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Dynamic Rearrangement of the Outer Mouth of a K^+ Channel during Gating

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Summary

With prolonged stimulation, voltage-activated K^+ channels close by a gating process called inactivation. This inactivation gating can occur by two distinct molecular mechanisms: N-type, in which a tethered particle blocks the intracellular mouth of the pore, and C-type, which involves a closure of the external mouth. The functional motion involved in C-type inactivation was studied by introducing cysteine residues at the outer mouth of Shaker K^+ channels through mutagenesis, and by measuring state-dependent changes in accessibility to chemical modification. Modification of three adjacent residues in the outer mouth was 130–10,000-fold faster in the C-type inactivated state than in the closed state. At one position, state-dependent bridging or disulfide crosslinking between subunits was also possible. These results give a consistent picture in which C-type inactivation promotes a local rearrangement and constriction of the channel at the outer mouth.

Introduction

In response to prolonged changes in transmembrane potential, voltage-activated ion channels undergo specific conformational changes called inactivation gating that restrict access to the ion selective pore. In voltage-activated K^+ channels, inactivation gating occurs by two distinct molecular mechanisms. N-type inactivation occurs by a "ball and chain" process in which a tethered blocking particle occludes the inner mouth of the channel pore (Hoshi et al., 1990; Zagotta et al., 1990). C-type inactivation proceeds by a different mechanism, involving the outer mouth of the pore (Choi et al., 1991; Yellen et al., 1994). C-type inactivation can occur alone (Grissmer and Cahalan, 1989), or in combination with N-type inactivation (Hoshi et al., 1991; Pardo et al., 1992).

In Shaker K^+ channels, C-type inactivation can be studied in isolation by removing the N-type inactivation particle at the N-terminus of the protein (through the deletion Shaker H4:Δ6–46, which, for simplicity, is referred to as ShakerΔ here [Hoshi et al., 1990; Hoshi et al., 1991]). The rate of C-type inactivation in such ShakerΔ channels is decreased by external tetraethylammonium (TEA) (Choi et al., 1991) or by external K^+ (López-Barneo et al., 1993; Baukrowitz and Yellen, 1995), as if the binding of TEA or K^+ to an ion site at or near the outer mouth prevents C-type inactivation.

C-type inactivation can also produce large changes in the binding of Cd^{2+} to an engineered binding site in the outer mouth of the channel. When a cysteine is introduced in all four subunits at position 449 in the outer mouth, the channel binds Cd^{2+} extremely well in the inactivated state, but much less well (45,000-fold) in the open state (Yellen et al., 1994). This result suggests that C-type inactivation might change the Cd^{2+} affinity by either of two (nonexclusive) structural changes: it might increase the accessibility of the cysteine side chains to the aqueous phase containing Cd^{2+}, or it might narrow the pore and bring the cysteines close together, forming a high affinity Cd^{2+} binding site.

To investigate the nature of the conformational change that occurs during C-type inactivation, we used the method of cysteine substitution mutagenesis and chemical modification. We introduced a cysteine at one of a series of positions in the outer mouth, and then assessed the rate of modification of this cysteine by a water-soluble sulfhydryl reagent in different gating states. The general approach of using chemical modification to determine the accessibility of introduced cysteines has been used for bacteriorhodopsin (Altenbach et al., 1990), lactose permease (Sahin Toth and Kaback, 1993), bacterial chemoreceptors (Falke et al., 1988), and now for many channel proteins (Akabas et al., 1992; Xu and Akabas, 1993; Stauffer and Karlin, 1994; Pascual et al., 1995; Yang and Horn, 1995; Kürz et al., 1995; Lü and Miller, 1995). In addition to providing information for building static structural models, the method can also be used to learn about the functional motions of proteins. Recent work on Na^+ channels indicates that the S4 sequence, which has long been implicated in voltage-dependent gating, probably moves when the channel is depolarized (Yang and Horn, 1995; Yang et al., 1996). The related method of disulfide trapping with introduced cysteines has been used to give dynamic information about flexibility of the D-galactose receptor protein, whose crystal structure is known (Falke and Koshland, 1987; Careaga and Falke, 1992).

We present evidence that during C-type inactivation, there is a structural rearrangement of the outer mouth of the channel protein, resulting in the exposure of side chains at three consecutive positions and a close apposition of the four subunits. This probably reflects a collapse of the pore structure, but this collapse may be rather local because peptide toxins can still bind well to the outer mouth. The very large state-dependent changes in cysteine accessibility show that the cysteine mutagenesis and modification method can be very sensitive to the conformation of the protein.

Results

State-Dependent Modification of Cysteines at Three Positions in the Outer Mouth

We substituted cysteines at amino acid positions 447–451 (one position at a time) in the outer mouth of the Drosophila ShakerΔ K^+ channel. As shown in Figure 1, these residues form part of the outer mouth of the pore. At two of these positions, substitution of cysteine at all four subunits resulted in either the loss of channel

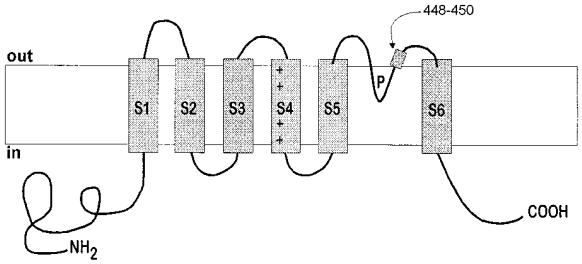

Figure 1. Schematic of the Putative Transmembrane Folding Pattern of a Shaker K^+ Channel Subunit

The S5-S6 linker is thought to dip into the membrane and form the channel pore (P) (MacKinnon and Yellen, 1990; Yellen et al., 1991; Hartmann et al., 1991; Yool and Schwarz, 1991). The approximate locations of the mutants we studied (448–450) are indicated.

function (D447C) or much slower recovery from inactivation than the wild-type channel (P450C). Therefore, we cotransfected the wild-type and mutant cDNA (1:1 ratio) to express heterotetramers that had relatively normal gating properties (for simplicity, they are still referred to as D447C and P450C below). Cysteine substitutions in all four subunits at the other positions also resulted in functional channels with relatively normal gating properties (the biggest change was found in M448C, which C-type inactivated with a time constant of 0.3 s at 0 mV, compared with 2.3 s in ShakerΔ; Table 1; data not shown).

