+. The observation that the small Cd^{2+} ion interferes with ion permeation as efficiently as the considerably larger MTSET molecule most likely reflects differences in binding stoichiometry or valence.

The *Shaker* B mutant T449C has a much higher affinity for Cd^{2+} in the C-type inactivated state than in the open state or closed state, indicating some structural change between the open and the inactivated states of the *Shaker* K^+ channel (Yellen et al., 1994). We could not detect a similar Cd^{2+} effect on the Y380C mutant of Kv2.1 (data not shown). The outer mouths of *Shaker* and Kv2.1 are not identical, and some sequence differences may cause different state-dependent affinities for Cd^{2+}. In a recent report, a histidine substitution at position 369 in a deletion variant of Kv2.1 was shown to render the channel sensitive to Zn^{2+} from the outside, and the authors proposed that residue 369 is located at a surface position near the outer channel mouth (De Biasi et al., 1993). In our hands, the mutant I369C is sensitive neither to externally applied Cd^{2+} (Table 2) nor to 1 mM Zn^{2+} (data not shown). It is difficult to explain this apparent discrepancy. A reason may be that the I369H mutant was made in a truncated channel variant of Kv2.1 lacking 318 amino acids but compared with the full length wild-type Kv2.1 rather than the truncated Kv2.1 with isoleucine at position 369 as the appropriate control channel.

In summary, we used several sulfhydryl-specific reagents in conjunction with cysteine-scanning mutagenesis to probe

side-chain accessibilities in the outer mouth of the voltage-gated K^+ channel Kv2.1 (DRK1). We identified five positions at which amino acid side chains are accessible to MTSET and Cd^{2+}. These results provide structural evidence that side chains at these positions face the aqueous lumen in the outer vestibule of the ion conduction pathway.

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