Molecular Motions of the Outer Ring of Charge of the Sodium Channel: Do They Couple to Slow Inactivation?

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ABSTRACT In contrast to fast inactivation, the molecular basis of sodium (Na) channel slow inactivation is poorly understood. It has been suggested that structural rearrangements in the outer pore mediate slow inactivation of Na channels similar to C-type inactivation in potassium (K) channels. We probed the role of the outer ring of charge in inactivation gating by paired cysteine mutagenesis in the rat skeletal muscle Na channel (rNav1.4). The outer charged ring residues were substituted with cysteine, paired with cysteine mutants at other positions in the external pore, and coexpressed with rat brain β_1 in *Xenopus* oocytes. Dithiolthreitol (DTT) markedly increased the current in E403C+E758C double mutant, indicating the spontaneous formation of a disulfide bond and proximity of the α carbons of these residues of no more than 7 Å. The redox catalyst Cu(II) (1,10-phenanthroline)$_3$ (Cu(phe)$_3$) reduced the peak current of double mutants (E403C+E758C, E403C+D1241C, E403C+D1532C, and D1241C+D1532C) at a rate proportional to the stimulation frequency. Voltage protocols that favored occupancy of slow inactivation states completely prevented Cu(phe)$_3$ modification of outer charged ring paired mutants E403C+E758C, E403C+D1241C, and E403C+D1532C. In contrast, voltage protocols that favored slow inactivation did not prevent Cu(phe)$_3$ modification of other double mutants such as E403C+W756C, E403C+W1239C, and E403C+W1531C. Our data suggest that slow inactivation of the Na channel is associated with a structural rearrangement of the outer ring of charge.

KEY WORDS: rNav1.4 channel • cysteine mutagenesis • disulfide bond • electrophysiology

INTRODUCTION

The voltage-gated Na channel mediates the rapid upstroke of action potential and is responsible for excitation and conduction in nerve and muscle. Upon depolarization, the channel activates a process that involves outward movement of all four charged S4 segments (Stuhmer et al., 1989; Hirschberg et al., 1995; Yang and Horn, 1995; Yang et al., 1996; Kontis et al., 1997). Na channel opening is transient, with inactivation rapidly ensuing. Na channels undergo several different kinetically distinct forms of inactivation. The time constants of recovery from inactivated states range from milliseconds (fast inactivation) to hundreds of milliseconds (intermediate inactivation) and seconds (slow inactivation) (Simoncini and Stuhmer, 1987; Ruff et al., 1988; Nuss et al., 1996; Kambouris et al., 1998).

In *Shaker* K channels, N-type inactivation is mediated by an intracellular region at the NH_2 terminus acting as "ball" occluding the pore (Hoshi et al., 1990). The analogous region in the Na channel is the cytoplasmic III-IV linker, which serves as a tethered pore blocker binding to an inactivation gate receptor in the intracellular mouth of the channel (Armstrong, 1981; Stuhmer et al., 1989; Vassilev et al., 1989; Patton et al., 1992; West et al., 1992). Unlike fast inactivation, the molecular basis of slow inactivation in Na channels is poorly understood. However, slow inactivation plays a critical role in membrane excitability and firing properties by governing the availability of Na channels in neurons and muscle (Ruff et al., 1988; Fleidervish et al., 1996). The physiological significance of slow inactivation in Na channels has been established by studies demonstrating that abnormalities of slow inactivation are associated with a variety of human diseases, including hyperkalemic periodic paralysis (Cummins and Sigworth, 1996; Hayward et al., 1999; Bendahhou et al., 2002), Brugada syndrome (Veldkamp et al., 2000), and epilepsy (Alekov et al., 2001; Spampanato et al., 2001).

In *Shaker* K channels, C-type inactivation, which can be slow, is a mechanism of inactivation that involves a conformational rearrangement of the outer pore. C-type inactivation persists when N-type inactivation is deleted (Hoshi et al., 1991), is inhibited by extracellular tetraethylammonium (Choi et al., 1991) and is sensitive to extracellular [K^+] and mutations in the outer pore (Lopez-Barneo et al., 1993).

The outer ring of charge (E403, E758, D1241, and D1532 in domains I-IV, respectively) is located three

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Abbreviations used in this paper: DTT, dithiolthreitol; MTS, methanethiosulfonate.

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to four residues external to the putative selectivity filter (D400, E755, K1237, and A1529) of the Na channel (Heinemann et al., 1992). Whether the conserved outer ring of charge in the external pore of the Na channel is involved in slow inactivation is not well defined, although charge neutralization of one residue (E403) in this ring markedly delays recovery from slow forms of inactivation (Zhang et al., 2003). We hypothesized that slow inactivation in the Na channel is associated with a structural rearrangement of this outer ring of charge. This rearrangement changes the relationship of side chains of residues that comprise the outer pore of Na channels, including those in the outer ring of charge. In the present study, we introduced pairs of cysteines into the outer ring of charge (EEDD) and other pore residues in each of the domains of rNav1.4 (Fig. 1). Paired-cysteine substitutions in the outer pore of Na channels can form disulfide bonds both spontaneously and in the presence of a redox catalyst (Benitah et al., 1996, 1997, 1999). The combination of paired-cysteine mutagenesis and disulfide trapping was employed to explore the changes in spatial relationships of residues in the outer pore that may participate in slow inactivation. We determined the conformation-dependent rate of disulfide bond formation in the paired-cysteine mutants and investigated the effects of the induction of slow inactivation on the extent of disulfide bond formation. Our results suggest that motion in the outer ring of charge is closely associated with slow inactivation gating of the Na channel.

