Phenylalanine 1489 in the inactivation gate of the rat brain IIA sodium channel α subunit is required for stable inactivation. It is proposed to move into the intracellular mouth of the pore and occlude it during inactivation, but direct evidence for movement of this residue during inactivation has not been presented. We used the substituted cysteine accessibility method to test the availability of a cysteine residue substituted at position 1489 to modification by methanethiosulfonate reagents applied from the cytoplasmic side. Mutation of Phe-1489 to modification by methanethiosulfonate reagents irreversibly slowed the inactivation rate and decreased the fraction of inactivating current of F1489C but not wild-type channels. Single channel analysis showed that modification slowed inactivation from both closed and open states and destabilized the inactivated state. Depolarization prevented rapid modification of Cys-1489 and the voltage dependence of their reaction rate correlated closely with steady-state inactivation. Modification was not detectably voltage-dependent at voltages more negative than channel gating. Our results show that, upon inactivation, Phe-1489 in the inactivation gate moves from an exposed and modifiable position outside the membrane electric field to a buried and inaccessible position, perhaps in or near the intracellular mouth of the channel pore.

Voltage-gated Na⁺ channels initiate the action potential in most excitable cells. Na⁺ channels open in response to depolarization, resulting in Na⁺ influx through a Na⁺-selective pore. Within a few ms after opening, the channels convert to a nonconducting inactivated state, from which they recover only on repolarization. The rat brain Na⁺ channel consists of three glycoprotein subunits: α (260 kDa), β1 (36 kDa), and β2 (33 kDa) (reviewed in Ref. 1). The α subunit is composed of four homologous domains (I–IV), each with six transmembrane segments (S1–S6; Refs. 2 and 3). Expression of the α subunit in mammalian cells (4, 5) or Xenopus oocytes (6, 7) yields functional Na⁺ channels, although co-expression of β1 and β2 accelerates activation and inactivation in oocytes (8, 9).

Treatment of the intracellular surface of Na⁺ channels with proteolytic enzymes specifically blocks inactivation, indicating that intracellular parts of the channel are required for inactivation (10, 11). Studies using site-specific antibodies and site-directed mutagenesis indicated that the intracellular loop between homologous domains III and IV (LIII-IV) is critically involved in inactivation (12–14). In LIII-IV of the rat brain type IIA α subunit, mutation of Phe-1489 in the center of a cluster of three hydrophobic amino acids, IFM, to glutamine almost completely prevents inactivation (15), and the analogous amino acid residues in cardiac Na⁺ channels are also critical for fast inactivation (16, 17). Peptides containing the IFM motif can block open Na⁺ channels, consistent with the idea that these residues serve as an inactivation particle that enters the intracellular mouth of the pore and blocks it during inactivation (18). Based on these studies, it has been proposed that the intracellular loop between domains III and IV serves as an inactivation gate, which closes over the intracellular mouth of the pore and binds to a putative inactivation gate receptor via the IFM motif when the channel inactivates (1, 15, 18). This mechanism of inactivation predicts that the amino acid residue at position 1489 should become inaccessible to reaction with intracellular reagents during the inactivation process. To test this idea, we have expressed a mutant Na⁺ channel with a cysteine residue at position 1489 and analyzed its reaction with charged methanethiosulfonate (MTS)¹ derivatives and with silver. The effect of these reagents on inactivation of F1489C depends on the size of the charged group introduced and on its distance from the polypeptide backbone. The accessibility of residue Cys-1489 to reaction with MTS compounds applied from the cytoplasmic side depends on the functional state of the channel and is greatly reduced during steady-state inactivation. Our results are consistent with movement of Phe-1489 in the inactivation gate from the cytoplasm into the channel protein upon inactivation.

EXPERIMENTAL PROCEDURES

Na⁺ Channel Expression—Site-directed mutagenesis was performed as described in Ref. 15. RNA encoding wild type (WT) and F1489C mutant Na⁺ channel α subunits and WT β1 subunits was synthesized in vitro using the Ambion mMessage mMachine kit. Isolation, purification, and maintenance of Xenopus oocytes was done as described (9). Healthy stage V and VI oocytes were pressure-injected with 50–100 nl of a solution containing a 1:1 ratio of α and β1 subunits at a concentration of 10–200 ng/µl. Electrophysiological recordings were carried out 2–8 days after injection.

Electrophysiological Recording—The excised inside-out configuration of the patch clamp technique (19) was used to obtain both macro-patch and single channel data. Before recording, the vitelline layer was removed as described (20). For macropatch recording, the bath solution contained 10 mM NaCl, 140 mM KCl, 1 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES, pH 7.4. For single channel patches KCl was reduced to 90 mM. For treatment with Ag⁺, the bath solution contained 150 mM potassium aspartate, 1 mM Mg(NO₃)₂, 10 mM EGTA, and 10 mM
HEPES, pH 7.4. For macropatch recording, the pipette solution contained 150 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES, pH 7.4. For single channel recording it contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.4. The volume of the bath chamber was about 0.3 ml and was perfused continuously with bath solution (3 ml/min). Excised patches were placed in a stream of the bath perfusate. Currents were recorded with a List EPC-7 patch clamp amplifier and filtered at 8 (macropatch currents) or 5 (single channel currents) Hz prior to digitization at 30 kHz. Aqueous stock solutions of MTS ethylammonium (MTSEA), MTS ethylsulfonate (MTSES), and MTS ethyltrimethylammonium (MTSET) (Toronto Research Chemicals) were prepared just prior to the experiment, maintained on ice, and diluted in the bath solution immediately before use.