We examined the state-dependence of the reactivity of substituted cysteines with thiol-specific methanethiosulfonate (MTS) reagents (Smith et al., 1975; Stauffer and Karlin, 1994). These reagents had only a small effect on the current through wild-type ShakerΔ channels (Table 1). However, modification of cysteines introduced at three consecutive positions (448, 449, and 450) resulted in substantial and irreversible reduction of the current (Table 1). The rate of this reduction was very state-dependent. An example is shown in Figure 2. When P450C mutant channels were treated for 25 s with the reagent MTS-ethylsulfonate (MTSES) in the closed state, there was no reduction in the current; however, when treatment was continued during a 6 s depolarization that opened and then inactivated the channels, there was a 70% irreversible reduction in the current (Figure 2A). To distinguish whether the rapid reaction involved the open or the inactivated state, we first inactivated the channels by a long depolarizing pulse (10 s), then immediately after repolarizing the membrane, applied the reagent for only 1 s to the inactivated channels (plus a small fraction of closed channels, Figure 2B). This briefer treatment of inactivated channels resulted in a similar reduction in the current.

Using state-selective treatments similar to that shown in Figure 2B, we systematically measured the rates of cysteine modification in the closed, open, and C-type inactivated states of the M448C, T449C, and P450C mutants. Figure 3A illustrates the protocols we used to modify channels in different states. Closed channel modification simply involved application of MTS reagent to the patch, while maintaining the membrane potential at −80 mV. Open channel modification required repeated treatments during brief depolarizing pulses, in order to avoid contamination by the inactivated state.

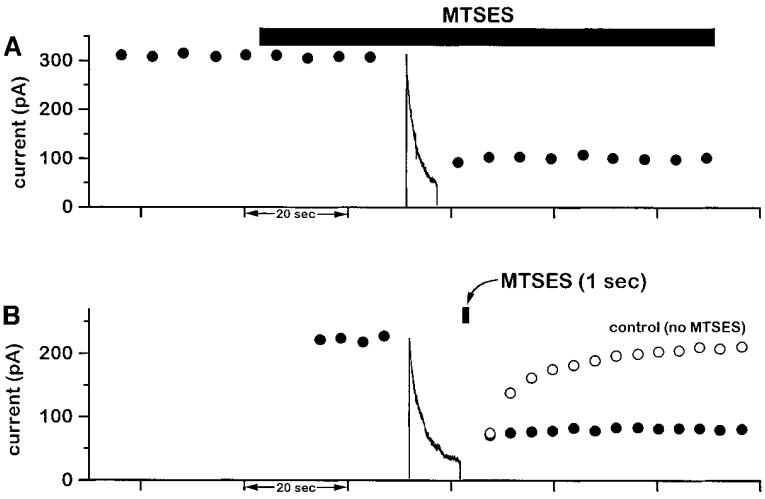

Figure 2. State-Dependent Modification of P450C with MTSES

(A) Solid circles represent the average current in the second half of a 10 ms voltage pulse (applied every 6 s) from −80 mV to 0 mV. The trace represents the current during a 6 s depolarization to 0 mV, applied at the time indicated by its position on the abscissa. MTSES (300 μM) was added during the time period indicated by the solid bar.

(B) Same as in (A), except that MTSES was applied for 1 s (solid bar) immediately following a 10 s depolarization. The 10 ms depolarizing pulses were applied every 4 s. Currents recovered fully after inactivation when no MTSES was applied (open circles).

Table 1. Properties of the Cysteine Mutants: Inactivation Kinetics and Maximal Inhibition of Current by Modifying Agents

Mutant Channels	Inactivation Time Constants (in seconds)		Reduction of Current with Maximal Modification (Current Remaining as Percent of Control)					
	τ_{inact} (at 0 mV)	τ_{recov} (at −80 mV)	MMTS	MTSEA	MTSET	MTSES	APA	Cu(II):phen
ShakerΔ	2.3 ± 0.1 (4)	8.3 ± 1.0 (4)	75.1 ± 5.0 (6)	84.2 ± 5.9 (4)	98.3 ± 1.2 (2)	90.6 ± 2.0 (3)	100 (2)	98.4 ± 1.6 (3)
M448C	0.3 ± 0.03 (6)	5.8 ± 0.5 (6)			2.2 ± 0.8 (8)		1.4 ± 0.7 (3)	2.9 ± 0.5 (6)
T449C	2.3 ± 0.2 (10)	9.6 ± 0.4 (10)	47.8 ± 0.5 (3)	23.3 ± 2.2 (2)	46.4 ± 2.4 (5)		93.7 ± 2.8 (4)	93.6 ± 1.0 (3)
P450C[a]	2.3 ± 0.2 (6)	14.7 ± 1.8 (6)			5.7 ± 1.9 (3)	5.6 ± 1.8 (4)		

[a] As a 1:1 mixture with ShakerΔ.