MATERIALS AND METHODS

Mutagenesis and Channel Expression

A 1.9Kb BamHI-SphI or 2.5kb SphI-KpnI fragment of the μ_1 skeletal muscle Na channel (rNav1.4 channel) cDNA (Trimmer et al., 1989) was subcloned into pGEM-11Zf$^+$ and pGEM-7Zf$^+$ (Promega), respectively. The 1.9- and 2.5-kb cassettes were employed for oligonucleotide-directed mutagenesis in domains I or II–IV, respectively (Kunkel, 1985). Mutations were confirmed by sequencing mutagenic cassettes, which were cloned backed into pSP64T (Krieg and Melton, 1984). Complementary RNA was prepared via in vitro transcription using SP6 RNA polymerase. The α subunit was coexpressed with the rat brain β_1 subunit (1:1 weight ratio) (Isom et al., 1992) in *Xenopus laevis* oocytes as described previously (Tomaselli et al., 1995; Perez-Garcia et al., 1996; Benitah et al., 1997). Oocytes were stored in the following solution (in mM): NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, Na Pyruvate 5, theophylline 0.5, supplemented with penicillin 100 U/ml and streptomycin 100 μg/ml (pH 7.6 with NaOH).

Electrophysiology and Data Analysis

Macroscopic Na currents were recorded 24–48 h after injection of cRNA using a two-microelectrode voltage-clamp (OC-725B; Warner Instrument Corp.) in frog Ringer's solution containing (in mM): NaCl 96, KCl 2, $MgCl_2$ 1, HEPES 5 (pH 7.6 with NaOH). All experiments were performed at room temperature. The stock solution of the redox catalyst Cu (II) (1,10-phenan-

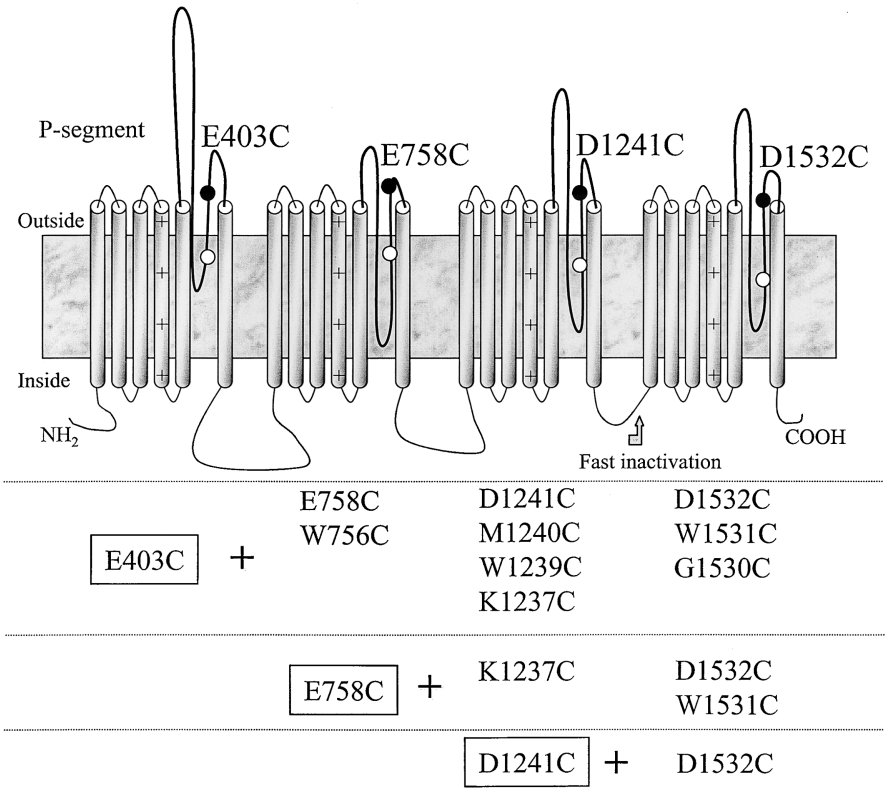

FIGURE 1. Schematic depiction of the Na channel α subunit. It consists of four domains, each of which has six transmembrane segments. The S4 segments represent at least part of the voltage sensor. The segments between S5 and S6 (P-segments) line the outer pore. The outer ring of negative charge (E403, E758, D1241, and D1532) is illustrated in filled circles. The open circles represent the putative selectivity filter residues (D400, E755, K1237, and A1529). The domain III-IV linker underlies fast inactivation. The 13 double cysteine mutants studied are shown below the schematic. E403C, E758C, and D1241C (enclosed in the boxes) were paired with other cysteine mutants in different domains of the outer pore.