**Data Analysis**—Single channel openings were detected using standard half-amplitude threshold analysis after digital filtering at 2 kHz (21). Patches contained one to four channels. Channel number was estimated from the maximum number of overlapping openings at potentials where the probability of opening was high (22). Open times, closed times, and first latencies were analyzed using PSTAT (Axon Instruments, Foster City, CA). Overlapping openings were omitted from analysis. Averaged data are presented as mean ± S.E., except for single channel data, which are given as mean ± S.D.

**Model Simulation**—Data predicted by particular models were simulated assuming one or two channels in the patch. For simulation, the minimum detectable duration was set at that observed experimentally in our recording system after filtering. Histograms of simulated data were generated using the same criteria used in generating histograms of experimental data. These predicted histograms were compared with the experimental data and rate constants adjusted to produce good fits to the data. In general the rate constants of Scheme 1 (see below) are highly determined by the data. The sum of the rates leaving the inactivated state, $f + h$, was chosen to fit the major component of closed times. The sum $d + g$, the two rates for leaving the open state, was determined from the open time. Values for $d, e,$ and $g$ were chosen to fit the burst duration and give the correct fraction of short closed times. The opening rate constant, $c$, was chosen to fit the duration of the rapid flickers in the bursts of MTSET-modified channels. Finally, the ratio of $c$ to $e$ was determined from the ratio of the rapidly versus slowly activating currents in the first latency histogram.

**RESULTS**

**Effects of Cysteine Reagents on Inactivation of the Mutant Na⁺ Channel F1489C**—Replacement of Phe-1489 by hydrophobic amino acids leaves inactivation almost unchanged, whereas replacement by hydrophilic or charged amino acids almost completely removes inactivation (48). ² We constructed mutant F1489C in order to examine the chemical reactivity of the amino acid residue at position 1489. Co-expression of the mutant α subunit F1489C together with the βι subunit in Xenopus oocytes resulted in Na⁺ channels with altered inactivation; 8% of the Na⁺ current failed to inactivate by the end of an 11-ms-long depolarization compared with 2% for the WT channel (Fig. 1A). The midpoint for the voltage dependence of activation of F1489C measured in cell-attached macropatches was not changed in comparison with WT, while the midpoint for the voltage dependence of inactivation was shifted from $-101 ± 6$ mV for WT (n = 12) to $-84 ± 4$ mV (n = 3) for F1489C.

Ag⁺ reacts with sulphhydryl groups to form a strong S–Ag bond (23). After reaction with Ag⁺, cysteines have increased hydrophilicity but are uncharged. When AgNO₃ (500 nM) was added to the cytoplasmic side of inside-out patches, inactivation was almost completely removed (Fig. 1B; 87 ± 6% noninactivating current, n = 4), and the peak current amplitude at $-20$ mV was increased by $49 ± 28%$ (n = 4). AgNO₃ did not affect inactivation of WT channels (data not shown), but application of AgNO₃ to the extracellular surface of WT or F1489C channels in outside-out patches reduced peak Na⁺ currents of both WT and F1489C substantially, suggesting that this reagent reacts with another cysteine residue in the Na⁺ channel in addition to the inserted Cys-1489. To avoid possible effects of reaction at this second site, we focused primarily on reaction with MTS reagents.

Charged methanethiosulfonate reagents can be used to add either positively or negatively charged groups to cysteine residues by disulfide exchange (24, 25). MTS reagents did not affect either the magnitude of WT Na⁺ currents or their rate of decay in four patches (data not shown). In contrast, the MTS reagents had marked effects on inactivation of F1489C mutant channels. Application of 50 nM MTSET, which adds a positively charged group, nearly completely blocked fast inactivation of F1489C Na⁺ channels (Fig. 1C). The smaller MTS reagents, MTSES (500 nM), which adds a negatively charged group, or MTSEA (150 nM), which adds a positively charged group, slowed inactivation significantly and also slightly increased the fraction of noninactivating Na⁺ current observed at the end of the pulse (Fig. 1, D and E). The difference between these results and those obtained with MTSET-treated channels was not caused by incomplete reaction of MTSES and MTSEA. Application of MTSET after MTSES treatment had no additional effect on inactivation, indicating that the reaction of F1489C with MTSES was complete (n = 2, not shown).

Treatment with MTSET reagents also increased peak sodium current (Fig. 1, C–E). The extent of increase was correlated with the magnitude of increase of mean open times that is observed with the different MTS reagents (see below), consistent with the conclusion that slowed inactivation allowed more superimposed channel openings and thereby increased peak current.

