N-type Inactivation and the S4-S5 Region of the Shaker K⁺ Channel

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ABSTRACT   The intracellular segment of the Shaker K⁺ channel between transmembrane domains S4 and S5 has been proposed to form at least part of the receptor for the tethered N-type inactivation "ball." We used the approach of cysteine substitution mutagenesis and chemical modification to test the importance of this region in N-type inactivation. We studied N-type inactivation or the block by a soluble inactivation peptide ("ball peptide") before and after chemical modification by methanethiosulfonate reagents. Particularly at position 391, chemical modification altered specifically the kinetics of ball peptide binding without altering other biophysical properties of the channel. Results with reagents that attach different charged groups at 391C suggested that there are both electrostatic and steric interactions between this site and the ball peptide. These findings identify this site to be in or near the receptor site for the inactivation ball. At many of the other positions studied, modification noticeably inhibited channel current. The accessible cysteines varied in the state-dependence of their modification, with five- to tenfold changes in reaction rate depending on the gating state of the channel.

INTRODUCTION

Shaker K⁺ channels respond to membrane depolarization by opening and then rapidly inactivating, with an inactivation time constant of 2–3 ms. This fast or N-type inactivation has been shown to arise from a tethered blocker mechanism analogous to the "ball and chain" model originally proposed for Na⁺ channel inactivation (Armstrong and Bezanilla, 1977). In Shaker channels, the "ball" is provided by the first ∼20 amino acids at the NH₂ terminus of the protein (Hoshi et al., 1990; Zagotta et al., 1990). Removal of this ball by trypsin treatment or by genetic deletion of 41 amino acids at the NH₂ terminus abolishes N-type inactivation (ShBΔ6-46; hereafter called ShΔ) (Hoshi et al., 1990).

Two lines of evidence support the hypothesis that the inactivation ball acts by occluding the pore of the channel. First, intracellular application of tetraethylammonium (TEA)[1] slows down N-type inactivation, in a manner consistent with competition between binding of TEA and the ball (Choi et al., 1991). Since TEA is known to block K⁺ channels by occluding the pore (Armstrong, 1975), this suggests that the ball binds at or near the intracellular mouth of the channel. Second, external K⁺ ions speed the recovery from N-type inactivation (i.e., the dissociation of the ball) as if ions coming through the channel from the opposite side

can repel or "knock-off" the ball from the pore (Demo and Yellen, 1991).

Although fast inactivation is abolished in the NH₂-terminal deletion mutant, it can be effectively reconstituted by intracellular application of a synthetic "ball peptide" with the sequence of the first 20 amino acids of the Shaker K⁺ channel (Zagotta et al., 1990). By studying the interaction between the channel and peptides with altered sequences, Murrell-Lagnado and Aldrich (1993a, b) have learned how the physicochemical properties of the ball peptides affect blockade. They found that changes in the hydrophobic leucine residue at position 7 produce a significant increase of the dissociation rate constants without appreciable changes in the association rates. On the other hand, increasing the net positive charge of the peptide causes an increase in the association rate constants without appreciable changes in the dissociation rates. The specific localization of these charges does not seem to be critical: mutations that move the charges around but maintain the same net charge do not alter the kinetics of the blockade. As the authors pointed out, these results suggest that the ball peptide behaves as a point charge. That is, the electrostatic interactions are long range rather than specific, and their role is probably to increase the rate of diffusion of the peptide to the binding site.

Shortly after the NH₂ terminus was associated with fast inactivation (Hoshi et al., 1990; Zagotta et al., 1990), Isacoff et al. (1991) reported that the intracellular loop between transmembrane segments S4 and S5 probably forms at least part of the receptor for the inactivation gate. From site-directed mutagenesis studies, they suggested that the glutamate at position 395 (E395) might be the counter-charge for the positively charged residues of the inactivation ball. Mutations at position 395 completely disrupted N-type inactivation. Moreover,

mutations at other positions of the S4-S5 linker also produced alterations in the rates of the N-type inactivation.

The activation gating of the Shaker channel is also very sensitive to mutations in this region (McCormack et al., 1991), and it is known that binding of the inactivation ball and other blockers is dependent on the activation state of the channel. Thus, mutations in this region might change the apparent affinity for the ball (or for other blockers) by a direct change in the binding site, or indirectly by changing gating (Choi et al., 1993). We have used the method of cysteine substitution and subsequent chemical modification to help distinguish direct and indirect effects, with the goal of identifying residues that directly interact with the inactivation gate. This technique has been used to explore amino acid surface exposure (Falke et al., 1988; Akabas et al., 1992; Lü and Miller, 1995; Pascual et al., 1995; Kürz et al., 1995; Sun et al., 1996), as well as to detect protein conformational changes (Slatin et al., 1994; Yang and Horn, 1995; Larsson et al., 1996; Liu et al., 1996; Yang et al., 1996). We made 19 single-cysteine substitutions in this region, and found a particularly strong effect of charge modification at position 391, consistent with a direct electrostatic interaction. We also found that the rate of chemical modification of some of these cysteines was dependent on the gating state of the channel. Finally, the overall accessibility pattern of the region was determined from the rates of modification, and it is consistent with most of the residues participating in an α-helix.

MATERIALS AND METHODS

Mutagenesis and Expression

Mutations were introduced in Shaker H4 potassium channel (Kamb et al., 1988) subcloned in the GW1-CMV expression vector (British Biotechnology, Oxford, UK). Oligonucleotide-directed mutagenesis were performed following Kunkel's method (Kunkel et al., 1991). Mutations were confirmed by sequencing the segment of interest (Sanger et al., 1977). In some channels, amino acids 6–46 were deleted to remove N-type inactivation (Hoshi et al., 1990).

Shaker K$^+$ channels were expressed in human embryonic 293 cell line (HEK293; American Type Culture Collection, Rockville, MD) using the expression vector GW1-CMV (British Biotechnology). The channel expression plasmid DNA (20 μg) was cotransfected with 1 μg of plasmid πH3-CD8 for the α subunit of the human CD8 lymphocyte antigen. Before recording, cells expressing the CD8 antigen were identified by decoration with antibody-coated beads (Dynal; Jurman et al., 1994). Cells were transfected by electroporation as previously described (Jurman et al., 1994).

Solutions, Chemical Reagents, and Electrophysiological Recordings

The internal solution contained (in mM): 160 KCl, 1 EGTA, 10 HEPES, and 0.5 MgCl$_2$, pH 7.4. The external solution contained (in mM): 10 KCl, 150 NaCl, 3 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH 7.4. These chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific Co. (Santa Clara, CA).

The chemical reagents used to modify cysteines were thiosulfonate derivatives and silver. [(2-trimethylammonium)ethyl]methanethiosulfonate bromide [MTSET; CH$_3$SO$_2$—SCH$_2$CH$_2$N$^+$(CH$_3$)$_3$ Br$^-$], sodium (2-sulfonato-ethyl)methanethiosulfonate (MTSES; CH$_3$SO$_2$—SCH$_2$CH$_2$SO$_3$$^-$ Na$^+$) and (2-aminoethyl)methanethiosulfonate hydrobromide (CH$_3$SO$_2$—SCH$_2$CH$_2$NH$_3^+$ Br$^-$) were acquired from Toronto Research Chemicals Inc. (Downsview, Canada). Methyl methanethiosulfonate (MMTS) was obtained from Sigma Chemical Co., [(2-triethylammonium)ethyl]methanethiosulfonate (MTSTEA; CH$_3$SO$_2$—SCH$_2$CH$_2$N$^+$(C$_2$H$_5$)$_3$ Br$^-$) was synthesized by C. Mossman and J. Aubé at the University of Kansas.