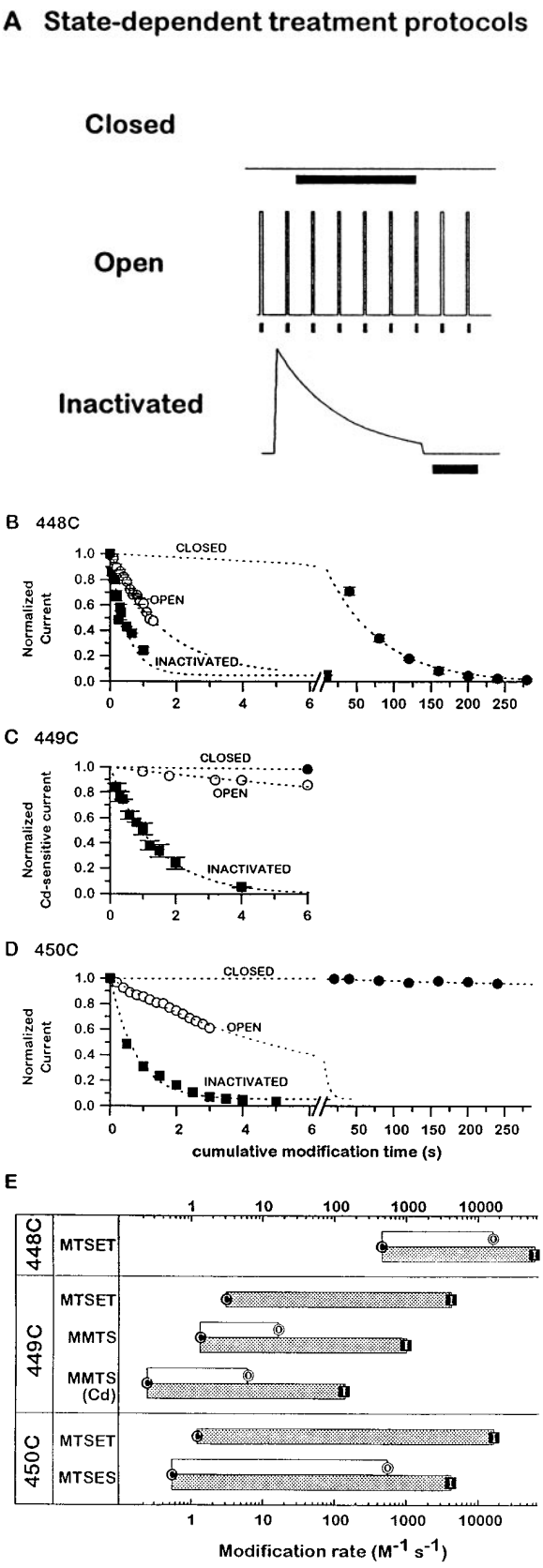

Figure 3. Chemical Modification of M448C, T449C, and P450C in the Closed, Open, and Inactivated States

(A) Perfusion protocols used for chemical modification in the three states. Solid bars under the simulated current traces indicate pulses containing modifying reagent.

(B–D) State-selective modification of M448C, T449C, and P450C with MTSET (30 μM), MMTS (5 mM; 10 mM for open state modification) and MTSES (300 μM), respectively. Symbols represent the fraction of current remaining (B and D) or of current still sensitive to Cd^{2+} (C) after the indicated duration of modification. Dotted lines are best fits to a single exponential function. Steady state values used in these fits are from Table 1. The time constants for the closed, open and inactivated states are (in seconds; numbers in parentheses are numbers of independent determinations): (B) 72.5 ± 2.1 (5), 1.9 ± 0.04 (4), 0.5 ± 0.04 (6); (C) 847.6 ± 185.3 (4), 14.6 ± 0.8 (5), 1.4 ± 0.03 (5); (D) 6317.5 ± 806.9 (3), 6.1 ± 0.1 (3), 0.8 ± 0.1 (4).

(E) Bar graph of the rates of modification for different MTS reagents. Solid circle (●), closed state; open circle (○), open state; solid square (■), inactivated state. The length of the bars (in log units) corresponds to the ratio of reaction rate in the inactivated or open state to that in the closed state.

Inactivated channel modification was done by brief treatment at −80 mV, immediately following a long depolarizing pulse. For all three positions, we measured the rate of modification by following current reduction over a series of repeated treatments. For T449C, we observed that prolonged modification with an uncharged MTS reagent, methyl methanethiosulfonate (MMTS), abolished the channel sensitivity to Cd^{2+}. Therefore, we also measured the rate at which T449C channels became completely insensitive to Cd^{2+}.

For each of these three residues, the pattern of modification was the same: very slow reaction in the closed state, very rapid reaction in the C-type inactivated state, and an intermediate rate in the open state (Figures 3B–3D). Depending on the position and the MTS reagent used, the reaction rates showed a dramatic 130–10,000-fold difference between the closed and inactivated states (Figure 3E).

The closed and inactivated state measurements have relatively little contamination from reaction that occurs in other states. However, we expect that the measurements in the open state may be contaminated by a small amount of inactivation, and that this contamination may dominate the measurement, because inactivated state modification is so fast. This contamination may be more pronounced for 448C, since inactivation is faster and more complete in this mutant than in 449C and 450C. We can say with confidence that modification is much slower in the open state than in the inactivated state.