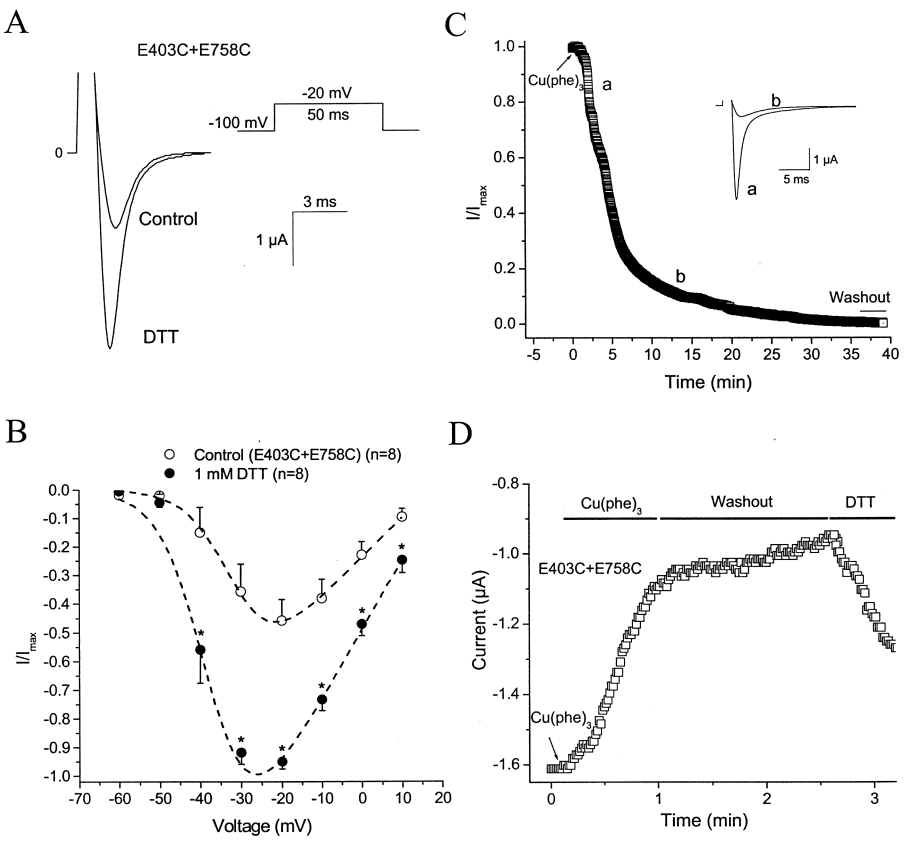

FIGURE 2. Evidence for disulfide bonding of residues in the outer ring of charge. (A) Representative traces show that current through E403C+E758C mutant Na channels increased more than twofold in magnitude in the presence of 1mM DTT. (B) The current-voltage relationship shows a similar increase in peak current amplitude of the double mutant by exposure to DTT over the entire voltage range ($P < 0.01$, $n = 8$). Peak currents measured at each voltage step were normalized to the maximum current observed in each cell, averaged and plotted as a function of voltage. (C) A plot of the time course of the decrease in peak current amplitude upon exposure to 100 μM $Cu(phe)_3$. The effects of $Cu(phe)_3$ were not reversed during washout. The inset shows currents recorded at time (a) and (b). (D) A plot of the peak Na current amplitude during application of $Cu(phe)_3$, washout, and subsequent treatment with 1 mM DTT.

throline) ($Cu(phe)_3$) was a mixture of 150 mM $Cu(II)SO_4$ and 500 mM 1,10-phenanthroline in a 4:1 water/ethanol solution (Careaga and Falke, 1992) and was diluted to its final concentration just before use. Dithiothreitol (DTT) was dissolved in extracellular solution just before use. Acquisition and analysis of whole-cell currents was performed with custom-written software. Current-voltage relationships were fitted to a transform of the Boltzmann distribution:

$$I/I_{max} = (V - V_{rev})\,G_{max}/[1 + \exp(V_{1/2} - V)/k],$$

where V_{rev} is the reversal potential, G_{max} the maximum normalized conductance, $V_{1/2}$ the potential of half activation, and k the slope factor. Average data are expressed as mean ± SEM. Statistical significance was evaluated by ANOVA or Student's t test (Origin; MicroCal) with $P < 0.05$ representing significance.

RESULTS

Disulfide Bonding of Paired Cysteine Substitution Mutants

Fig. 1 shows a schematic depiction of rNav1.4 with the residues of the selectivity filter and the outer ring of charge highlighted. Amino acids in the outer ring of charge (E403, E758, D1241, and D1532) in the ascending limb of the P-segment (SS2 region) were substituted singly with cysteine and paired with other cysteine substitutions in the external pore. 13 paired cysteine substitution mutants (Fig. 1, bottom) were studied and exhibited distinct phenotypes. For example, Fig. 2 A shows the Na currents elicited by a voltage step from −100 to −20 mV through E403C+E758C mutant channels. The magnitude of the Na current was markedly increased after a 1–1.5 min exposure to the reducing agent DTT (1 mM) (Fig. 2 A). The mean data shows that the peak Na current at −20 mV more than doubled in the presence of DTT and the increase was observed over a wide range of voltages ($n = 8$, $P < 0.01$) (Fig. 2 B). The enhancement of Na currents upon exposure to DTT suggests that a disulfide bond forms spontaneously between the cysteines at the 403 and 758 positions, cross-linking the pore-lining (P) segments of domains I and II, and partially occluding the pore. DTT did not increase the magnitude of the basal current of any of the other paired mutants examined in this study.

The redox catalyst $Cu(phe)_3$ facilitates disulfide bond formation in paired cysteine mutant channels, but at concentrations of 100 μM or less has no effect on either the wild-type Na channel or single-cysteine pore mutant channels (Benitah et al., 1997, 1999). Disulfide bond formation was also induced by $Cu(phe)_3$ in E403C+E758C mutant channels. The modification by $Cu(phe)_3$ was irreversible during washout (Fig. 2 C). However, the application of DTT, at least partially, restored the Na current through E403C+E758C mutant channels (Fig. 2 D).