Currents from inside-out excised patches (Hamill et al., 1981) were recorded between 1 and 3 d after transfection. Voltage was controlled by our own software (VectorView, written by G. Yellen) using a Pentium computer (Gateway 2000), a 12 bit National Instrument A/D-D/A converter, and an Axopatch 1D amplifier with an integrating headstage. Usually, currents were sampled between 2.5 and 5 kHz and filtered between 1 and 2 kHz. Macroscopic (100 to 2,000 pA) currents were recorded using electrodes with a resistance of 2–2.5 MΩ. To obtain single channel recordings, HEK293 cells were transfected with 5 μg of DNA channel and 15 μg of Bluescript plasmid. Single channel currents were recorded with smaller patch electrodes (7–10 MΩ) to avoid endogenous K$^+$ channels. Experiments on the state dependence of chemical modification used a computer-controlled solenoid to perform rapid perfusion switches (Brett et al., 1986). The rate of perfusion change was tested by application of TEA. Internal TEA is known to block K$^+$ channels with very fast kinetics, therefore the time constant of TEA blockade observed (usually ~10 ms) reflects the time course of solution change by our perfusion system. In other experiments, exchange of solutions was done by a manual switch.

Synthetic Ball Peptides

Shaker ball peptide consisted of the first 20 amino acids of the wild type Shaker K$^+$ channel, i.e., MAAVAGLYGLGEDRQHRKKQ (Zagotta et al., 1990). It was synthesized, HPLC purified, and the COOH-terminal amidated by the Massachusetts General Hospital peptide synthesis facilities. We are indebted to Dr. R.W. Aldrich (Stanford University, Stanford, CA) for kindly providing us with a sample of this peptide to perform our preliminary experiments.

E12K,D13K ball peptide is a double mutation of the Shaker ball peptide. Residues 12 and 13 were mutated to lysine (Murrell-Lagnado and Aldrich, 1993a). This peptide was synthesized, HPLC purified, and the COOH-terminal amidated by the Biopolymer Facility of the Howard Hughes Medical Institute in Baltimore.

Genetic Background for the Cysteine Mutants

Internal modification of the wild-type Shaker K$^+$ channel with any thiosulfonate derivative resulted in a substantial and irreversible reduction in current. The Shaker K$^+$ channel possesses four cysteines facing the intracellular environment (at positions 96, 301, 308, and 505). When these cysteines were substituted by serine and valine, the mutant channel was insensitive to internal modification, and the level of expression was good. Unfortunately, when additional mutations are made on this background, they usually express poorly. We found out that restoring the cysteine before the first transmembrane domain (C96) and the one after the last transmembrane domain (C505) does not restore the sensitivity to MTS reagents but does improve the expression of other mutants. We therefore used channels with the mutations C301S and C308S in all of our experiments.
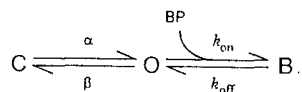
In ShΔ6-46 channels, C-type inactivation is speeded up by a number of cysteine mutations (unpublished observations). In

some of them, C-type inactivation becomes so fast and recovery from it so slow that experiments become tedious and are difficult to interpret. Mutations in threonine 449 have been shown to alter the rate of C-type inactivation (López-Barneo et al., 1993). In particular, T449V produced a channel that does not have C-type inactivation when expressed in oocytes. For reasons we do not understand, this mutant does show C-type inactivation when expressed in HEK293 cells. The rate of C-type inactivation is approximately the same as wild-type, but the extent of inactivation is much smaller (data not shown).

Our background channel has cysteines 301 and 308 substituted by serine and position 449 mutated to valine. We have two versions of this background: a wild type and a ShΔ6-46 channel. In this background channel, we have introduced cysteines, one at a time, between residues 380 and 398 (S4-S5 linker). Only one mutant (386C) did not give functional expression.

Model for Ball Peptide Blockade

The following model was used to analyze the kinetics of ball peptide blockade:

$$C \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O \overset{BP}{\underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}}} B.$$

Ball peptide (*BP*) blocks the channel only in the open state (*O*); at the concentrations we used, this results in a relaxation of the current after a rapid opening phase (e.g., see Fig. 3 *A*, *lower trace*). The relaxation was fitted by a sum of two exponentials. The time constant (τ) and the fraction of current remained (f_b) of the fast component was used to estimate the k_{on} and k_{off} of the ball peptide as follows (Choi et al., 1993):

$$k_{on} = \frac{f_b}{\tau [BP]},$$

and

$$k_{off} = \tau^{-1} - k_{on} [BP].$$

Our model is supported by three separate lines of evidence. First, the tethered inactivation gate has been shown to block the pore once the channel is opened (Demo and Yellen, 1991). Second, only one of the four tethered inactivation particles is necessary to block the pore and produce inactivation (MacKinnon et al., 1993). Third, there is a linear increase of the blocking rate with the concentration of ball peptide, suggesting a bimolecular reaction mechanism (Murrell-Lagnado and Aldrich, 1993a).

RESULTS

Chemical Modification of Cysteines in the S4-S5 Linker Produced Either Current Reduction or Changes in N-Type Inactivation

For all these studies we used the Shaker H4 channel with two of the native cysteines removed (C301S, C308S) and with another mutation to disable C-type inactivation (T449V; López-Barneo et al., 1993). Removal of the two cysteines at 301 and 308 abolished the effects of intracellular modification seen with wild-type channels (which ranged from 20 to 40%; data not shown). In some cases we left the NH_2 terminus intact (Sh^+) and observed the native N-type inactivation process; in others, we used the NH_2-terminal deletion mutant (ShΔ) and measured the affinity and rate of ball peptide bind-

ing. On this genetic background, residues at nineteen positions (380–398) were changed one at a time to cysteine. We used the methanethiosulfonate (MTS) reagents of Kenyon (Smith et al., 1975) and of Stauffer and Karlin (1994), which specifically donate a thioalkyl group to accessible cysteines.

For each cysteine mutant, we measured the ability of trimethylaminoethyl-MTS (MTSET) to reduce the current through the ShΔ channel or to alter its affinity for a high-affinity Shaker ball peptide (Fig. 1). We expected that attachment of the positively charged trimethylaminoethylthio-group would significantly reduce the affinity of the positively charged ball peptide, if it was located at a position in or near the ball peptide binding site. Modification of one cysteine mutant, A391C, produced a particularly strong reduction in the ball peptide affinity (21 ± 2-fold), with no reduction in the total current. We focused on the effects of 391C modification on the interaction between the ball peptide and the channel.