Table I summarizes the effects of MTS reagents and Ag⁺ on voltage-dependent channel gating. Treatment of F1489C with MTS reagents led to a 4–9 mV negative shift in the apparent voltage dependence of activation. Treatment with AgNO₃ resulted in a 14-mV negative shift. These apparent voltage shifts may be caused indirectly by slowing of inactivation, as observed for α-scorpion toxins and proteolytic enzyme treatment (26, 27). The voltage dependence of steady-state inactivation was unchanged after MTSEA and MTSES treatment.

**Single Channel Analysis of the Effects of MTSEA and MTSET**—To understand the microscopic state transitions un-
underlying the macroscopic behavior of MTS-modified Na\(^+\) channels, single channel experiments were carried out with the two positively charged MTS derivatives, MTSEA and MTSET. WT type IIA Na\(^+\) channels open only once or twice at the beginning of depolarizing pulses, inactivate, and rarely reopen later in depolarizations (e.g. Refs. 28 and 29). Fig. 2 shows representative single channel traces and the corresponding ensemble averages (last traces in each column) at −20 mV for F1489C Na\(^+\) channels before and after reaction with MTS reagents. The ensemble averages were derived from all analyzed single channel openings at −20 mV and were similar in time course to the macropatch currents shown in Fig. 1. Unmodified F1489C channels open soon after depolarization and reopen several times during a 40-ms depolarization, consistent with the small fraction of noninactivating current observed in macropatch voltage clamp records (Fig. 2). Single channel open times are well fit by a single exponential with a time constant of 0.27 ms (Fig. 3A, Table II), consistent with the presence of a single predominant open state. The closed time distributions showed two components, which had time constants of 0.75 and 8.6 ms at −20 mV in patches containing two Na\(^+\) channels (Figs. 2 and 3B, Table II). The frequent reopening of F1489C channels indicates that fast inactivation of these channels is reversible and that the longer components of closed times represent sojourns in the destabilized inactivated state.

Modification of F1489C channels by MTSEA increased channel open times approximately 2-fold (Figs. 2 and 3A, Table II). This increase in mean open time is an important contributor to the slowed decay of the Na\(^+\) current following reaction with MTSEA (Fig. 1E). The major component of the closed time distribution was similar in duration to the predominant closed state in unmodified F1489C channels (Fig. 2). Thus, the increased open time is primarily responsible for the slight increase in sustained Na\(^+\) current after reaction with MTSEA (Fig. 1E). These results indicate that the rate of inactivation was substantially decreased by modification with MTSEA, but the stability of the inactivated state was nearly unaffected.

Modification of F1489C channels by MTSET caused even longer open times (Fig. 2). A single time constant of 1.47 ms was observed, approximately 4.6-fold longer than unmodified F1489C (Fig. 3A). MTSET-modified channels showed long bursts of openings and both long and short channel closures were evident. Within bursts, the openings were interrupted by short closings, and bursts were separated by longer closings. The two main closed times estimated by fits to closed time histograms were 0.1 and 0.75 ms at −20 mV, far shorter than the corresponding values for unmodified F1489C channels (Fig. 2). Single channel traces and ensemble averages of unmodified and MTSEA- and MTSET-treated F1489C channels. Traces of single channel activity and ensemble averages (last trace in each column) from excised inside-out patches are shown. The arrows indicate the beginning of 40-ms depolarizations to −20 mV from a holding potential of −140 mV. The vertical calibration bar is 1 pA for single channel traces and 0.5 pA for ensemble averages. Numbers of channels in the patches for single channel traces shown were two for F1489C and MTSEA-modified F1489C and one for MTSET-modified F1489C. Ensemble average traces are the average of all current traces analyzed in Fig. 3. The dotted traces are the ensemble average of F1489C for comparison.

Effects of MTSEA and MTSET on Inactivation Rate Con-
FIG. 3. Single channel properties of unmodified and MTSEA- and MTSET-treated channels. Analysis of single channel kinetics. For modified channels, recordings were made before and after 3-min perfusion with 1.5 μM MTSEA or 500 nM MTSET. Data are from six (F1489C), two (F1489C + MTSEA) and two patches (F1489C + MTSET). A, single channel open time histograms at −20 mV. Patches containing one to four channels were used for F1489C and MTSEA-modified F1489C open time analysis. Patches containing one or two channels were used for MTSET. The solid lines are fits of single exponentials to the log binned data (47) for durations >100 μs (Table II). The dashed lines are single exponentials with the F1489C time constant. The dotted lines show simulated open time distributions calculated using the parameters given in Table III. B, closed time histograms at −20 mV. Only closed times occurring after the first opening of a depolarization were analyzed. The solid lines are fits of the sum of two (F1489C, F1489C + MTSEA) or three (F1489C + MTSET) exponentials to the log binned data with durations >100 μs (Ref. 47, Table II). The dotted lines show closed time distributions calculated from model simulations using the parameters given in Table III. Data are from patches with two channels for F1489C and F1489C + MTSEA. With F1489C + MTSET, data are from patches with one or two channels, but only sweeps with one active channel were analyzed. Sweeps with two active channels were readily detected due to the high probability of opening after MTSET modification. C, cumulative first latency distributions at −40 mV displayed on two different time scales. Distributions were corrected for the channel number (30). The dotted lines are fits to the double exponential equation \( p = f \times (1 - \exp(-x/\alpha)) + (1 - f) \times (1 - \exp(-x/\beta)) \), where \( p \) is the probability, \( f \) is the fraction of the fast component, \( \beta \) is the lag, and \( \alpha \) and \( \beta \) are the time constants of the fast and slow components, respectively. \( f, \beta, \alpha, \) and \( b \) were 0.30 ms, 0.44, 0.52 ms, and 25.37 ms (F1489C); 0.30 ms, 0.63, 0.43 ms, and 19.37 ms (F1489C + MTSEA); and 0.21 ms, 0.65, 0.45 ms, and 4.00 ms (F1489C + MTSET). Data are from patches with two channels (F1489C, F1489C + MTSEA) and one or two channels (F1489C + MTSET). Null sweeps generally occurred in long runs and were omitted from this analysis. The numbers of events fitted were 555 (F1489C), 450 (F1489C + MTSEA), and 236 (MTSET).