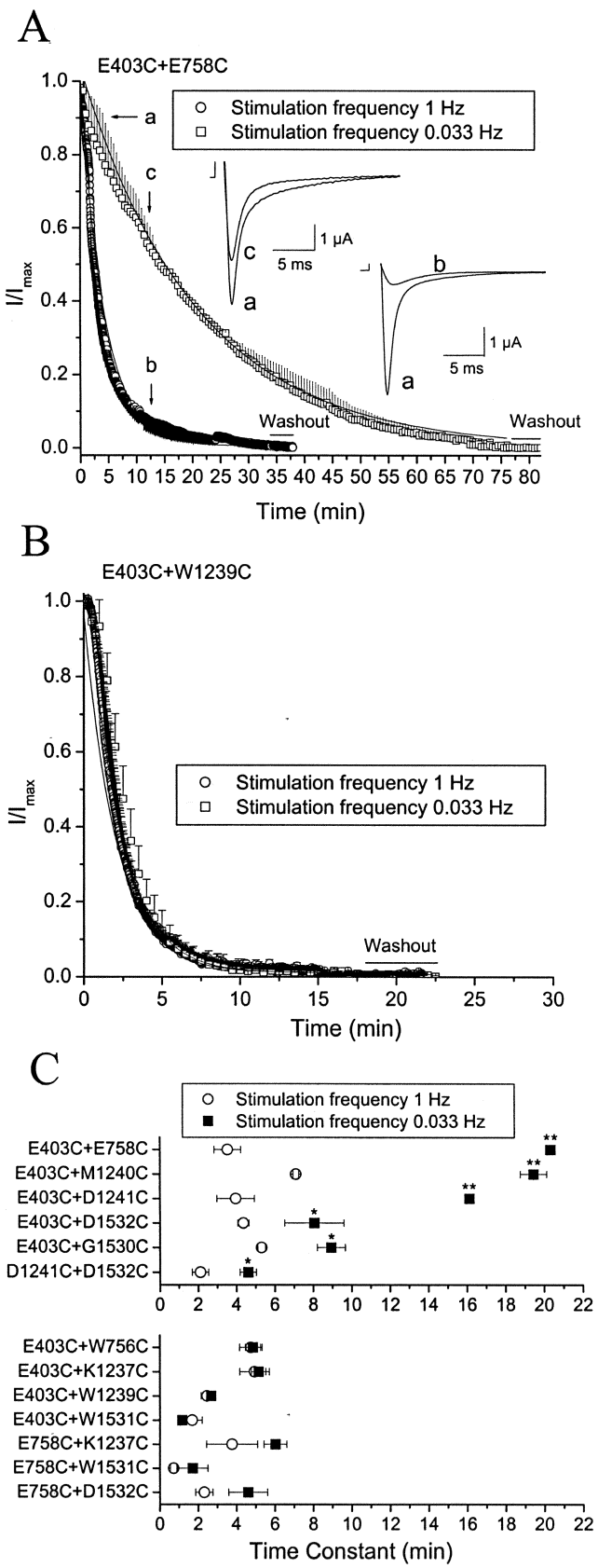

FIGURE 3. Rate-dependent modification by $Cu(phe)_3$ in paired mutant channels. (A) Rate-dependent modification by $Cu(phe)_3$ of E403C+E758C mutant channels. Increasing stimulation frequency (from 0.033 Hz to 1 Hz) enhanced the rate of current reduction of E403C+E758C, suggesting rate-dependent modification of $Cu(phe)_3$ in E403C+E758C mutant channels. The inset shows representative currents at 2 min (a) and 12.5 min at stimulation frequencies of 1 Hz (b) and 0.033 Hz (c) after the application of $Cu(phe)_3$. (B) In contrast, current reduction was rapid and not dependent on stimulation frequency in the E403C+W1239C mutant channels. (C) Plots of the rates of reduction in peak current amplitude at different stimulation frequencies in the presence of $Cu(phe)_3$ (n = 2–7). Single exponential fits of the form $y = Ae^{(-t/\tau)}$ to plots like those shown in A and B were used to determine the time constants, τ. A is the amplitude. The top panel shows the time constants of current decay of mutant channels that were significantly increased at slower stimulation frequencies. The bottom panel is a plot of the time constants of double mutant channels that did not exhibit stimulation frequency dependent rates of modification. (*P < 0.05, **P < 0.01).

Conformation-dependent Rate of Catalyzed Disulfide Bond Formation

We used different voltage protocols to examine the conformation-dependent rate of disulfide bond formation of the paired cysteine mutants. The rates of modification of paired mutants were determined using depolarizing test pulses to −20 mV for 50 ms from a holding potential of −100 mV at stimulation frequencies of 1 Hz or 0.033 Hz in the presence of $Cu(phe)_3$. Using this protocol at 1 Hz, voltages that favored the activated and fast inactivated states (−20 mV) occupied 4.76% duty cycle, compared with only 0.17% of the duty cycle at 0.033 Hz. At test pulse durations of 50 ms very little slow inactivation was recruited in the wild-type or any of the paired mutant channels. The rates of disulfide bond formation were indexed by the peak current reduction curves and the time constants were determined by single exponential fits to the plots of current reduction (Fig. 3, A and B). A plot of the time constants of the modification rates demonstrates that cysteine substitutions of the outer ring of charge residues exhibit specific patterns of frequency-dependent disulfide bond formation when combined with other P-segment cysteine substitutions (Fig. 3 C). Paired cysteine substitutions of outer charged ring residues in domains I and II (E403C+E758C), I and III (E403C+D1241C), I and IV (E403C+D1532C), and III and IV (D1241C+D1532C) exhibited significantly faster rates of catalyzed disulfide bond formation at 1 Hz compared with 0.033 Hz stimulation frequencies (Fig. 3, A and C). Similarly, the other paired outer charged ring mutant (II+IV E758C+D1532C) exhibited a more rapid rate of modification with 1 Hz stimulation frequency compared with 0.033Hz, but this difference did not reach statistical significance (Fig. 3 C). The pairing of charge ring mutants with other cysteine mutations in the P-segments, but not in the outer charged ring, generally produced double mutants with rapid $Cu(phe)_3$-induced disulfide bond formation insensitive to the stimulation frequency (Fig. 3, B and C). The exceptions were when E403C was combined with M1240C (in