Cysteines at the Deepest Position Can Be Crosslinked between Subunits in the Inactivated State

Another aspect of the conformational change during C-type inactivation was revealed by additional behavior of the cysteines at position 448, which lies deeper in the pore than 449 and 450. Upon initial transfection in HEK293 cells, this mutant channel did not give functional expression. We suspected that this might arise from disulfide formation during channel assembly. Consistent with this idea, we found that pretreatment of cells with

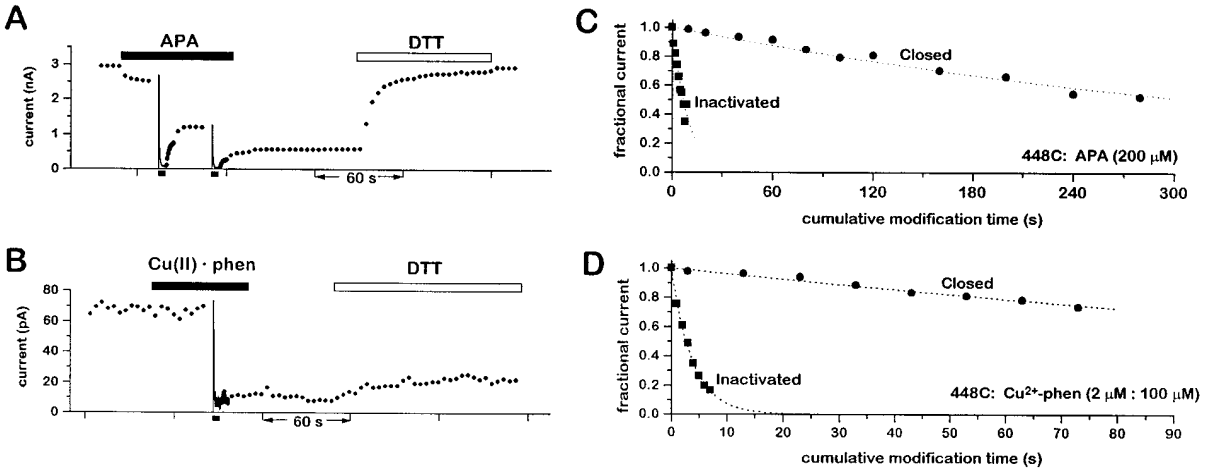

Figure 4. State-Dependent Bridging and Crosslinking Between M448C Subunits

(A) Effect of p-aminophenyl-dichloroarsine (APA). Solid circles represent the average current in the second half of a 10 ms voltage pulse (applied every 4 s) from −80 mV to 0 mV. The solid bar indicates the duration of 100 μM APA application. Current was hardly decreased during the application of APA in the closed state (25 s) but was significantly diminished after 10 s depolarizations to 0 mV (short solid bars under the current traces). Current reversal was rapid in 10 mM DTT (open bar).

(B) Effects of the oxidizing agent Cu(II):phenanthroline (2 μM:100 μM). Details as in (A).

(C and D) Time course of current reduction for closed and inactivated state treatments with APA (200 μM) and Cu(II):phenanthroline (2 mM:100 mM), respectively. Symbols represent the fraction of current remaining after the indicated duration of APA or Cu(II):phenanthroline application. Dotted lines are best fits to a single exponential function. Steady state values are from Table 1. The time constants for the closed and inactivated states are (in seconds): (C) 452.4 ± 16.2 (7), 9.4 ± 0.4 (6); (D) 252.6 ± 7.9 (2), 3.8 ± 0.1 (3).

10 mM dithiothreitol (DTT) for 20 min allowed measurement of large Shaker K^+ currents (this pretreatment was used for all of the electrophysiological experiments performed on the 448C mutant). Therefore, we wanted to see whether the four cysteines at the 448 positions might lie very close together, particularly in the inactivated state.

To test for juxtaposition of the cysteines at position 448, we first used the trivalent arsenical reagent p-aminophenyl-dichloroarsine (APA), which selectively interacts with nearby pairs of thiols, but not with single thiols (Loring et al., 1992). APA (100 μM) had little effect on closed channels, but rapidly diminished currents when treatment was continued during 5 s depolarizations that opened and subsequently inactivated the channels (Figure 4A). As further illustrated in Figure 4C, using state-selective treatments, the rate of current reduction is ~50-fold faster in the inactivated state than in the closed state, consistent with the idea that a pair of nearby cysteines is exposed in the inactivated state. Figure 4A also shows that the effect of APA on 448C does not reverse spontaneously, but can be rapidly reversed by 10 mM DTT (a dithiol).

It was also possible to form inter-subunit disulfide bonds between the cysteines at position 448. Application of an oxidizing agent, copper(II):phenanthroline (2 μM:100 μM), produced a state-dependent elimination of current in the 448C mutant (65 times faster in the inactivated state than in the closed state; Figures 4B and 4D) but not in wild-type or 449C mutant channels (even when they were pretreated with DTT; Table 1). Reversal of current by 10 mM DTT was incomplete on the time scale of the recording (Figure 4B). Similar results were obtained using another oxidizing agent, dithionitrobenzoic acid (DTNB, data not shown).

Polyacrylamide gel electrophoresis in the absence of reducing agent confirmed the presence of a crosslink between 448C monomers. As shown in Figure 5, 448C

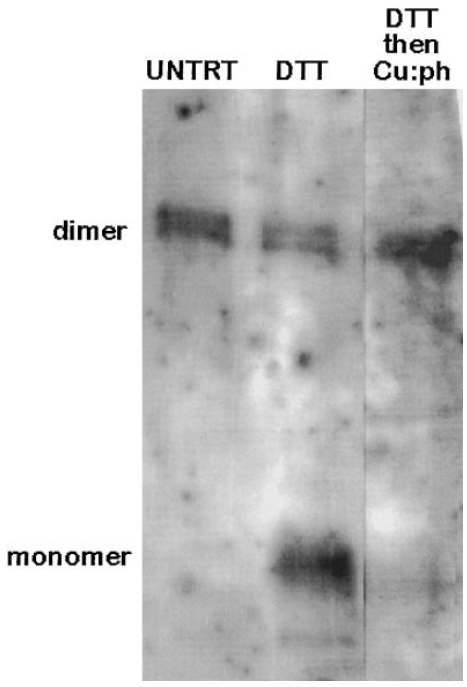

Figure 5. Dimer Formation by 448C Channels

Upon channel assembly, M448C existed mainly as dimers, as seen on this nonreducing polyacrylamide gel (left lane). DTT treatment (10 mM, 20 min) reduced many of the dimers to monomers (middle lane). Brief reoxidation with Cu(II):phenanthroline (1 μM:50 μM for 10 s) reformed dimers (right lane).