Quantitative description of the changes in single channel gating properties requires fitting to a specific model of the state transitions that underlie the opening and closing of the channel. The simple kinetic model shown in Scheme 1 has been used successfully to describe the gating of single Na\(^+\) channels during depolarizations to −40 mV or more positive membrane potentials (31–34).

\[ C_1 \xrightarrow{a} C_2 \xrightarrow{b} C_3 \xrightarrow{c} O \]

**SCHEME 1**

Upon depolarization, Na\(^+\) channels undergo voltage-dependent transitions through multiple closed states (states \( C_1 \) through \( C_n \)) and then inactivate (state I). Channels can also pass directly from one (or more) closed states to the inactivated state as illustrated. The simple model shown in Scheme 1 is advantageous for simulation and fitting of single channel data because the rate constants are constrained by the requirement for microscopic reversibility in the C-O-I loop.

We used the model in Scheme 1 to simulate our data for depolarizations to −20 mV, incorporating corrections for the number of channels in the patch and missed transitions due to the limited frequency response of the recording system. Open time distributions were well fit by a single exponential with increased mean open times after reaction with MTS reagents (Fig. 3A). The results indicate that a single predominant open state is achieved by both unmodified and MTS reagent-modified channels, but the lifetime of the open state is prolonged by reaction with MTS reagents.

The effects of MTSEA modification on our data were reproduced (Fig. 3, A and B, dotted lines) by changes in the time constants governing the rate of inactivation from open state (g) and the rate of inactivation from closed states (e) with comparatively little change in the rate constants that govern the stability of the inactivated state once formed (f and h; see best fit parameters given in Table III). The rate of activation, measured from the rapid component of the first latency distribution, did not vary detectably upon reaction with MTS reagents (Fig. 3C and similar results at −20 mV; data not shown), dictating a constant value of the rate constant c (Table III). The increase in mean open time upon MTSEA treatment is reproduced by a reduction in the transition rate from O to I (g) from 3450 s\(^{-1}\) in F1489C to 1380 s\(^{-1}\) in the MTSEA-modified channel. The reduced fraction of slowly opening channels in the first latency histogram is reproduced by reducing the transition rate for \( C_n \) to I (e) 1.3-fold from 9300 s\(^{-1}\) to 7080 s\(^{-1}\). The changes in mean open times and time course of the first latency distribution caused by reaction with MTSET could be reproduced by larger changes in the same rate constants, indicating more extreme slowing of the rate of channel inactivation.
from open and closed states and substantial destabilization of
the inactivated state by reaction with this reagent. The rate of
inactivation from closed states, while MTSET destabilizes the inactivated
state, was only a small effect on the stability of the
Cys-1489.

Kinetics of Reaction with Cys-1489—The dramatic effects of
MTSET and MTSES on inactivation allowed us to accurately
measure the time course of the modification of Cys-1489 by
these reagents. Fig. 4 shows the time course of reaction of
Cys-1489 during cytoplasmic application of 500 nM MTSES to
excised inside-out patches. Quantification of the modification is
described in the legend to Fig. 4. Patchs were held at −140
mV, and 15-ms test pulses to −20 mV were applied every 10 s
to determine the inactivation kinetics of the Na+ current. The
reaction was 90% complete after 3 min. The rate of MTSES
modification at −140 mV could be fit by a function containing
a lag, b, followed by an exponential time course with rate a (Fig.
4). For modification by 500 nM MTSES (Fig. 4), the fit parameters
were \(a = 0.0133 \pm 0.0007 \text{ s}^{-1}\) and \(b = 40 \pm 7 \text{ s} (n = 3)\).
The lag did not vary with different reagents or between
experiments. These kinetics are consistent with a perfusion-dependent
lag followed by bimolecular reaction of MTSET with
Cys-1489.