Chemical Modification of 391C Alters the Interaction Between the Tethered Inactivation Gate and its Receptor

Fig. 2 *A* shows the effect of MTSET modification of 391C on intact N-type inactivation. Complete modification produced a parallel increase of the steady state and the peak current. A simple interpretation of this result would be that modification mainly decreased the
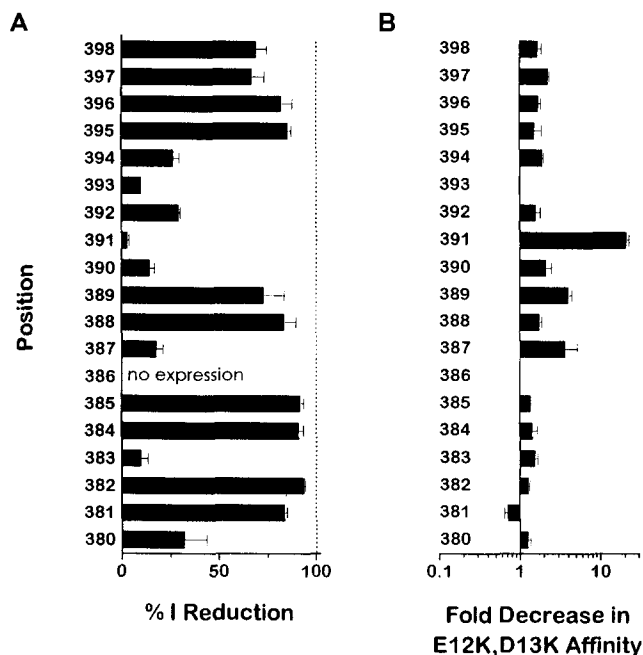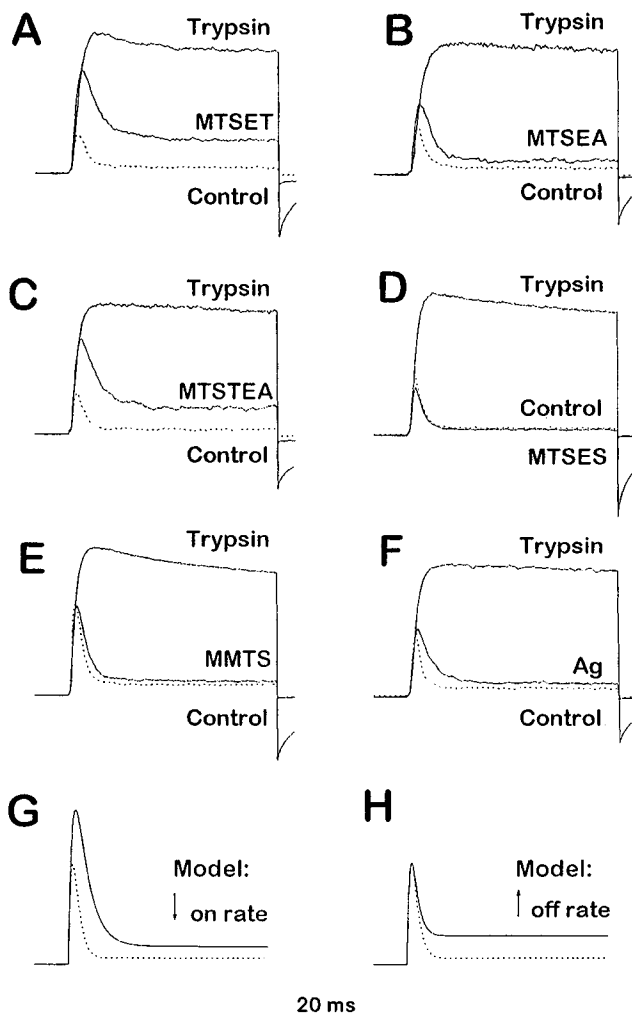

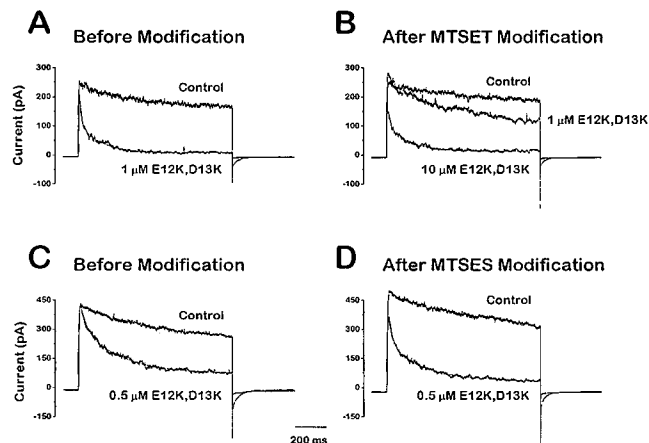FIGURE 1. MTSET modification of cysteines in the S4-S5 linker of the ShΔ channel. (*A*) Percent of current reduction after complete modification with MTSET. (*B*) Fold decrease in E12K,D13K ball peptide affinity. In positions where chemical modification produced complete current reduction, ball peptide affinity after chemical modification was tested after ~50% of current reduction ($n \geq 3$).

A Trypsin / MTSET / Control

B Trypsin / MTSEA / Control

C Trypsin / MTSTEA / Control

D Trypsin / Control / MTSES

E Trypsin / MMTS / Control

F Trypsin / Ag / Control

G Model: ↓ on rate

H Model: ↑ off rate

20 ms

FIGURE 2. Chemical modification of 391C alters N-type inactivation. (A–F) Each panel shows three current traces in response to a 50-ms voltage jump from −80 to 0 mV under different experimental conditions: (a) control, (b) after complete chemical modification with the reagent specified in each panel, and (c) after removing the tethered inactivation gate by perfusing the inside-out patch with 0.0025% trypsin. Current traces were normalized to the average current value from the last 20 ms of the trace after trypsin application. Complete chemical modification was monitored by pulsing at 0.25 Hz in the following particular conditions: MMTS, 2 mM for 1 min; Ag⁺, 20 nM for 2 min; MTSEA, 100 μM for 30 s; MTSET, 100 μM for 30 s; MTSTEA, 100 μM for 30 s; MTSES, 100 μM for 40 s. Data were acquired at 5 kHz and filtered at 2 kHz. (G and H) A simplified model was simulated to qualitatively describe the effect of chemical modification. (G) A decrease of the forward rate constant O → I$_N$ produced a noticeable increase of the peak and the steady state current levels as experimentally observed after chemical modification with reagents that added positively charged moieties (A, B, and C). (H) Increasing the backward rate constant I$_N$ → O produced a considerably increase in the steady-state current level without effects on the peak current, as empirically observed with chemical reagents that added a neutral moiety to the cysteine side chain (E and F).

A Before Modification — Control — 1 μM E12K,D13K

B After MTSET Modification — Control — 1 μM E12K,D13K — 10 μM E12K,D13K

C Before Modification — Control — 0.5 μM E12K,D13K

D After MTSES Modification — Control — 0.5 μM E12K,D13K

200 ms

FIGURE 3. Modification of 391C in the ShΔ K⁺ channel alters the kinetics of blockade by the ball peptide. The current traces shown in this figure were acquired in response to 1-s voltage steps from −80 to 0 mV. (A) Current traces acquired before chemical modification in the absence or presence of 1 μM E12K,D13K ball peptide. (B) Current traces after complete chemical modification with MTSET (50 μM for 40 s). An additional current trace in the presence of 10 μM of E12K,D13K ball peptide is shown. Superimposed on the current traces in the presence of 1 μM ball peptide before modification (A) and 10 μM ball peptide after modification (B) are solid lines obtained from the best-fit of the current values to the following function:

$$f(t) = y_0 + A\exp\left(\frac{-t}{\tau_1}\right) + B\exp\left(\frac{-t}{\tau_2}\right),$$