channels without DTT treatment have mainly a molecular weight corresponding to dimers. Treatment with DTT reduced most of these dimers to monomer form, and brief reoxidation with copper(II):phenanthroline reformed dimers. Formation of dimers required the cysteines at position 448, but did not require any of the native cysteines. Two multiple mutants that lack the native cysteines in the transmembrane region, (C245V:C286V:M448C) and (M448C:C462A), gave similar electrophysiological and biochemical results (data not shown). Together with the absence of trimers and tetramers, these results argue that the dimers result from disulfide formation between two 448C cysteines in different subunits, and thus, that the 448C cysteines are very closely juxtaposed in the inactivated state.

Discussion

Modification Occurs at the Site of the Introduced Cysteines

We examined the state-dependence of reactivity of three cysteine-substituted mutant channels with MTS reagents. We think that these introduced cysteines are the site of modification. The MTS reagents are highly selective for cysteine (Smith et al., 1975). There are seven cysteine residues in each wild-type subunit (four are intracellular and three are in the transmembrane regions), but the reagents had only small, state-independent effects on wild-type channels (Table 1). In contrast, all of the reagents reduced the current of the mutant channels substantially (by 50% or greater; Table 1). Moreover, even when the three native cysteines in the transmembrane region (at positions 245, 286, and 462) were removed (individually or in combination) from the mutant channels, the effects on the mutants persisted. Therefore, native cysteines do not appear to be involved in modification. In addition, modification of M448C by MTS-ethylammonium (MTSEA) and of T449C by MTS-ethyltrimethylammonium (MTSET) (both reagents are positively charged) resulted in reduced sensitivity of the remaining current to the external cation blocker TEA (data not shown), consistent with modification at the position of the mutant cysteines, rather than elsewhere.

Because the Shaker K^+ channel is a homotetramer (MacKinnon, 1991), cysteine substitution puts a new cysteine residue into each of the four subunits at the position of interest. Reaction with MTS reagents can result in partial modification of 1–3 of these cysteines, or complete modification of all four of them. We found that when T449C mutant channels are incompletely reacted with MMTS, they have a reduced (but not absent) Cd^{2+} sensitivity, which is comparable to that of channels with two cysteines at 449 (using tandem-linked dimers; Y. L. and G. Y., unpublished data). With prolonged application of MMTS, they lose their Cd^{2+} sensitivity completely. We suspect that the T449C channels with reduced Cd^{2+} sensitivity have been modified at only one or two cysteines, but that Cd^{2+} sensitivity is lost when three or four cysteines are modified. On the other hand, the reduction in channel current produced by this same reagent appears to be faster than the loss of Cd^{2+} sensitivity (Figure 3E), suggesting that maximal current reduction is achieved with modification of fewer than four cysteines.

In general, we do not know how current reduction is correlated with the number of cysteines modified. In one special case, however, we have evidence that modification of a single cysteine is probably sufficient to eliminate channel current. We observed that complete modification of 448C by positively charged MTSET resulted in virtual elimination of current. At different stages of the current reduction, the sensitivity of the residual current to TEA remained unchanged (data not shown). If there were a partially modified channel that still conducted current, we would expect the TEA sensitivity to be substantially altered, so the lack of a change suggests that there is no such intermediate form, and that modification of a single 448C cysteine is enough to completely abolish current.

The Large Change in Reactivity Probably Reflects a Local Rearrangement and Exposure of the Side Chains

There is a large increase in the rate of cysteine modification at three consecutive positions of the outer mouth during C-type inactivation. We think that this large difference in reaction rate between the closed and inactivated states results from a conformation-dependent change in the folding pattern of the thiol side chains of the substituted cysteines (Figure 6). In the closed and open states, the side chains of residues 448, 449, and 450 are hidden from the aqueous phase; upon C-type inactivation, they become exposed and probably project into the channel pore, allowing fast reaction with modifying reagents. An alternative scenario for the change in reaction rate would involve an inactivation-induced change in the pKa of the thiol side chain, and thus, a change in the concentration of the reactive thiolate species (Roberts et al., 1986). To account for the 10,000-fold difference in reactivity, there would have to be a large change in the pKa upon channel inactivation. This would likely be accompanied by a change in the local electrostatic potential. If this were true, then positively charged MTSET and negatively charged MTSES should be affected quite differently. Because both reagents highly

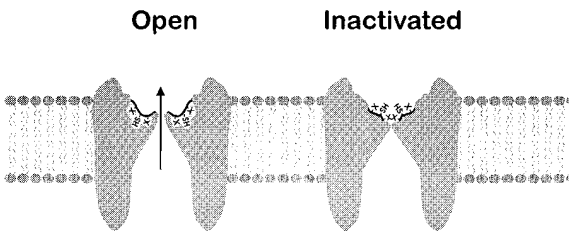

Figure 6. Cartoon Showing C-type Inactivation-Induced Movement of the Side Chains of Residues 448, 449, and 450 at the Outer Mouth of the Shaker K^+ Channel

Illustrated here as an example is the T449C mutant channel. The balls represent the backbone of the residues. The side chains are labeled as X's or SH's. In the open and closed states, the side chains are hidden from the aqueous pore, facing toward the bulk of the protein. In the inactivated state, these same side chains become exposed to the pore and project into the aqueous phase. At the deepest and presumably the narrowest position of the three, position 448, this movement allows contact between 448 side chains in different subunits.

favored modification in the inactivated state, we think that this possibility is unlikely.