State Dependence of Cys-1489 Accessibility—Having defined
the effects of reaction of Cys-1489 with the MTS reagents, we
next examined whether their rate of reaction was dependent on
channel state. We hypothesized that accessibility of Cys-1489
during cytoplasmic application of 500 nM MTSET
and MTSES on inactivation allowed us to accurately
measure the time course of the modification of Cys-1489 by
these reagents. Fig. 4 shows the time course of reaction of
Cys-1489.
Modification of Cys-1489 at a given reagent concentration is proportional to the fraction of channels in which Cys-1489 is available for reaction, providing a direct estimate of accessibility.

To test the accessibility of Cys-1489 in different channel conformations, F1489C channels were modified by brief exposure to MTSES at different holding potentials (Fig. 5). First, patches were held at $-140 \text{ mV}$, and test pulses were applied every $10 \text{ s}$ (until time $1$ in Fig. 5A; trace 1 in Fig. 5B). The holding potential was then either maintained at $-140 \text{ mV}$ or changed to $0 \text{ mV}$ without pulsing for $5 \text{ min}$ ($V_H$ in Fig. 5A). During this time, the patch was exposed to $500 \text{ nM}$ MTSES for $3 \text{ min}$, followed by a $2\text{-min}$ wash with reagent-free medium. The membrane potential was then returned to $-140 \text{ mV}$, and test pulses to $-20 \text{ mV}$ were applied every $10 \text{ s}$ to assay the effect of modification. Channels held at $-140 \text{ mV}$ had been almost completely modified when pulses were resumed (Fig. 5B, trace 2; Fig. 5A, filled circles). In contrast, only $5\%$ of channels exposed to MTSES at $0 \text{ mV}$ had been modified (Fig. 5C, trace 2, Fig. 5A, open circles). To ensure that the unmodified channels could be modified at a negative membrane potential, the patch was then re-exposed to MTSES at a holding potential of $-140 \text{ mV}$ (Fig. 5A, asterisk). This resulted in rapid modification (Fig. 5, B and C, traces 3), confirming that a reversible, voltage-dependent change in channel state was responsible for the change in the MTSES reaction. In all experiments with $V_H = 0 \text{ mV}$, a slight increase in modification was seen during the interval between the first and second applications of MTSES reagent when no reagent was present. This could have been due to residual reagent remaining in the membrane after washing. Analogous experiments using MTSET as the modifying agent gave similar results (Fig. 5, D–F). These experiments show clearly that the reactivity of Cys-1489 for modification by MTSES and MTSET is greatly reduced when channels are depolarized to $0 \text{ mV}$.

**Correlation of Reaction Rate with Extent of Inactivation—**

The results of Fig. 5 show that Cys-1489 is accessible at $-140 \text{ mV}$, where there is no steady-state inactivation, whereas it is hidden from reaction at $0 \text{ mV}$, where inactivation is complete. In order to further examine the correlation of reaction rate with inactivation, we determined the voltage dependence of the MTSES modification rate at intermediate levels of inactivation. Inactivation curves using $50\text{-ms}$ conditioning pulses were measured in each experiment to estimate the fraction of channels inactivated at $V_H$. The extent of modification was measured $1.5 \text{ min}$ after repolarization from $V_H$ (e.g. at time $2$ in Fig. 5). With $V_H = -140 \text{ mV}$, where $0\%$ of the channels were inactivated, the fraction of modified channels during the $3\text{-min}$ MTSES application was $0.83 \pm 0.03$ ($n = 5$). For $V_H$ at which inactivation was $47 \pm 5\%$, modification was $0.58 \pm 0.05$ ($n = 4$), and for $V_H$ at which inactivation was $73 \pm 2\%$, modification was $0.15 \pm 0.07$ ($n = 4$). At $0 \text{ mV}$, steady-state inactivation was $90\%$. With $V_H = 0 \text{ mV}$, modification was $0.05 \pm 0.08$ ($n = 8$).

Second order rate constants of modification were calculated for the reaction at each holding potential using the exponential rate equation in Fig. 4. The extent of modification after $3 \text{ min}$ at $V_H = -140 \text{ mV}$ from the experiments of Fig. 4 with stimuli every $10 \text{ s}$ agrees well with the mean fractional MTSES modification after a $3\text{-min}$ exposure at $-140 \text{ mV}$ from the data of Fig. 5A without repetitive stimulation ($0.86 \pm 0.08$ compared with $0.83 \pm 0.03$). Thus, the time course of modification during the $3\text{-min}$ MTSES application was the same whether test pulses were given every $10 \text{ s}$ or not, indicating that the short depolarizing pulses in time course experiments like the one in Fig. 4 did not significantly change the accessibility of Cys-1489 over the $3\text{-min}$ test interval. Therefore, the second order rate equa-