where y$_0$ is the steady-state value of current, and A and B are the amount of current from the component with fast (τ$_1$) and slow (τ$_2$) time constants, respectively. The best-fit parameter values before modification were: y$_0$ = 8.2 ± 0.1 pA, A = 187 ± 2 pA, τ$_1$ = 13.3 ± 0.2 ms, B = 101 ± 1 pA, τ$_2$ = 139 ± 1 ms. The best-fit parameter values after MTSET modification were: y$_0$ = 14.4 ± 0.1 pA, A = 100 ± 2 pA, τ$_1$ = 15.8 ± 0.4 ms, B = 89 ± 1 pA, τ$_2$ = 142 ± 1 ms. Before modification, k$_{on}$ and k$_{off}$ were 4.7 × 10⁷ M⁻¹ s⁻¹ and 28 s⁻¹, respectively. After MTSET modification k$_{on}$ was 3.1 × 10⁶ M⁻¹ s⁻¹ and k$_{off}$ was 3.2 s⁻¹. (C) Current traces before chemical modification in the absence or presence of 0.5 μM E12K,D13K ball peptide. Superimposed on the lower record is a solid line representing the best-fit of the data to the double exponential function shown above. The best-fit parameter values were: y$_0$ = 71.9 ± 0.4 pA, A = 158 ± 3 pA, τ$_1$ = 37 ± 1 ms, B = 259 ± 2 pA, τ$_2$ = 249 ± 3 ms. (D) After complete chemical modification with MTSES, current traces were acquired in the absence or presence of 0.5 μM E12K,D13K ball peptide. The best-fit parameter values were: y$_0$ = 33.3 ± 0.3 pA, A = 239 ± 3 pA, τ$_1$ = 24.9 ± 0.5 ms, B = 202 ± 1 pA, τ$_2$ = 232 ± 2 ms. The values for k$_{on}$ increased from 1.8 × 10⁷ to 4.9 × 10⁷ M⁻¹s⁻¹. The values of k$_{off}$ remained practically unchanged (1.8 and 1.6 s⁻¹ before and after modification, respectively). Data were sampled at 5 kHz and filtered at 2 kHz.

binding rate of the attached inactivation ball. Consistent with this interpretation, decreasing the binding rate to zero by removing the inactivation gate with trypsin produced a larger non-inactivated current level (Fig. 2 A). In a simple three-state model, these results could be reproduced by reducing the on rate for the attached ball (Sh⁺, Fig. 2 G). Modification with two other

positively charged reagents, MTSEA and MTSTEA, produced qualitatively similar results (Fig. 2, *B* and *C*, respectively). A negatively charged reagent, MTSES, produced the opposite effect, i.e., a decrease of the peak and the steady state current levels (Fig. 2 *D*). These changes were smaller but quite reproducible.

Reagents that combine with cysteine to give an electroneutral product did produce some change in the ball peptide kinetics, but the changes were mostly in the attached-ball dissociation rate. Modification of 391C with MMTS or Ag^+ produced an increase in the steady-state current level without much change in the peak current (Fig. 2, *E* and *F*). This effect could be replicated by the model when the dissociation rate was increased, as shown in Fig. 2 *H*. With Ag^+ there was an additional effect on the association rate.

Chemical Modification of 391C in ShΔ K^+ Channel Alters the Interactions Between Synthetic Ball Peptides and the Modified Residue

Modification of 391C had similar effects on the channel's interaction with the soluble ball peptide, studied in the ShΔ version of the mutant. Because we could change the ball peptide concentration, this approach allowed us to determine the association and dissociation rates more accurately. We could also use ball peptides with different net charge to assess the role of electrostatic interactions. Fig. 3 shows the effect of chemical modification with MTSET (*A* and *B*) and MTSES (*C* and *D*) on the blockade properties of the E12K,D13K synthetic ball peptide. As observed for the native inactivation process, MTSET produced a reduction in the affinity of the ball peptide mainly by a 15.3-fold decrease in the k_{on}. Modification with MTSES, which introduces a negatively charged moiety, produced a 2.8-fold increase in the k_{on}.

The effects of chemical modification with different methanethiosulfonate derivatives on the k_{on} and k_{off} for Shaker and E12K,D13K ball peptides are shown in Fig. 4. Three main conclusions could be extracted from these results. First, the biggest effect of chemical modification is to change the k_{on} of the ball peptides. Second, the direction of the change in k_{on} depends on the electrical charge of the group attached to the thiol. Finally, the E12K,D13K ball peptide (net charge +7) seems to be more sensitive than the Shaker ball peptide (net charge +3) to modification with reagents that introduce positively charged moieties. These results combined suggest an electrostatic interaction between the modified residue at 391C and the ball peptides.

Does the chemical modification directly alter the binding and unbinding kinetics of the ball peptide, or does it change these kinetics indirectly through a primary effect on the gating properties of the channel? We recorded single mutant channels before and after complete modification with MTSET (Fig. 5, *A* and *B*). Neither the probability of opening in the absence of ball peptide (Fig. 5, *A* and *B*, *left*) nor the single channel conductance (Fig. 5 *C*) changed with MTSET treatment. Modification of the channel clearly occurred, though addition of 1 μM E12K,D13K ball peptide produced many fewer long closures (blockade events) after modification, as is apparent from the effect of ball peptide on the ensemble average current (Fig. 5, *A* and *B*, *right*). Thus, the change in ball peptide interaction occurs without any detectable change in gating, ruling out an indirect effect through gating.

State-dependent Modification of 391C

We looked for state dependence of the rate of modification of 391C, which would indicate changes in the accessibility of 391C resulting from the conformational

Shaker Ball Peptide
(net charge: +3)

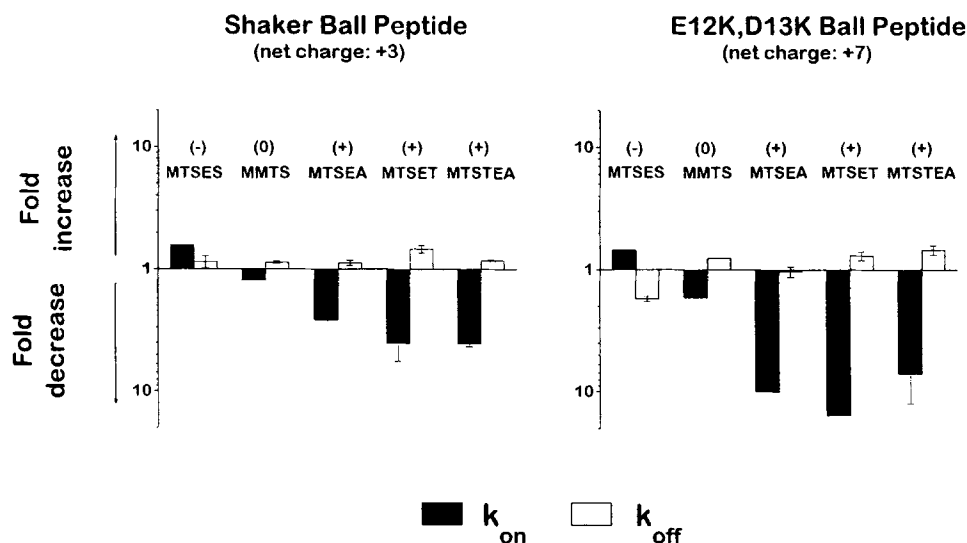**E12K,D13K Ball Peptide**
(net charge: +7)