The finding that a cysteine substituted at position 449 becomes exposed during C-type inactivation is generally consistent with the effects of 449 mutations on the inactivation rate: mutants with charged residues (which would prefer to be exposed) have fast inactivation rates, whereas mutants with hydrophobic residues like valine, tyrosine, or phenylalanine, show very little inactivation (López-Barneo et al., 1993). The histidine mutant inactivates quickly when it is charged and slowly when it is not. On the other hand, aromatic residues at this position are thought to interact directly with externally-applied TEA (Heginbotham and MacKinnon, 1992), which is inconsistent with the idea that these tyrosines are buried in the closed and open states of the channel. This may indicate a difference in the structure or in the structural details of inactivation gating between the cysteine- and tyrosine-containing channel proteins. It may also be that tyrosine is partially exposed, or that the tyrosine is in equilibrium between buried and exposed (in which case TEA binding might stabilize the exposed state).

The Cysteines at Position 448 Are Either Close or Flexible Enough to Crosslink with Each Other

In a delayed rectifier K^+ channel, Kv2.1, oxidation was reported to reduce current when the amino acid residue at position 379 (Shaker equivalent position 448) was replaced with a cysteine (Joho et al., 1995, Soc. Neurosci., abstract). These effects were attributed to disulfide formation between 379C and a native cysteine in the adjacent S6 segment, 394C (Shaker equivalent position 463), because mutation of this native cysteine eliminated the oxidation effects. In the Shaker K^+ channel, there is an alanine at position 463. The nearest native cysteine is at the neighboring position 462. Mutation of this cysteine (or the other two native cysteines at positions 245 and 286 in the transmembrane region) to either alanine or serine in the 448C mutant channel, had no effect on the state-dependent modification and oxidation. Pretreatment of these "cysteine-less" mutants with DTT was still necessary for functional expression. In addition, we saw no higher molecular weight forms on the gel, either before reduction with DTT, or after reoxidation with Cu(II):phenanthroline, further supporting the idea that disulfide formation occurs between two 448C cysteines in the Shaker mutant channel.

How close together are these 448C cysteines in the inactivated state of the channel? Cysteine residues that are near each other in a protein collide through thermal motions and then can form disulfide bonds. The rate of disulfide formation depends critically on the average distance between the disulfide-forming sulfhydryl pair, as well as on the amplitude of their thermal motion. The shorter the distance, or the larger the amplitude, the easier it is to form disulfide bonds. In one study on the amplitude of thermal motions between two adjacent α-helices in the D-galactose receptor of E. coli, crosslinking occurred with a time constant of $\sim$5 s between a pair of cysteines 4.5 Å apart in the crystal structure (Careaga and Falke, 1992). This rate is comparable to the rate of 448C oxidation in the inactivated state. Given our generally less-favorable conditions for disulfide formation than those in the galactose receptor study (room temperature vs. 37°C, 750-fold lower Cu(II):phenanthroline concentration, but slightly higher pH: 7.4 vs. 7.0), we think that in the inactivated state, the 448C cysteines either are very close to one another or experience larger translational movements than those in the D-galactose receptor.

How Local Is the Conformational Change at the Outer Mouth?

C-type inactivation thus involves a conformational change at the outer mouth that exposes the side-chains of three consecutive residues to the solvent phase, and constricts the pore enough to permit direct disulfide formation at the 448 position. How local is this conformational change? Unfortunately, experiments on the neighboring residues were not as informative. For D447C and V451C, the MTS reagents affected neither the total current nor the affinity for external TEA in any state. We suspect that in the case of 447C, the pore is too narrow for MTS reagents to reach, since treatment of this same mutant with the smaller Ag^+ ion did give current reduction (Lü and Miller, 1995). In the case of 451C, reaction probably occurs but has no effect on conductance or TEA affinity. Gross and MacKinnon (1996) observed effects of 451C modification on the affinity of scorpion toxin. However, the low affinity and inconsistent effect of toxin on 451C expressed in HEK293 cells prevented us from using toxin to monitor the state-dependence of modification at this position.

Several findings argue that C-type inactivation is not completely localized to these residues. C-type inactivation can be prevented not just by external TEA, but also (under specific circumstances) by K^+ ions in the pore, or by internal TEA (Baukrowitz and Yellen, 1995, 1996). It seems that the open channel structure is stabilized by the binding of ligands within the pore or to either end; when all of the ligand sites are empty, this structure is destabilized and C-type inactivation proceeds at its maximum rate. Also, it appears that C-type inactivation is coupled to activation (Yellen et al., 1994; Olcese et al., 1994, Biophys. J., abstract).

Nevertheless, we did find evidence that the change at the outer mouth may be surprisingly local. The binding of a scorpion toxin, agitoxin 2, to the channel, has been shown to be quite sensitive to the identity of individual amino acids at the positions we studied (MacKinnon et al., 1990; Gross et al., 1994). We assessed toxin binding by briefly exposing channels in a particular state to a high concentration of toxin, and then measuring the fraction of the channels that recover with the slow time course characteristic of toxin dissociation. If C-type inactivation involves a major rearrangement of the peptide chain at the outer mouth (as might be concluded from the exposure of three consecutive residues), then the binding of toxin ought to be quite poor in this state. On the contrary, we found that agitoxin 2 bound equally rapidly to the closed state and the inactivated state (Figures 7A and 7B). For 449C channels, when 4 μM agitoxin was applied for 200 ms, it bound to 41.8 $\pm$