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**Fig. 5. Control of Cys-1489 accessibility to MTSE modification by holding potential.** A, time course of channel modification by $500 \text{ nM}$ MTSES at $-140 \text{ or } 0 \text{ mV}$. The patch was initially at a holding potential of $-140 \text{ mV}$ and exposed to the control bathing solution. After a series of pulses to $-20 \text{ mV}$ every $10 \text{ s}$, the holding potential was set to $V_H$ (indicated by the bar), and pulsing was stopped. The bath perfusate was changed to one containing $500 \text{ nM}$ MTSES for $3 \text{ min}$ (indicated by the bar) followed by a $2\text{-min}$ wash with control solution. Then the holding potential was switched back to $-140 \text{ mV}$, and pulses were resumed. After $5.5 \text{ min}$, the patch was re-exposed to $500 \text{ nM}$ MTSES (asterisk), while pulses were continued from a holding potential of $-140 \text{ mV}$. Quantification of the extent of modification is described in the legend to Fig. 4. The filled circles represent five experiments with $V_H = -140 \text{ mV}$, and the open circles represent eight experiments with $V_H = 0 \text{ mV}$. In some experiments the time between resuming the pulses and the start of the second MTSES addition was longer than $5.5 \text{ min}$. In these cases the final data points before the second MTSES addition were omitted so that the second MTSES addition was synchronized at the asterisk for all experiments. B and C, representative current traces from experiments with $V_H = -140 \text{ mV} (B)$ and $V_H = 0 \text{ mV} (C)$. The traces are averages of five $15\text{-ms}$ pulses to $-20 \text{ mV}$ at the times indicated by the corresponding number in A. D, time course of $50 \text{ nM}$ MTSET modification at holding potentials of $-140$ and $0 \text{ mV}$. The protocol was the same for the reaction with MTSET except that the MTSET application was only $2 \text{ min}$ long. The fraction of noninactivating current was quantitated as the ratio of the current at the end of the $15\text{-ms}$ depolarization/peak current during the pulse. The normalized modification is the change in the fraction of noninactivating current, normalized to the beginning and the end of the experiment. The filled circles represent three experiments with $V_H = 0 \text{ mV}$, and the open circles represent three experiments with $V_H = -140 \text{ mV}$ and $V_H = 0 \text{ mV}$, respectively. The traces are averages of five $15\text{-ms}$ pulses to $-20 \text{ mV}$ at the times indicated by the corresponding number in D. 0 mV. However, even at positive potentials, the channels could be modified at long times or high MTSES concentrations, so the reduction in the degree of MTSES modification by depolarization was caused by slowing of the reaction with MTSE reagents rather than by preventing a fraction of F1489C channels from reacting (data not shown; see below). In this case, the rate of
FIG. 6. Correlation of the voltage dependence of reaction rates with the extent of inactivation. Estimated rate constants were calculated from the fractional modification in 3 min at different holding potentials as described under “Results.” Those rate constants are plotted versus the fraction of channels not inactivated at a given $V_H$, as estimated from inactivation curves. Steady-state inactivation curves for experiments at intermediate levels of inactivation were recorded in each experiment immediately prior to the MTSES paradigm. Steady-state inactivation for $V_H = 0$ mV was determined from pooled data (Table I). Estimated rate constants were $0.0005 \pm 0.0006 \text{s}^{-1}$ (90 ± 6% inactivated, $n = 8$), $0.0012 \pm 0.0006 \text{s}^{-1}$ (73 ± 2% inactivated, $n = 4$), $0.0059 \pm 0.0007 \text{s}^{-1}$ (47 ± 5% inactivated, $n = 4$), and $0.0133 \pm 0.0013 \text{s}^{-1}$ (0% inactivated, $n = 5$). The dotted line represents the linear regression to the data points.

The rates of reaction at each $V_H$ derived by fitting to the exponential rate equation for a second order binding reaction illustrated in Fig. 4 are plotted versus the fraction of channels not inactivated at each $V_H$ in Fig. 6. There is a nearly linear correlation between the rate observed for reaction with MTSES at each $V_H$ and the fraction of channels not inactivated at that $V_H$. The reaction rate at $V_H$ corresponding to 47% steady-state inactivation (53% of the channels not inactivated) was 0.45 times the reaction rate at $V_H = -140$ mV (0% inactivated). The reaction rate at $V_H$ corresponding to 73% inactivation was 0.098 times the reaction rate at $V_H = -140$ mV, and the reaction rate derived from experiments at $V_H$ corresponding to 90% steady-state inactivation was 0.04 times that at $-140$ mV. The rates of reaction at 73 and 90% inactivation were difficult to estimate accurately due to the low absolute modification during these experiments. Modification was also measured for maximally inactivated channels using a 40-fold higher concentration of MTSES to obtain faster reaction rates (see below). After correcting for the concentration difference, a relative reaction rate of 0.013 times the rate at $-140$ mV was obtained, in good agreement with the results using lower reagent concentrations. Thus, there is a good linear correlation between the extent of inactivation and the rate of reaction of Cys-1489 with MTSES reagents. These results support the conclusion that the inactivation process itself is responsible for the voltage-dependent change in reactivity of Cys-1489.