TABLE I

Extent of Current Recovery

Mutant	Extent of current recovery			
	Protocol A (1 Hz) $Cu(phe)_3$	Protocol B (0.033 Hz) $Cu(phe)_3$	Protocol C (slow inact) Control	Protocol C (slow inact) $Cu(phe)_3$
	%	%	%	%
E403C+E758C	0.16 ± 0.03	0.14 ± 0.02	96.55 ± 0.55	88.64 ± 5.09
E403C+D1241C	0.18 ± 0.04	0.16 ± 0.02	93.14 ± 3.34	81.01 ± 6.26
E403C+D1532C	0.15 ± 0.02	0.18 ± 0.04	97.28 ± 2.72	94.43 ± 2.27
E403C+M1240C	0.14 ± 0.03	0.12 ± 0.03	93.88 ± 4.30	84.95 ± 8.54
E403C+K1237C	0.12 ± 0.03	0.15 ± 0.03	97.94 ± 2.73	83.21 ± 3.31
D1241C+D1532C	0.15 ± 0.02	0.13 ± 0.02	97.33 ± 1.67	60.69 ± 1.38
E758C+K1237C	0.14 ± 0.02	0.13 ± 0.03	98.02 ± 0.91	57.75 ± 9.76
E403C+G1530C	0.16 ± 0.02	0.19 ± 0.04	95.13 ± 1.15	47.22 ± 1.56
E403C+W756C	0.13 ± 0.02	0.15 ± 0.03	96.02 ± 2.80	40.04 ± 3.85
E403C+W1239C	0.19 ± 0.04	0.16 ± 0.02	96.13 ± 3.87	37.52 ± 4.85
E758C+D1532C	0.17 ± 0.03	0.15 ± 0.02	94.02 ± 2.80	33.02 ± 3.15
E403C+W1531C	0.15 ± 0.02	0.17 ± 0.02	97.91 ± 1.10	10.16 ± 6.40
E758C+W1531C	0.12 ± 0.03	0.15 ± 0.03	98.13 ± 0.76	1.76 ± 1.41

(Protocols A and B) Depolarizing test pulses to −20 mV for 50 ms from a holding potential of −100 mV at stimulation frequencies of 1 Hz or 0.033 Hz, respectively (n = 2–7 cells). (Protocol C) Slow inactivation was induced by voltage clamping cells at −20 mV and then repolarizing to −100 mV for 10 ms every 5 s (Fig. 4 A). Recovery at −100 mV was measured by voltage steps to −20 mV for 50 ms at 1 Hz after washout of $Cu(phe)_3$ (n = 3–6 cells).

domain III) and G1530C (in domain IV) (Fig. 3 C). In all the paired mutant channels examined, the modification by $Cu(phe)_3$ was irreversible in the absence of a reducing agent (Table I), consistent with disulfide bond formation (Benitah et al., 1997, 1999).

Slow Inactivation and $Cu(phe)_3$-induced Disulfide Bond Formation

We used two different protocols to induce slow inactivation in the presence and absence of $Cu(phe)_3$ to investigate the effect of conformational changes of the outer pore associated with slow inactivation on the extent of disulfide bond formation. Slowly recovering inactivated states were populated by holding the cells at −20 mV and then repolarizing to −100 mV for 10 ms every 5 s. The brief repolarization permitted recovery from fast inactivation and monitoring of the residual peak current amplitude. $Cu(phe)_3$ was washed out for 4 min before returning the channels to a holding potential of −100 mV. The recovery component after washout represented channels that had slow inactivated but had not undergone catalyzed disulfide bond formation. Fig. 4 A shows that the rate of development of slow inactivation of E403C+E758C mutant channels was not significantly different in the presence and absence of $Cu(phe)_3$. The reduction of current was largely attributed to cumulative slow inactivation, rather than induced disulfide bond formation by $Cu(phe)_3$. Fig. 4 B shows that the recovery of the Na current was >90% complete by 1 min of washout independent of the presence of $Cu(phe)_3$. Similarly, E403C+E758C mutant channels exhibited complete recovery from slow inactivation, which was induced by holding the channels at −20 mV and pulsing to 0 mV at a frequency of 1 Hz before and during exposure to $Cu(phe)_3$ (Fig. 4 C). Slow inactivated E403C+E758C mutant channels recovered with a similar time course independent of prior exposure to $Cu(phe)_3$ ($P > 0.05$) (Fig. 4, B and C). These results demonstrate that slow inactivation in E403C+E758C mutant channels can completely prevent the formation of a disulfide bond between the cysteines at the 403 and 758 positions in the presence of $Cu(phe)_3$. In contrast, there was no significant recovery of the Na current through E403C+W1531C mutant channels in the presence of $Cu(phe)_3$ (Fig. 4 D).

The induction of slow inactivation prevented catalyzed disulfide bond formation in specific patterns with paired mutants in the outer pore of the channel. When the domain I outer-charged ring mutant E403C was combined with a mutation in any one of the charge residue positions in the other domains (domain II E758C, domain III D1241C, and domain IV D1532C), disulfide bond formation was prevented by voltage protocols that populated slow inactivated states (Fig. 5). In contrast, the pairing of E403C with other cysteine mutations in the P-segments generated double mutants (e.g., E403C+W756C, E403C+W1239C, and E403C+W1531C) that were not protected from modification by $Cu(phe)_3$ during slow inactivation. Interestingly, slow inactivation in other paired mutants in the outer ring (III+IV D1241C+D1532C, II+IV E758C+D1532C) provided less protection from Cu $(phe)_3$-induced disulfide bond formation compared with the mutants containing E403C (Fig. 5). However, slow inactivation does not appear to render the E403C side chain completely inaccessible, since it remains able to form disulfide bonds with other cysteine substitutions in the pore (W1531C and possibly W1239C and W756C).