FIGURE 4. Changes of the binding and unbinding kinetics of the ball peptides by modification with different methanethiosulfonate derivatives. The electrical charge of the moiety added by each reagent is shown. The rates were extracted from the current relaxation as described in MATERIALS AND METHODS and in Fig. 3.
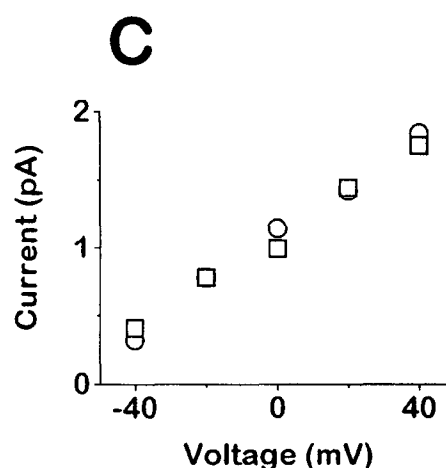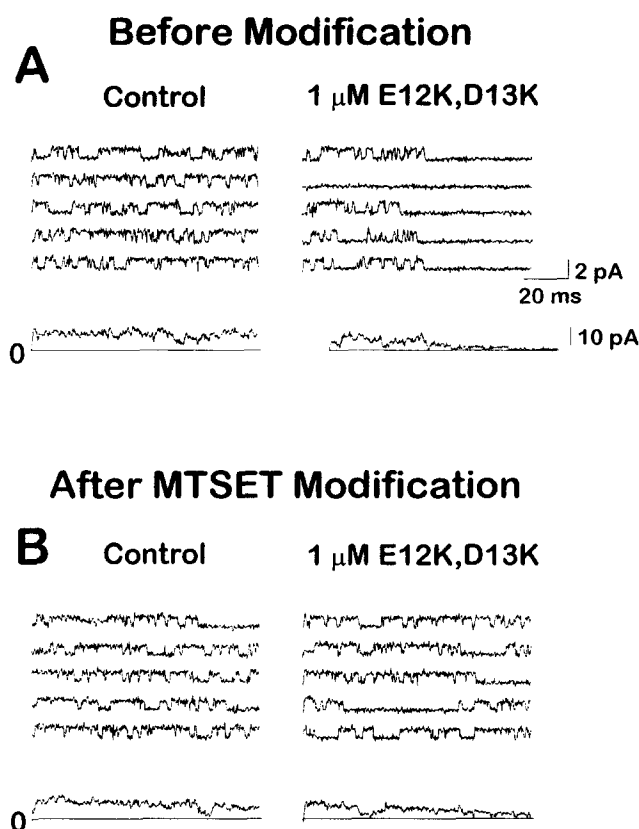
Before Modification

A

Control   **1 μM E12K,D13K**

2 pA
20 ms

10 pA

0

After MTSET Modification

B   **Control**   **1 μM E12K,D13K**

0

C

FIGURE 5. Modification of 391C in the ShΔ K⁺ channel does not alter channel gating or conductance. (A) Five consecutive single channel recordings in the absence (*left*) and in the presence of 1 μM E12K,D13K ball peptide (*right*) are displayed. The sum of ten such records are shown in the bottom panels. (B) After complete MTSET modification (100 μM for 40 s), the same experimental protocol was repeated. (C) In the same patch, single channel I-V curve before (*open circles*) and after (*open squares*) MTSET modification were obtained. Data were sampled at 5 kHz and filtered at 1 kHz.

changes during gating. To measure state-dependent accessibility, we tested the binding of 1 μM E12K,D13K ball peptide before and after controlled modification. As an example, Fig. 6 *A* shows current traces before modification (time 0) and after 3 and 13 s of cumulative exposure to 35 μM of MTSET in the closed state at −80 mV. Control currents (without ball peptide), before and after complete modification, are indicated by dotted traces (Fig. 6 *A*). As an empirical approximation of the time course of modification, we tracked the change in the average current during the last 50 ms of the current traces, as shown in Fig. 6 *B*. The time course of chemical modification could be well described by a single exponential function.

We did similar experiments at different voltages to assess the voltage and state dependence of modification. These results are summarized in Fig. 6 *C* and compared to the relative probability of opening at the different voltages (calculated from the tail currents). The modification rate was higher at positive voltages, consistent either with faster reaction in the open state or with a direct effect of voltage on the modification rate. Because the voltage dependence is steep only in the range of voltages in which gating occurs, we suspect that the changes in MTSET reactivity are due to differences in the accessibility of the cysteine in different gating states: that is, the side chain of 391C is more accessible in the open than in the closed state. Similar results

were obtained for the 391C channel with intact N-type inactivation (data not shown).

Modification Reduces the Current for Cysteine Mutants at Many Other Positions

We also looked at the state-dependence of modification for cysteines at two other positions with relatively rapid rates of modification, positions 381 and 382. For cysteines at these positions, we observed a reduction in current after chemical modification. Although we do not know the specific mechanisms by which the current reduction occurred, we measured the rates of this current reduction as an indication of the state-dependent changes in surface accessibility.

In the 381C mutant, modification by MTSET reduced the current by >80% (Fig. 7, *A* and *B*), and the rates of chemical modification were voltage-dependent. Fig. 7 *B* shows the time course of modification at 0 mV (*open symbols*) and at −120 mV (*filled symbols*). The overall voltage dependence of the rates is shown in Fig. 7 *C*. This voltage-dependent change in k_{app} is apparently a consequence of the channel gating, since modification with the negatively charged MTSES showed the same voltage-dependence (Fig. 7 *D*). This rules out a direct effect of voltage on the local concentration of reagent, since such an effect would be opposite for the two reagents. Moreover, the dependence on voltage is bipha-
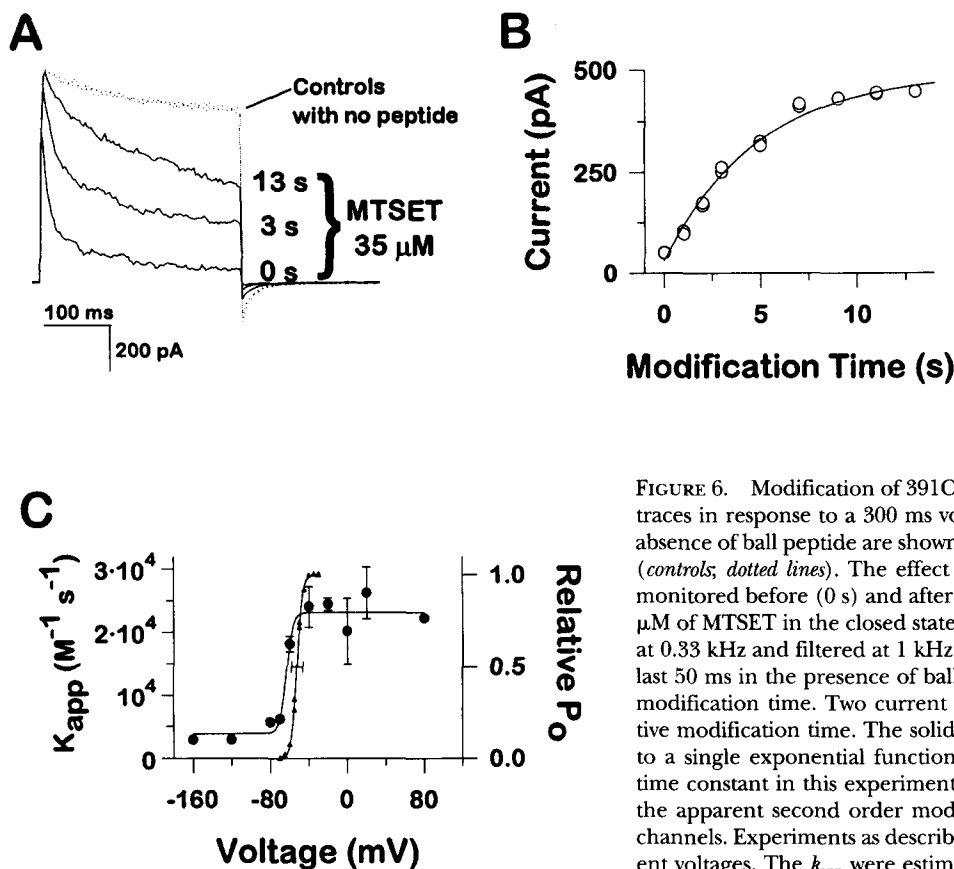
A