Voltage Dependence of Reaction of Cys-1489 in the Resting and Inactivated States—Reaction rates of Cys-1489 with negatively charged MTSES would be voltage-dependent if the charge had to enter the electrical field to react. If this were the case, the reaction would be faster at more negative potentials. To measure the voltage dependence of MTSES reaction with channels in the resting state, 500 nM MTSES was applied at three holding potentials over a voltage range where there is no inactivation in the steady state, and the reaction time course was measured. Fits to the time course of experiments at holding potentials of $-140$ mV ($n = 8$), $-150$ mV ($n = 3$), and $-180$ mV ($n = 2$) yielded reaction rates that were indistinguishable from each other (Fig. 7). We estimate that a 1.5-fold change in reaction rate over 40 mV would have been readily detected. A change of this magnitude would have reflected a requirement for the charged reagent to pass 25% of the way through the electric field in order to react (35). Therefore, the charge on MTSES does not deeply enter the electric field to react with Cys-1489 of resting channels.

We also considered whether Cys-1489 might move into the field during channel inactivation, causing reaction of MTSES with the inactivated state to become voltage-dependent. As noted above, MTSES can modify inactivated channels but at a slower rate than it modifies resting channels. Therefore, a higher MTSES concentration was chosen for these experiments (20 μM), resulting in modification of about 60% of the channels during a 3-min application at a depolarized potential. The rate of reaction was determined at two holding potentials at which inactivation was maximal, $-20$ mV and $+40$ mV. The modification measured was 0.66 ± 0.06 ($V_H = -20$ mV, $n = 2$) and 0.62 ± 0.03 ($V_H = +40$ mV, $n = 2$), and the estimated reaction rates were 0.008 ± 0.002 s$^{-1}$ and 0.007 ± 0.001 s$^{-1}$. Thus, reaction with inactivated channels also is not detectably voltage-dependent.

To interpret the apparent lack of voltage dependence of the reaction with inactivated channels at depolarized potentials, it is necessary to consider the factors responsible for the finite reaction rate at these potentials. The inactivated state is destabilized in F1489C mutant channels, and even at depolarized potentials channels spend 8% of the time in noninactivated states. The rate of reaction of MTSES at depolarized potentials was 1.3–4.0% of the rate at $-140$ mV. Thus, it is possible that all modification of Cys-1489 at depolarized potentials occurs during these openings of the inactivation gate and that F1489C is completely inaccessible when it is inactivated. In this case, the inactivation gate would not be in the electric field when it opened, and therefore its reaction rate would not be voltage-dependent. Thus, the fact that no voltage-dependent reaction rate was detected at depolarized potentials cannot exclude entry of the inactivation gate into the electrical field when the channel is inactivated. Nevertheless, the lack of voltage dependence of MTS reaction in the positive and negative potential ranges where no gating occurs further supports the conclusion that the voltage dependence of reaction between $-140$ and 0 mV is caused by a preferential state-dependent reaction with the closed state in comparison with the inactivated state.

DISCUSSION

Modification of both Forward and Reverse Inactivation Rates of Mutant F1489C by Sulfhydryl Reagents—Mutation of Phe-1489 to hydrophilic amino acids causes a strong destabilization of the inactivated state and has smaller effects on the rate of entry into the inactivated state (15, 48).2 Similarly, the reac-
tion of Cys-1489 with Ag⁺ essentially completely prevents inactivation, suggesting profound destabilization of the inactivated state by the hydrophilic silver moiety. In contrast, our macropatch and single channel recordings indicate that reaction of Cys-1489 in the inactivation gate with the MTS reagents MTSEA and MTSES slows the rate of entry into the inactivated state and has relatively little effect on the stability of the inactivated state. The effects of reaction with these two reagents are similar, although they have opposite charge. After MTSEA or MTSES modification of cysteine residues, the hydrophilic group is more distant from the polypeptide backbone than it is in charged natural amino acids or in the silver-cysteine adduct, and it is shielded by two large sulfur atoms. Therefore, the modified cysteine residue in the MTSEA-substituted or MTSES-substituted F1489C channel might still be able to make a hydrophobic interaction effectively with the inactivation gate receptor with relatively little interference from the distal hydrophilic group.

In contrast to the reaction with MTSEA and MTSES, the reaction with MTSET had dramatic effects on both the forward and reverse rates of inactivation. At −20 mV, the rates of entry into the inactivated state from the final closed state and the open state were slowed 7- and 12-fold, respectively, and the rates of return to those states from the inactivated state were accelerated 35- and 12-fold. Thus, the magnitude of the effect on inactivation is smaller with the smaller reagents MTSEA (66 Å³) and MTSES (90 Å³) and greater with the larger reagent, MTSET (109 Å³), and does not correlate with the sign of the added charge. It is likely that the size or shape of the hydrophilic group determines the rate of entry into the inactivated state, while its size, hydrophilicity, and position in the side chain all contribute to the stability of the inactivated state. The larger charged group of MTSET may slow entry into the inactivated state most strongly because of steric hindrance of the tetraethylammonium group and may destabilize the inactivated state most strongly because its positive charge cannot be accommodated sterically in the inactivated state without energetically costly interactions of the charged tetraethylammonium group with the hydrophobic inactivation gate receptor site.