Interestingly, E403C+K1237C Na currents were also protected from $Cu(phe)_3$-induced disulfide bond formation (83%) by slow inactivation. These results, along with the findings that charge-altering mutations at residue K1237 favor slow forms of inactivation (Todt et al., 1999), suggest a role for K1237 in slow inactivation. With the exception of E403C+K1237C mutant, double mutants that have more protection (i.e., 60% or more) from catalyzed modification in slow inactivated states (Fig. 5) also exhibit significant differences in the modification rate at different stimulation frequencies from the −100 mV holding potential (Fig. 3). These findings suggest that in these mutant channels that exhibit a high degree of flexibility of P-segments during gating,

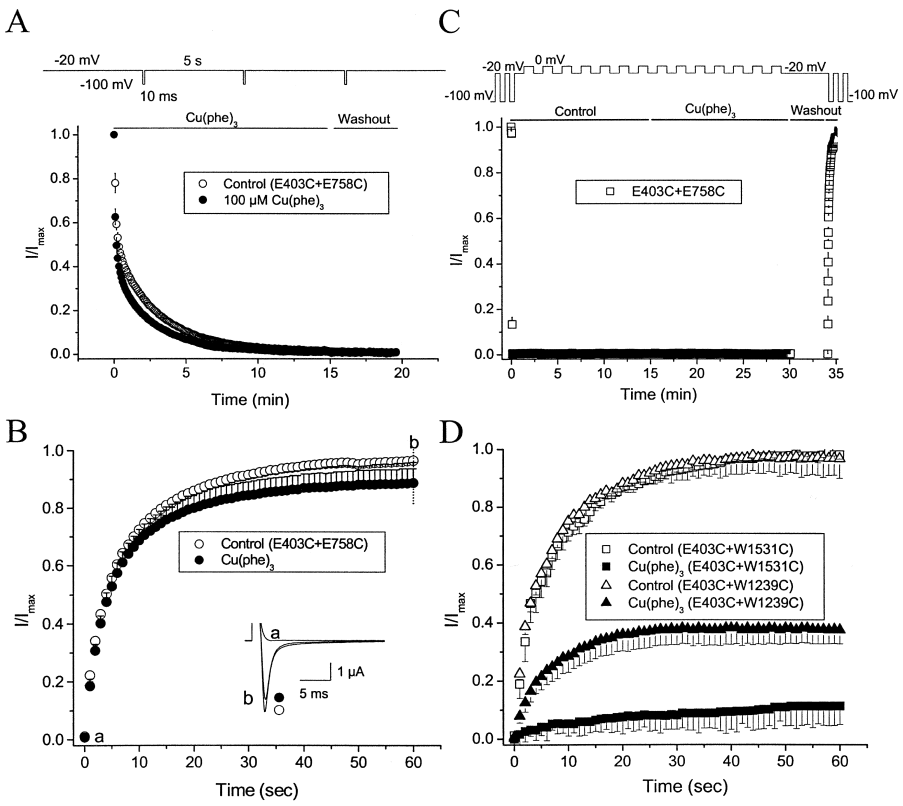

FIGURE 4. Slow inactivation prevented the modification of E403C+E758C, but not E403C+W1531C mutant channels by $Cu(phe)_3$. (A). Slow inactivation was induced by holding the cells at −20 mV for 15 min with brief repolarizations to −100 mV for 10 ms to elicit currents after recovery from fast inactivation. The mean data shows that channels entered slow-inactivated states at approximately the same rate regardless the presence (filled circles) or absence (open circles) of $Cu(phe)_3$. (B) Upon washout, Na currents nearly completely recovered from slow inactivation induced by the voltage protocol used in A independent of prior exposure to $Cu(phe)_3$ ($P > 0.05$, $n = 6$). The inset shows representative currents before (a) and 60 s after initiation of recovery (b) with (filled circle) or without (open circle) prior exposure to $Cu(phe)_3$. (C) A complementary voltage protocol was employed to induce slow inactivation before application of $Cu(phe)_3$. Channels were held at −20 mV and pulsed for 50 ms to 0 mV at 1 Hz for 30 min. After 15 min of pulsing, $Cu(phe)_3$ (100 μM) was applied for 15 min before washout. The oocytes were voltage clamped at −20 mV during the 4 min washout, then they were stimulated at 1 Hz from a holding potential of −100 to −20 mV to elicit Na currents. The Na currents completely recovered from slow inactivation compared with control ($P > 0.05$) (B). (D) After washout of $Cu(phe)_3$, Na currents in E403C+W1531C mutant channels did not significantly recover from slow inactivation induced by the voltage protocol used in A ($P < 0.05$, $n = 3$). E403C+W1239C mutant channels were also subjected to modification by $Cu(phe)_3$ and recovered incompletely ($P < 0.05$, $n = 3$).

neither closed state nor slow inactivated states favor catalyzed disulfide bond formation (Fig. 6).

In summary, the side chains of amino acid residues in the outer ring of charge of the voltage-gated Na channel (especially other outer ring mutants paired with E403C) undergo unique motions in slow inactivation that prevents $Cu(phe)_3$-induced modification (Fig. 5).