Controls
with no peptide

13 s
3 s } MTSET
0 s } 35 μM

100 ms

200 pA

B

Current (pA)

500

250

0

0   5   10

Modification Time (s)

C

K_{app} (M^{-1} s^{-1})

$3 \cdot 10^4$
$2 \cdot 10^4$
10^4
0

Relative P_O

1.0
0.5
0.0

-160   -80   0   80

Voltage (mV)

FIGURE 6. Modification of 391C is faster in the open state. (A) Current traces in response to a 300 ms voltage jumps from −80 to 0 mV in the absence of ball peptide are shown before and after MTSET modification (*controls; dotted lines*). The effect of 1 μM E12K,D13K ball peptide was monitored before (0 s) and after computer-controlled application of 35 μM of MTSET in the closed state at −80 mV. Current data was sampled at 0.33 kHz and filtered at 1 kHz. (B) The average current values of the last 50 ms in the presence of ball peptide are plotted vs the cumulative modification time. Two current traces were sampled for each cumulative modification time. The solid line represents the best-fit of the data to a single exponential function. The best-fit parameter value for the time constant in this experiment was 4.8 s. (C) Voltage dependence of the apparent second order modification rate constant (k_{app}) for 391C channels. Experiments as described in A and B were performed at different voltages. The k_{app} were estimated by the reciprocal of the modification time constant multiplied by the [MTSET] (*filled circles*). The relative probability of opening (P_o) estimated from the tail currents at −55 mV are displayed (*filled upward triangles*). The solid line represents the best-fit of the data to a Boltzmann function:

$$P_O = \frac{1}{1 + \exp\{\frac{zF}{RT}(V_{1/2} - V)\}},$$

where z is the charge valence, $V_{1/2}$ is the mid-point voltage, and R, T, and F have their usual meanings. The best-fit parameter value for z was 8.9 and for $V_{1/2}$ was −53 mV. The horizontal bar represents the standard deviation (5.8) from the mean of $V_{1/2}$ (−52.4).

sic, with the fastest modification in the same range of voltages over which the gating occurs. The 382C mutant shows a similar biphasic voltage dependence, but with the voltage dependence for both gating and modification shifted toward more positive voltages by ~50 mV (compare Fig. 7, C and E).

Because there is little or no C-type inactivation in the T449V-substituted channels that we use, the reduced reaction at positive voltages is unlikely to result from inactivation. We suspected instead that this unusual voltage dependence for the modification rate at these two positions might indicate that their accessibility was highest not in the steady-state open state or in the resting closed state, but in some intermediate state whose population was highest at this intermediate voltage. To test this idea, we used pulse trains with a constant duty cycle (i.e., a constant average open probability), but with different frequencies. At the higher frequencies, the chan-

nel would presumably spend more of its time in states that are intermediate between the open and closed states. The apparent rate of modification for the 381C mutant indeed increases with a rapidly switching stimulus (Fig. 7 F). For instance, using steps from −100 to 0 mV, the rate of modification was about threefold faster if we applied 50 10-ms pulses rather than 5 100-ms pulses. Our ability to study the kinetics of the most rapidly modified state(s) is limited by the relatively high reactivity of the closed state, but the results are certainly consistent with more rapid modification of a state that is visited mostly during the transition between the closed and open state.

Overall Modification Pattern of the S4-S5 Linker

Fig. 8 A shows the maximal modification rates for mutants in which chemical modification was detected. The measured rates varied over four orders of magnitude,
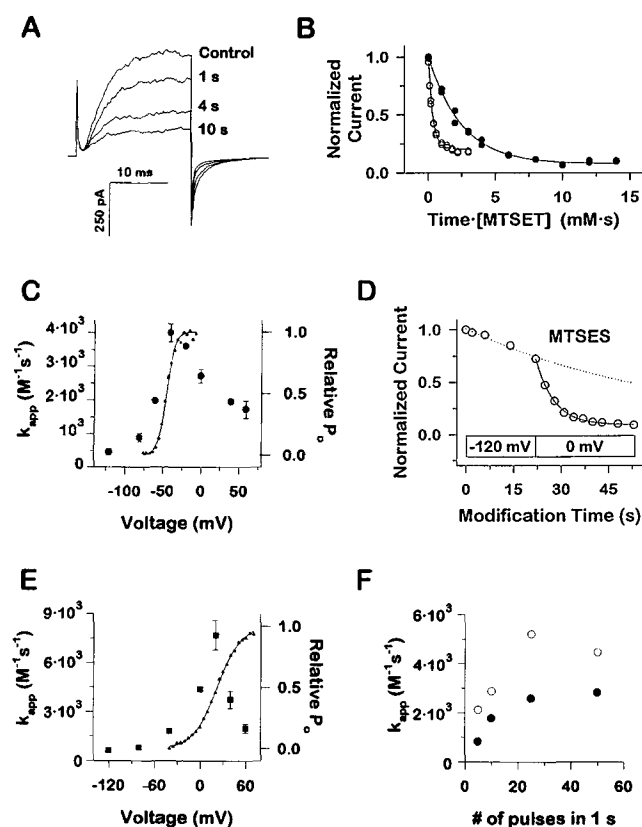
FIGURE 7. Modification of many S4-S5 cysteines produced substantial current reduction. (*A–D*). Chemical modification of 381C. (*A*) Current traces in response to a 20 ms voltage step to 0 mV from a holding potential of −100 mV before (*control*) and after chemical modification. Computer-controlled MTSET applications were performed for 1 or 2 s starting ∼500 ms after the onset of the voltage jump to 0 mV. Beside each current record is indicated the cumulative modification time. Data was acquired at 5 kHz and filtered at 1 kHz. (*B*) Time course of the normalized current reduction of modifications performed at 0 mV (*open circles*; data from *A*) and at −120 mV (*filled symbols*) are shown. Since these experiments at different voltages were performed with two different [MTSET], the time axis was normalized to the [MTSET]. Solid lines represent the best-fit of the current reduction data to single exponential functions. The best-fit parameter values for the slopes were 0.33 s·mM at 0 mV and 2.47 s·mM at −120 mV. (*C*) Voltage dependence of the k_{app}. Open symbols represent the k_{app} obtained from the reciprocal of the calculated slope values of cumulative modification (e.g., *B*), as in Fig. 6 *C*. The small dots show the P_o estimated from the tail currents at −55 mV. The solid line represents the best-fit of the data to a Boltzmann function. The best-fit parameter value for *z* was 4.6 and for $V_{1/2}$ was −45 mV. (*D*) Temporal course of modification with 100 μM MTSES. The first 22 s of cumulative modification time was performed at −120 mV. The time constant of modification was 63 s (*dotted line*). In the same patch, modification was continued at 0 mV. The modification rate (time constant of 5.7 s; continuous line) was ∼10 times faster than at −120 mV. (*E*) Voltage dependence of the k_{app} for chemical modification of 382C. The small dots show the P_o estimated from the tail currents at −15 mV. The best-fit parameter value for *z* was 1.6 and for $V_{1/2}$ was 22 mV. (*F*) Frequency dependence of modification rates for 381C. The voltage was changed between −100 and 0 mV (*open circles*) or between −100 and +60 mV (*filled circles*). The ratio of the time at both potentials was always equal to one.