**Multiple Inactivated States after Reaction with MTSET**—Closed time distributions were well fit by the simple gating model of Scheme 1 for the F1489C mutant and for mutant channels modified by MTSEA but not for MTSET-modified F1489C channels. For MTSET, at least one component of long closed times was not well fit, and an additional more stable closed state is suggested by the truncated closures at the end of our test pulses (Fig. 2, Table II). These closures were longer than the fast component of first latencies, indicating that they arise from transitions not included in the first latency and therefore not on the direct pathway from closed to open channels. The model of Scheme 1 could not reproduce such data, since it includes only one closed state that is not in the direct pathway from Cᵢ to O, the inactivated state, I. Additional inactivated states, which are postulated for Na⁺ channels (Refs. 16 and 36 and references therein), may cause this long closed time component, which is observed after reaction with MTSET. These additional inactivated states may exist in unmodified F1489C channels but may be obscured by the similar or longer closed time of the major fast inactivated state of F1489C or missed because they are rarely entered when channels have effective fast inactivation. Alternatively, the MTSET-modified inactivation gate may be able to close and bind in more than one conformation, most often in a destabilized conformation, but occasionally in a conformation that is more stable, approaching the stability of unmodified F1489C and therefore producing long closed times. Whatever the detailed mechanism of action of the MTS reagents on inactivation of F1489C, their strong effects allowed us to measure the rate of their reaction and thereby to determine the state-dependent accessibility of Cys-1489 to modification.

**Movement of the Inactivation Gate during Inactivation**—Current models of sodium channel inactivation are based on the ball-and-chain model of Armstrong and Bezanilla (31). This model proposed that depolarization causes activation gates to move, ultimately creating a favorable binding site for a tethered cytoplasmic inactivation particle that acts as an open channel blocker. This mechanism may accurately describe the coupling between membrane potential and N-type inactivation in potassium channels (37–41). However, in the case of Na⁺ channels, the IFM motif is tethered on both ends to the channel core. This structure closely resembles the hinged lids of allosteric enzymes, which control substrate access to their active sites (15). Therefore, in analogy with the hinged lids of enzymes, the inactivation gate of the Na⁺ channel may undergo a concerted conformational change that places the IFM motif in its hydrophobic receptor site and blocks ion movement through the channel. This movement may be coupled to activation of the channel by movement of the S4 segment in domain IV (42–44).

Our results provide direct evidence for the relative movement of the IFM motif in the inactivation gate into the structure of the channel during inactivation. Using a mutant sodium channel with inactivation properties similar to WT, we show that the accessibility of Cys-1489 to hydrophilic cysteine reagents is dramatically reduced upon inactivation. The concentration of MTS reagents needed for complete reaction with Phe-1489 at negative membrane potential (50–500 nM) is 2000–20,000-fold lower than in typical cysteine-scanning mutagenesis applications (e.g. Ref. 24), indicating that Cys-1489 is readily accessible to intracellular MTS reagents when Na⁺ channels are in the resting state. During incubation at 0 mV, the rate of reaction with MTS reagents is reduced 25–80-fold (Fig. 5). The extent of slowing of the reaction rate is closely correlated with the extent of channel inactivation at different membrane potentials (Fig. 6). Control experiments demonstrate that the local concentration of MTSES is not voltage-dependent, supporting the conclusion that the change of reaction rate is due to a change in the accessibility of Cys-1489 to the MTS reagents (Fig. 7).

In addition to the fast inactivated state considered here, Na⁺ channels also enter a slow inactivated state during depolarizations of 1 s or more. Slow inactivation is a separate molecular process that does not require Phe-1489 (45). Therefore, it is unlikely that slow inactivation can affect reaction of sulphydryl reagents at Cys-1489. The voltage-dependent reaction rates observed in our experiments are most likely to be caused by movement of the inactivation gate during the fast inactivation process.

We have described our data as if channel inactivation makes the sulphydryl group of Cys-1489 physically inaccessible, as has been the assumption in reports using MTS reagents to probe ion channel function (e.g. Ref. 24). It is also possible that the reduced reactivity results from a change in the local protein environment subsequent to a conformational change (e.g. Ref. 46). The reduction in the modification rate of Cys-1489 upon inactivation might be also caused in part by the binding of the inactivation gate to its receptor. Experiments with site-specific antibodies (12, 13) support the conclusion that movement of the gate does indeed reduce its accessibility, at least to macromolecular reagents. Intracellular application of an antibody (anti-
directed against a peptide from the C-terminal portion of LIII-IV that does not include the IFM-motif (SP19), slowed inactivation. The time course of modification by the antibody was faster at more negative potentials, with a dependence on inactivation similar to that seen in our experiments. Since IFM was not included in the epitope recognized by the antibody and a polyclonal antibody that would have reactivity against most was not included in the epitope recognized by the antibody and inactivations similar to that seen in our experiments. Since IFM was faster at more negative potentials, with a dependence on inactivation. The time course of modification by the antibody of LIII-IV that does not include the IFM-motif (SP19), directed against a peptide from the C-terminal portion of which LIII-IV undergoes a conformational change upon inactivation that makes the entire loop less accessible to reaction with large antibody reagents and makes Cys-1489 inaccessible to reaction with even small MTS reagents.

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