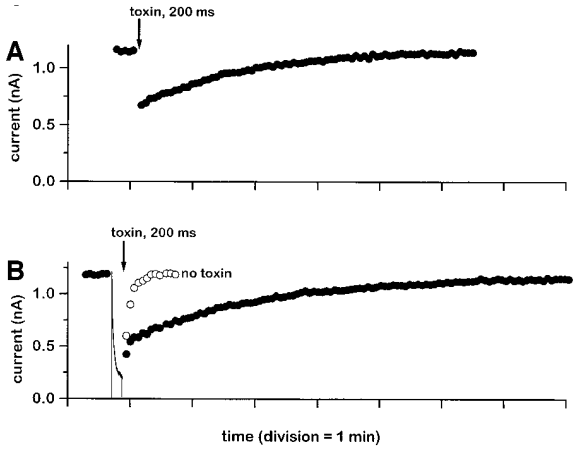

Figure 7. Agitoxin 2 Binds Equally Rapidly to the Closed and Inactivated States

(A) Binding of agitoxin 2 to the closed state of T449C. Solid circles represent the average current in the second half of a 10 ms voltage pulse (applied every 4 s) from −80 mV to 0 mV. At the time indicated by the arrow, toxin (4 μM) was applied for 200 ms at −80 mV; the first test pulse after toxin application showed that 42% of the current was blocked.

(B) Binding of agitoxin 2 to the inactivated state of T449C. Same patch as in (A). The trace represents currents during a 10 s depolarization to 0 mV. At the time indicated by the arrow (immediately following repolarization to −80 mV), toxin (4 μM) was applied for 200 ms. Currents recovered with two phases: a fast phase characteristic of normal recovery from C-type inactivation, and a slow phase characteristic of toxin dissociation (as in [A]). The recovery trace following the toxin application was fitted with a double exponential function. The amplitude of the slow component is 53% of the current before toxin binding. Open circles represent recovery from inactivation without toxin application.

1.0% (n = 2) of the closed channels, or 46.2 ± 3.9% (n = 5) of the inactivated channels (the latter was corrected for the fraction of channels that were not inactivated at the end of a long depolarizing pulse and were therefore closed immediately following repolarization). Similar results were obtained for the wild-type channel (data not shown).

Relationship to Other Work

Probing with a cysteine-reactive monovalent cation, Ag^+, Lü and Miller (1995) studied the accessibility of substituted cysteines in Shaker K^+ channels expressed in *Xenopus* oocytes. They showed that 447, 448, 449, and 450 were all readily accessible to Ag^+ under conditions that should favor the closed state. We examined the Ag^+ reactivity with T449C and P450C expressed in HEK293 cells and found large state-dependence similar to our results using the MTS reagents (data not shown). It is not clear at this time whether the disparity arose from the different expression systems (mammalian HEK 293 cells vs. *Xenopus* oocytes), recording and perfusion techniques used (patch-clamp vs. two-electrode voltage clamp; fast perfusion of patches vs. perfusion of whole oocytes), or from other sources. One possible explanation is that oocyte-expressed Shaker K^+ channels may have a significant probability of being in the C-type inactivated state, even at negative membrane potentials. In Kv2.1 expressed in *Xenopus* oocytes, residues at the Shaker equivalent positions 447, 448, 449, and 451 were also found to be exposed to the aqueous phase at negative potentials in the closed state, based on modification of the substituted cysteines at these positions with MTS reagents (Pascual et al., 1995; Kürz et al., 1995). The rate of modification was dependent on neither the frequency and duration of the testing pulse, nor the holding potential.

Schlief et al. (1996) have observed current reduction and acceleration of C-type inactivation in Shaker K^+ channels by chloramine-T, an oxidizing agent; these effects occurred much faster in the C-type inactivated state. In addition, there was a linear correlation between the rate of chloramine-T induced current reduction and the rate of C-type inactivation in several mutant channels. These effects were largely attributed to the state-dependent, irreversible oxidation of the native methionine residue at position 448, since mutating this position to an isoleucine substantially diminished these effects. These findings give a picture consistent with our results of state-dependent oxidation and chemical modification of M448C.

Although it is slow in ShakerΔ channels, C-type inactivation may be rapid and physiologically important in the presence of N-type inactivation (Baukrowitz and Yellen, 1995) or pore blockers (Baukrowitz and Yellen, 1996). Also, it appears that a very rapid version of C-type inactivation is responsible for the inward rectification behavior of another K^+ channel, HERG, which is involved in the repolarization phase of the cardiac action potential, and is defective in an inherited cardiac arrhythmia (Smith et al., 1996).

Implications of Large State-Dependent Differences in Reactivity

In the most susceptible state of M448C, T449C, and P450C mutant channels, the reaction rates are very high: treatment of M448C with 30 μM MTSET for 2 s gave complete reaction. The existence of fast and highly state-dependent reaction rates argues for caution in the use of the cysteine accessibility method to probe secondary structure by a go/no-go measurement, particularly when millimolar reagent is applied for minutes. The use of such treatments runs the risk of including substantial contributions from conformational states with low population but high rates of reaction, thus compromising conclusions about secondary structures based on the assumption of a single underlying structure.

Experimental Procedures

Molecular Biology and Channel Expression

Shaker mutant cDNA were constructed using the oligonucleotide-directed mutagenesis method described by Kunkel (1991). cDNA coding for Shaker H4:Δ6–46 and the mutant channels were then subcloned into the expression vector GW1-CMV (British Biotech, Cowley, Oxford, UK). For channel expression, we used the mammalian human embryonic kidney 293 cells (HEK293; American Type Culture Collection no. CRL-1533, Rockville, MD). Briefly, cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM-F12; GIBCO BRL, Gaithersburg, MD) with 10% fetal bovine serum (Sigma Chemical Company, St. Louis, MO) to ~50% confluence on the day

of transfection. Right before transfection, cells were treated with 0.05% trypsin, 0.53 mM EDTA, washed once, and resuspended in HEPES-buffered saline at 2×10^6 cells/ml. Electroporation was done at 300–350 V (Electroporator, Invitrogen, San Diego, CA) in 0.4-cm cuvettes containing 200 μl of cells, 10–25 μg of channel plasmid, 1 mg of an SV40 T antigen expression plasmid, and 3–5 μl of CD8, an expression plasmid that encodes the α subunit of the human CD8 lymphocyte surface antigen (gift from Dr. Brian Seed). Cells were maintained in growth medium at 37°C with 5% CO_2 until use.