DISCUSSION

Slow inactivation is a collection of distinct gating processes. However, the molecular determinants of slow inactivation remain unknown. Site-directed mutagenesis suggests that residues in the S4 (Mitrovic et al., 2000), S4-S5 linkers (Bendahhou et al., 2002), S5 (Bendahhou et al., 1999), and S6 segments (Hayward et al., 1997; Wang and Wang, 1997) may play a role in slow inactivation. Moreover, several lines of evidence have supported a role for the outer pore in slow inactivation. First, mutations in the P-segments disrupt slow inactivation in Na channels (Tomaselli et al., 1995; Balser et al., 1996; Kambouris et al., 1998; Todt et al., 1999; Ong et al., 2000; Hilber et al., 2001, 2002; Vilin et al., 2001; Zhang et al., 2003). Second, alkali metal cations applied to the external surface inhibit slow inactivation (Townsend and Horn, 1997), similar to observations for C-type inactivation in *Shaker* K channels (Pardo et al., 1992; Lopez-Barneo et al., 1993; Baukrowitz and Yellen, 1995). Third, residues in the outer pore of Na channels may underlie isoform-specific differences in slow inactivation (Vilin et al., 2001).

In the K channel, C-type inactivation is clearly associated with constriction of the outer mouth of the pore (Liu et al., 1996). In the Na channel, whether a similar constriction of the outer pore occurs is not certain. Townsend and Horn showed that increasing the extracellular Na^+ concentration inhibits slow inactivation, suggesting that binding of Na^+ to a site near the outer mouth of the pore inhibits closing of the slow inactivation gate (Townsend and Horn, 1997). On the other hand, gating- and conformation-independent modification of cysteine mutants in the outer pore by methanethiosulfonate (MTS) reagents argues that the outer pore remains largely unobstructed when slow inactivated (Struyk and Cannon, 2002). However, in the latter study, only a limited number of cysteine mutations

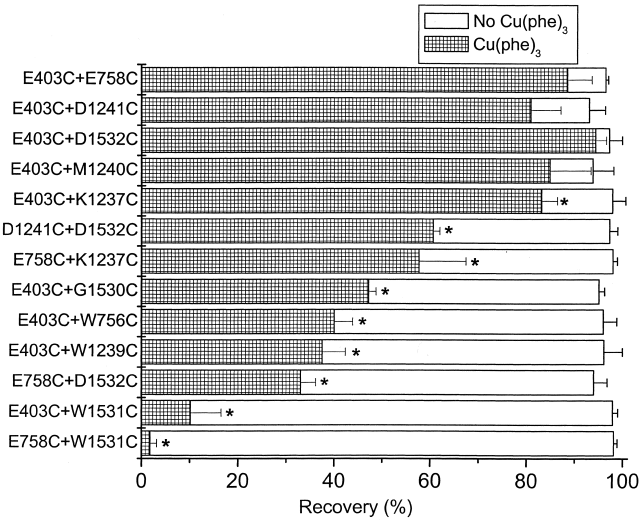

FIGURE 5. A bar plot of the percent recovery from slow inactivation elicited by the voltage protocol in Fig. 4 A. The percent recoveries of E403C+E758C, E403C+K1240C, E403C+D1241C, and E403C+D1532C channels were not significantly changed in the presence of $Cu(phe)_3$ ($P > 0.05$, $n = 3–6$). Significant $Cu(phe)_3$-induced modification of the other paired mutants occurred during induction of slow inactivation ($P < 0.05$, $n = 3–4$).

were informative and most of these were superficial in the pore (Struyk and Cannon, 2002), thus a dynamic constriction of the deeper reaches of the pore during slow inactivation cannot be excluded. Indeed, we observed reduced accessibility to the side chain of F1236C to (2-aminoethyl)methanethiosulfonate (MTSEA) with voltage protocols favoring slow inactivation (Ong et al., 2000). Therefore, a structural rearrangement of the outer pore is likely during slow inactivation of Na channels, although it is uncertain whether such a conformation change completely prevents ion flux.

The P-segments are asymmetrically organized in the Na channel and each segment in domains I–IV makes a unique contribution to the formation of the pore (Chiamvimonvat et al., 1996). Unexpected flexibility of P-segments of Na channels allows disulfide formation between the side chains of paired engineered cysteines in P-segments of different domains (Benitah et al., 1997; Tsushima et al., 1997). The unique ability of cysteine mutants in domains I and II to form spontaneous disulfide bonds suggests that these two domains are in close proximity (Benitah et al., 1997). The spontaneous or catalyzed disulfide bonding of E403C and E758C in this study is consistent with this close proximity of the domains I and II P-segments and a separation of no more than 7 Å between the α carbons.

The outer charged ring appears to exhibit a high degree of flexibility during channel gating, consistent with previous studies (Benitah et al., 1996, 1997, 1999; Tsushima et al., 1997). Paired cysteine mutants in the outer ring exhibited conformationally sensitive rates of modification in the presence of a redox catalyst (Fig. 3 C). At the faster stimulation frequencies from a holding voltage of −100 mV, the channels spend a greater proportion of time in the activated and fast inactivated states compared with the closed state. The reactive thiol side chains in paired mutant channels that exhibited faster rates of modification at 1 Hz compared with 0.033 Hz stimulation frequencies may come into close proximity during activation or fast inactivation. Notably, even those mutant channels, which did not exhibit stimulation frequency–dependent differences in the rate of disulfide bond formation, were rapidly modified by $Cu(phe)_3$ (Fig. 3 C). This is consistent with preferential modification of closed channels or nearly equal rates of modification of closed and activated/fast inactivated channels.