from ∼8 M^{-1} s^{-1} to ∼20,000 M^{-1} s^{-1}, with no signs of a correlation between the extent of current reduction and the modification rate. Since this region has been hypothesized to fold as an α-helix (Isacoff et al., 1991) and perhaps to participate in a leucine-zipper (McCormack et al., 1991), we arranged our results in a helical wheel representation, considering both the reaction rates for those residues that were reactive (Fig. 8 *B*) and the degree of current reduction produced by modification (Fig. 8 *C*). In most cases where current reduction was <25%, we could not measure reaction rates, nor could we conclude firmly that these residues were inaccessible to reaction (since it might be that reaction occurred but had no effect on the channel current). For example, by the criterion of current reduction, the cysteine at 391 would be judged unreactive, but we actually found using a different assay (effects on binding of ball peptide) that this residue is highly reactive. Conversely, some positions at which modification produced a large reduction in the current were only very slowly reactive (389 and 397).

DISCUSSION

Electrostatic Interaction between S4-S5 and the Ball Peptides

Murrell-Lagnado and Aldrich (1993*a, b*) found that increasing the net positive charge of the ball peptide produces an increase in the association rate. This effect could be explained by supposing a simple long range electrostatic interaction between the peptide and its receptor. Our results on the chemical modification of 391C support this hypothesis and identify this residue as being at or near the receptor site for the ball peptide. We found that k_{on} was sensitive to the electrical charge of the moiety added by modification. Adding a negative charge increased k_{on}, while adding a positive charge decreased it. Chemical modification with MMTS, which does not change the formal charge on the thiol, produced less than a twofold decrease in k_{on}. This small effect might be explained by the partial negative charge on the cysteine thiol group (due to deprotonation) at pH 7.4.

The electrostatic nature of the modification effect was also tested by using two ball peptides of different net charges. In both peptides, the COOH terminals were amidated and the NH_2 terminals remained positively charged, giving a net charge for the Shaker ball peptide and E12K,D13K ball peptide of +3 and +7, respectively. Maximal modification of 391C with positively charged reagents reduced k_{on} by 2.5–4-fold for Shaker ball peptide and by 5–15-fold for E12K,D13K ball peptide. This 2 to 3 times larger effect for the more positively charged peptide is comparable with the difference in the net charge between the two ball peptides. Chemical modification of 391C with MTSES, which introduces a
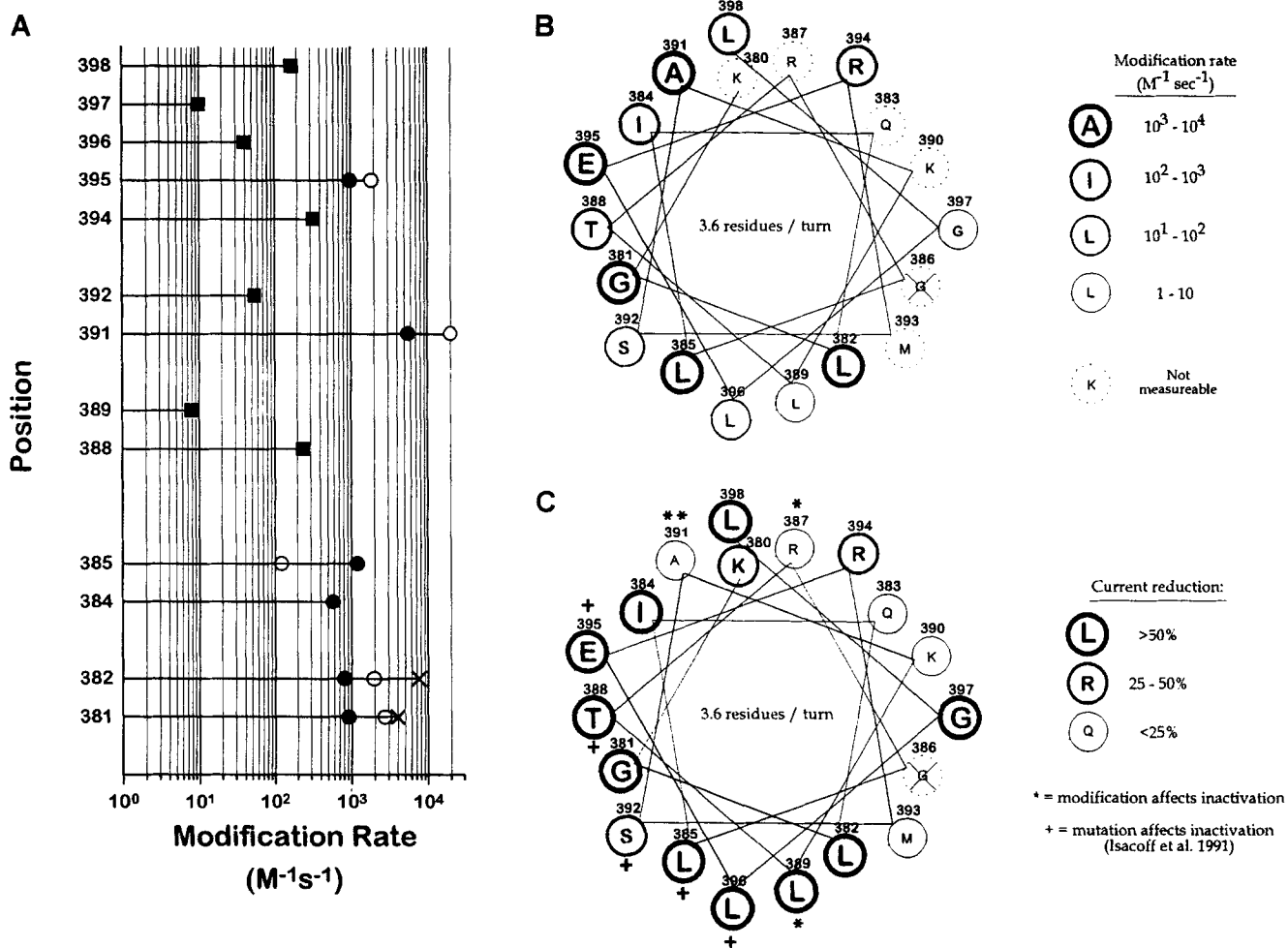
FIGURE 8. MTSET modification rates of residues in the S4-S5 linker. (A) Modification rates by MTSET in positions where chemical modification was detected and substantial (i.e., current reduction >25%; at position 391, the modification rate was determined from changes in ball peptide affinity). The symbol indicates the conditions for modification: ■, steady brief pulses (usually a 20-ms pulse presented every 4 s); ●, closed state (−80 mV); ○, open state (0 mV); ×, flicker protocol (Fig. 7 F). (B and C) Helical wheel representation of the reaction rates (B) and the degree of current reduction with maximal modification (C; from Fig. 1) for the cysteine mutants. The wild-type residue is indicated by the single-letter code in each circle. The three positions at which modification had substantial effects on inactivation are indicated with stars (*); positions at which Isacoff et al. (1991) found a substantial effect of mutation on N-type inactivation are marked with +. The G386C mutant did not express measurable currents.