Visual Identification of Transfected Cells for Electrophysiological Recording

Cells were cotransfected with CD8 antigen and incubated a few minutes before use with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M-450 CD8, Dynal, Great Neck, NY). Beading occurred preferentially on cells that expressed the CD8 antigen. Most of the cells that were beaded also had channel expression (Jurman et al., 1994). Only beaded cells were used for electrophysiological recording.

Electrophysiology

Cells typically expressed functional channels within 15–20 hr after transfection. Currents from excised outside-out patches were recorded using standard patch-clamp methods (Hamill et al., 1981). The extracellular bathing solution contained 150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 1 mM EGTA (3 mM $CaCl_2$ and 0 mM EGTA when Cd^{2+}, Cu^{2+}, or APA was added) (pH 7.4). The pipette solution contained 160 mM KCl, 1 mM EGTA, 0.5 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). MTS reagents (MTSEA, MTSET, and MTSES from Toronto Research Chemicals, Ontario, Canada; MMTS from Sigma) were directly dissolved or diluted in the extracellular solution immediately before application. APA was prepared from aminophenylarsine oxide (Aldrich), as described by Loring et al., (1992), and was dissolved in 100% dry ethanol before being added to the extracellular solution. The phenanthroline (Aldrich) stock solution was prepared by dissolving phenanthroline in 100% dry ethanol. The stock solutions of phenanthroline and $CuSO_4$ (Sigma) were diluted to the experimental concentrations in the extracellular solution at the time of use. For accurate control of the application of reagents, we used a technique for fast perfusion of solutions described by Liu and Dilger (1991). We routinely achieved solution exchange times of about 1 ms.

Protein Sample Preparation

Tissue culture dishes containing transfected cells in media were cooled for 10 min. to room temperature. Intact cells were collected from the dishes by repeated pipetting, and then were washed and resuspended in buffer containing 150 mM NaCl, 10 mM KCl, 10 mM HEPES, 4 mM $CaCl_2$, and 3 mM $MgCl_2$ (pH 7.4). They were then either left untreated or were exposed to 10 mM DTT in 3 ml of the above buffer, with shaking for 30 min. at room temperature.

Following incubation in DTT, cells were washed four times with buffer. In some experiments, cells were further treated with copper (II):phenanthroline (1 μM:50 μM) for 10 s. Reaction with copper was quenched by the addition of Na_2EDTA (10 mM final). Cells under each condition were washed four times and resuspended in 1 ml PBS (pH 7.4) supplemented with protease inhibitors (15 μM antipain, 20 μM bestatin, 16 μM chymostatin, 16 μM leupeptin, 15 μM pepstatin, 1.2 μM phenylmethyl sulfonylfluoride [PMSF]), and 20 mM N-ethylmaleimide (NEM).

Crude cell membranes were prepared with an initial set of three freeze/thaw cycles followed by homogenation on ice under a stream of nitrogen gas, using a dounce tissue grinder (30 strokes with tight pestle). The homogenate was centrifuged at 70k rpm 4°C for 10 min in a Beckman TL100 ultracentrifuge. The pellet was resuspended in 300 μl PBS (with protease inhibitors and 20 mM NEM) by bath sonication under nitrogen (Branson Sonifier). Total protein was quantified by using the BCA protein assay method (Pierce).

Protein Gel Electrophoresis and Immunoblotting

For each sample, an aliquot containing 3 μg total protein was combined with a buffer containing 65 mM Tris (pH 6.8), 10% glycerol, 2% SDS, and 0.00025% pyronin Y. The samples were heated to 70°C for 5 min, loaded onto a 7.5% polyacrylamide gel (Bio-Rad Mini Protean II), and electrophoresed for 1.5 hr at 170–200 V. Protein was transferred by electroblotting (16 hr, 4°C, 30 mA) to a polyvinylidene fluoride (PVDF) hydrophobic membrane (Immobilon-P, Millipore).

Following transfer, the PVDF membrane was fixed for 15 min in 10% acetic acid, 30% methanol. The membrane was blocked for 1 hr with Tris-buffered-saline (TBS) containing 2% nonfat dry milk and 0.1% Tween-20, and then was incubated for 1 hr in a blocking solution containing a 1:10,000 dilution of a polyclonal rabbit antiserum against the Shaker protein (gift from Dr. Christopher Miller). The membranes were then washed three times for 5 min each in TBS, and reblocked for 1 hr. Protein was detected using the Attophos Substrate detection system (JBL Scientific, 1960A). The secondary antibody (fluoroscein-conjugated IgG, 1:600) was linked to an anti-fluorescein alkaline phosphatase IgG (1:2500). The blot was washed three times for 5 min each with TBS, and protein was then visualized by scanning the immunoblot into a Molecular Dynamics FluorImager 575.

Data Acquisition and Analysis

Experimental data were acquired on a 486 personal computer with user-designed, Windows-supported software (VectorView; Yellen, unpublished data). Currents were filtered at 2 kHz and digitized at 0.1–2 kHz. Fitting of current traces to a single or double exponential function was performed using Levenberg-Marquardt minimization method (Origin software, MicroCal, Northampton, MA). Results are expressed as mean ± sem.

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