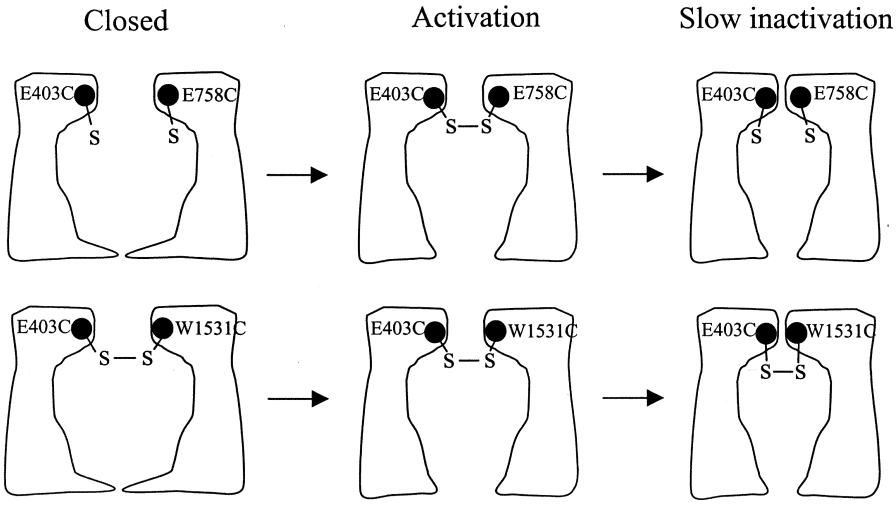

FIGURE 6. A schematic model showing how gating influences the rate and extent of disulfide bond formation of double mutants in the external pore of the Na channel. In the case of E403C+E758C, enhanced rates of current reduction with higher stimulation frequencies suggest that the activated or fast inactivated states are modified more readily than the rested state. In contrast, double mutant channels such as E403C+W1531C (Fig. 3 C) are rapidly modified at both slow and fast stimulation rates, suggesting that the rested state may be accessible to modification by $Cu(phe)_3$. Slow inactivation protects E403C+E758C channels (and E403C+D1241C and E403C+D1532C in the outer ring of charge) but not E403C+W1531C from $Cu(phe)_3$-induced modification. The results are consistent with a change in the conformation of the outer ring of charge during slow inactivation of Na channels.

W756C was previously shown to be insensitive to bulky charged MTS reagents (Perez-Garcia et al., 1996), but in this study E403C forms a disulfide bond with W756C in the presence of $Cu(phe)_3$. This apparent discrepancy is not well understood. MTS agents may modify W756C without a significant effect on the current amplitude. Alternatively, the combination of both mutations may alter the pore structure so that the side chain of W756C is accessible for disulfide bond formation.

Residues in the outer ring of charge are also involved in the conformational changes in the channel pore associated with slow inactivation. For example, Na currents through E403C+E758C mutant channels that were slow inactivated before the application of $Cu(phe)_3$ completely recovered (Fig. 4 C), suggesting that slow inactivation protects the channels from modification by the redox catalyst. The structural rearrangement in the outer pore during slow inactivation appears to alter the spatial relationships of the thiol side chains of E403C (domain I) and E758C (domain II) (Fig. 6). Similar protection was afforded to E403C+D1241C (domain III) and E403C+D1532C (domain IV) channels. An alternative explanation is the accessibility of the redox catalyst to the outer ring of charge changed with slow inactivation. It is possible that the redox catalyst was excluded from the pore; however, this would have to be a selective exclusion because some double mutant channels involving residues deep in the pore (e.g., E403C+W756C, E403C+W1531C, E758C+W1531C, Fig. 5) were modified by $Cu(phe)_3$. Furthermore, the same paired cysteine mutants in the outer ring of charge underwent catalyzed disulfide bond formation by $Cu(phe)_3$ when cells were pulsed from a holding potential of −100 mV (Fig. 3). In any case, slow inactivation appears to involve movements of the P-segments and changes in the spatial relationships of the side chains of the outer charged ring residues.

In addition to residues in the external charge ring, two other cysteine mutants when combined with E403C provided nearly complete protection from disulfide bond formation when the channels were slow inactivated. Na currents through E403C+K1237C mutant channels exhibited comparable recovery from slow inactivation independent of prior exposure to $Cu(phe)_3$, suggesting a role for K1237 in slow forms of inactivation. These data support the study by Todt et al. (1999) that charge-altering mutations at position 1237 favor occupancy of the ultra-slow inactivation state, similar to C-type inactivation in shaker K channels. Slow inactivation also protected E403C+M1240C from oxidation; however, M1240C undergoes extraordinarily slow modification by MTS agents that is independent of the channel conformation (Struyk and Cannon, 2002). We cannot exclude this as the cause of the redox insensitivity of this paired mutant channel.

The varying susceptibility of different outer ring double mutants further highlights the asymmetry of the Na channel pore (Chiamvimonvat et al., 1996; Perez-Garcia et al., 1996). The S4 segment, half of a "voltage-sensor paddle" (Jiang et al., 2003), may also play a significant role in slow inactivation of the Na channel (Kontis and Goldin, 1997; Mitrovic et al., 2000). It has been proposed that if slow inactivation depends on the mobility of S4 segments, S4 immobilization by fast inactivation may inhibit slow inactivation (Featherstone et al., 1996; Richmond et al., 1998). Indeed, Cha et al. (1999) have shown that S4 voltage sensors in domains III and IV, but not I and II, are immobilized by Na channel fast inactivation. Furthermore, the largest effects on Na channel slow inactivation result from mutations of S4 in domains I and II (Kontis and Goldin, 1997; see also Mitrovic et al., 2000). Thus, it is likely that the S4 segments in domains I and II make significantly different contributions to slow inactivation compared with domains III and IV. However, whether the effects of each S4 on slow inactivation transfer to its outer pore segments remains unknown.

In summary, our findings demonstrate that the role of the outer ring of charge is not limited to conventional notion that it increases the conductance by attracting cations into the channel entryway via its electrostatic action (Bell and Miller, 1984; Green et al., 1987). The molecular motions of the outer ring of charge (especially E403) suggest an unexpected role in slow inactivation of the Na channel.

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