negative charge, produced a 1.5–2-fold increase in the k_{on} of the ball peptides. Although compatible with electrostatic interactions between the modified channels and the ball peptide, these changes were smaller than those observed with positively charged moieties. These discrepancies might be explained by supposing that after modification with MTSES, another step becomes rate limiting in the process of ball peptide blockade. All these results are consistent with the idea that the ball peptide behaves as a point charge (that is, there are no specific charge-charge interactions), and that long-range electrostatic interactions increase the rate of diffusion of the peptide to the binding site (Murrell-Lagnado and Aldrich, 1993a, b). As suggested by Murrell-Lagnado and Aldrich, it appears that dissociation is

governed not by long-range electrostatic interactions but primarily by close interactions like the hydrophobic effect.

By performing modification experiments during single channel recordings, we have shown the effect of modification at 391C on ball-peptide binding and inactivation is probably direct rather than allosteric. Chemical modification did not change either the gating properties of the channel or the single channel conductance. Therefore, the changes of k_{on} observed with chemical modification probably resulted from changes in the local potential at the vicinity of the binding site.

Because the early studies on N-type inactivation showed the importance of the positively charged residues in the inactivation domain (Hoshi et al., 1990), the nega-

tively charged aspartate and glutamate residues in the core region of the channel were suspected as possible countercharges for the ball domain. Isacoff et al. (1991) found that mutating glutamate 395 to glutamine (E395Q) practically eliminates the fast component of inactivation. Similarly, we found that the E395C mutant binds the E12K,D13K ball peptide very poorly (K_d ~17 μM, compared to 0.5 μM for the wild type). This ~30-fold difference in affinity could be ascribed exclusively to changes in the k_{on} (data not shown).

Why is N-type inactivation impaired when glutamate 395 is mutated? To address this question, we also examined the sensitivity of E395C to two other internal open channel blockers: tetraethylammonium (TEA) and decyltriethylammonium (C_{10}-TEA). The affinity of the E395C mutant for both TEA and C_{10}-TEA was reduced fourfold compared with the wild-type channel. One possibility is that this region is the receptor for all three blockers, and mutations in 395 affect the binding affinity directly. However, single channel recordings of mutants at position 395 show constant flickering between the open and closed states (data not shown), with a substantial reduction in open probability. (It is difficult to get a good estimate of the open probability since the flickering makes it impossible to measure the true open channel current.) Because TEA, C_{10}-TEA and the ball peptide are all open channel blockers, their apparent affinity will depend on the open probability of the channel. Thus, the flicker with reduced open probability may account for the $\geq$fourfold reduction in blocker affinity. The observation that the ball peptide affinity is reduced even more (~30-fold) suggests that in addition there may be a direct effect on the receptor site for the ball.

We suggest that mutations at residue E395 somehow destabilize the open state, producing a "flickering channel." This glutamate residue is conserved in all K^+ channels, and even the charge-conservative mutation of this residue to aspartate disrupts N-type inactivation (Isacoff et al., 1991). The nearby mutation L398C produces similar effects (data not shown). Besides being so critical for the stability of the channel, the negative charge of E395 might also play a role in the N-type inactivation (though this is difficult to evaluate in the face of the other changes).

State-dependent Modification of Cysteines Introduced in the S4-S5 Linker

State-dependent cysteine modification has been used successfully to detect protein conformational changes (Slatin et al., 1994; Yang and Horn, 1995; Liu et al., 1996; Yang et al., 1996). We found state-dependent changes in the rate of chemical modification for the mutants 381C, 382C, and 391C, indicative of a conformational change in the S4-S5 region with activation gat-

ing. These residues are always accessible to modification but, when the channel is the open state and/or an almost-open state, these positions become approximately five times more reactive than they were in the resting state. Several reasons suggest that the differences in reactivity reflect an actual movement of the region. First, state-dependent modification of 381C with different MTS derivatives were similar regardless of the charge of the reagent. This rules out any direct effect of voltage on the accessibility of the reagent to the sulfhydryl side chain, or a change in the local electrostatic potential (which could change the pKa of the cysteine and thus the fraction of the reactive thiolate species). Second, the ascending part of the voltage dependence of modification of 381C and 382C differ in the steepness and the voltage at which the maximal modification rate was reached. These differences parallel the difference in gating between the two mutants, suggesting that the changes in reactivity are a consequence of gating.

Recent experimental results on chemical modification of residues in the transmembrane segment S4 have shown a strong state dependence indicating a significant movement of the S4 during activation gating (Larsson et al., 1996; Mannuzzu et al., 1996; and unpublished results; for Na^+ channels, see Yang and Horn, 1995; Yang et al., 1996). The apparent movement of the S4-S5 region detected in our experiments might be a consequence of coupling with the voltage driven movement of the transmembrane S4 domain.

With gating, we see changes of 4- to 10-fold in the modification rates. These changes might exaggerate the size of the movement, since even a very small motion might change the reaction rate severalfold. On the other hand, they might understate the differences in accessibility of the various gating states. Our measurements are made under conditions that favor occupancy of a particular state, but we cannot guarantee that only one gating state is occupied. It might be that we cannot reduce occupancy of an undesired (but rapidly reactive) gating state below 10%. This would prevent us from ever seeing changes larger than 10-fold. Although at negative voltages the channel open probability may be much below 1%, that does not mean that the individual subunits are never in their activated state. For instance, if the channels are open only when all four subunits are in the activated position, and if the subunits gate independently, then a "leaky" process that leaves 10% of the subunits in the activated position will nevertheless ensure that the channels are 99.99% closed.

Pattern of MTSET Modification of the S4-S5 Linker

Cysteine site-directed mutagenesis combined with chemical modification has been used to delineate accessibility patterns in small portions of channel proteins (Akabas et al., 1992; Kürz et al., 1995; Lü and Miller, 1995;

Pascual et al., 1995). We have explored the modification properties of 19 consecutive residues in the S4-S5 linker of Shaker K$^+$ channel. As Fig. 8 *B* and *C* illustrates, most of the highly reactive residues would be located on one face of an α-helix, consistent with earlier suggestions about the structure of this region (Isacoff et al., 1991; McCormack et al., 1991). The last five residues (394–398) can be all modified. This could mean either the α-helix is broken somewhere around 393 or the α-helix is maintained but that all the side chains of the last turn are accessible to the sulfhydryl reagent. Any conclusions about secondary structure must be taken with caution, because the lack of an effect by modification does not necessarily mean that a residue is buried. The 391C mutant is particularly striking in this respect, because modification produces no reduction in current, but from the effect on binding of the ball peptide we know that the rate of modification is very fast.

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