Disulfide trapping studies have revealed that the pore-lining (P) segments of voltage-dependent sodium channels undergo sizable motions on a subsecond time scale. Such motions of the pore may be necessary for selective ion translocation. Although traditionally viewed as separable properties, gating and permeation are now known to interact extensively in various classes of channels. We have investigated the interaction of pore motions and voltage-dependent gating in μ1 sodium channels engineered to contain two cysteines within the P segments. Rates of catalyzed internal disulfide formation ($k_{iSS}$) were measured in K1237C+W1531C mutant channels expressed in oocytes. During repetitive voltage-clamp depolarizations, increasing the pulse duration had biphasic effects on the $k_{iSS}$, which first increased to a maximum at 200 msec and then decreased with longer depolarizations. This result suggested that occupancy of an intermediate inactivation state ($I_m$) facilitates pore motions. Consistent with the known antagonism between alkali metals and a component of slow inactivation, $k_{iSS}$ varied inversely with external [Na $^+$]. We examined the converse relationship, namely the effect of pore flexibility on gating, by measuring recovery from inactivation in Y401C+E758C (YC/EC) channels. Under oxidative conditions, recovery from inactivation was slower than in a reduced environment in which the spontaneous YC/EC cross-link is disrupted. The most prominent effects were slowing of a component with intermediate recovery kinetics, with diminution of its relative amplitude. We conclude that occupancy of an intermediate inactivation state facilitates motions of the P segments; conversely, flexibility of the P segments alters an intermediate component of inactivation.

**Key words:** sodium channel; inactivation; permeation; cysteine mutagenesis; disulfide bond
We find that gating influences motions and vice versa. The direction of the effects is as follows. Greater flexibility decreases the occupancy of a particular inactivated state and slows its recovery kinetics; conversely, pulse protocols that favor the occupancy of this inactivated state facilitate KC/WC disulfide formation.

**MATERIALS AND METHODS**

*Mutagenesis and channel expression.* Site-directed mutagenesis of the α subunit of the α Na channel cDNA was performed as described previously (Bénitah et al., 1997a). Briefly, mutations were introduced by recombinant PCR on the double-stranded cDNA using *pfu* DNA polymerase and verified by sequencing. Oocytes were removed through an abdominal incision from ovaries of adult female *Xenopus laevis* (*Xenopus* I, Ann Arbor, MI, or Nasco, Ft. Atkinson, WI) under anesthesia and then treated for 1 hr with collagenase (Type 1A, Sigma, St. Louis, MO) in modified Barth’s solution containing (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO3, 15 Tris base, 0.8 Ca(NO3)2–4 H2O, 0.41 CaCl2–6 H2O, 0.82 MgSO4–7 H2O, supplemented with 100 U/ml penicillin, 100 μg streptomycin, 250 ng fungizone, and 50 μg/ml gentamicin. Oocytes (stages V and VI) were coinjected with cRNA for the α Na channel α subunit and the rat brain β1 subunit in an equimolar ratio (Bénitah et al., 1997a).

*Electrophysiology and data analysis.* Membrane currents were recorded 24–72 hr after injection using two microelectrodes, as described previously (Bénitah et al., 1997b), in frog Ringer’s solution containing (in mM): 96 NaCl, 1 KCl, 1 MgCl2, 5 HEPES, pH 7.6. Dithiothreitol (DTT; 1 mM) and Cu(II)(1, 10-phenanthroline) 3 [Cu(phe)3 ; 100 mM): 96 NaCl, 2 KCl, 1 MgCl2, 5 HEPES, pH 7.6. Dithiothreitol (DTT; 1 mM) and Cu(II)(1, 10-phenanthroline)3 [Cu(phe)3 ; 100 mM] were dissolved in the external solution as described previously (Bénitah et al., 1997a). Voltage protocols and data acquisition were managed by custom-written software as described previously (Bénitah et al., 1997b). Linear leak and capacitive currents were corrected using a P/4 protocol. Best data fits were performed using a nonlinear least-squares Marquardt-Levenberg algorithm (Origin, MicroCal, Northampton, MA). Average data are expressed as means ± SEM. Statistical significance levels were evaluated by ANOVA and paired *t* (Origin), as appropriate. A *p* value of < 0.05 was considered significant.

**RESULTS**

**Cu(phe)3 catalyzes disulfide formation**

We showed previously that multiple double-cysteine mutants of the α Na channel can form internal cross-links, either spontaneously or in the presence of the redox catalyst Cu(phe)3. Because of the patterns and rates of the reactions, we concluded that the P segments that form the structural basis of the permeation pathway are flexible (Bénitah et al., 1997a). Figure 1 shows an example of the occlusion of the pore by catalyzed internal disulfide formation. The panels show representative Na currents in the P-segment. Furthermore, recent studies in Shaker potassium channels (Li et al., 1996), a slow (C-type) inactivation process has been linked to dynamic rearrangement of residues in the P-segment. Furthermore, recent studies in α Na+ channels (Balser et al., 1996) have shown that cysteine substitution of a single P-segment tryptophan (W402) reduces slow inactivation. Given the spatial proximity between W402 and both K1237 and W1531 (Bénitah et al., 1997a), we examined the relationship between slow inactivation and K1237C–W1531C disulfide bond formation. Our previous work (Bénitah et al., 1997a) showed that the effect of Cu(phe)3 on disulfide formation is not overtly voltage dependent; however, these experiments were not designed to discriminate among particular inactivated states. Figure 3 illustrates that the time course of Cu(phe)3 inhibition depends on the duration of depolarization. Macroscopic currents from oocytes expressing KC/WC channels were monitored during a 0.05 Hz pulse train with depolarizations from −100 to −40 mV before and after addition of 100 μM Cu(phe)3 to the bath solution. Although with 50 or 5000 msec pulse duration complete inhibition requires >9 min, with 200 msec pulses the Cu(phe)3 effect is nearly complete within 5 min.

Figure 4 summarizes the rate constants of redox catalysis determined from a number of oocytes exposed to Cu(phe)3 and several other engineered cysteine pairs (Bénitah et al., 1997a). These observations imply the existence of motions in the pore; constraining such motions through disulfide bond formation eliminates or reduces ion flux.

To quantitate the thermal backbone motions revealed by disulfide trapping methods, the oxidation catalysis reactions were analyzed according to Careaga and Falke (1992) as described previously (Bénitah et al., 1997a). Briefly, the oxidation chemistry of the Cu(phe)3 catalysis yields two competing reactions that generate the intended disulfide bond product and undesired higher oxidation products of cysteine. Higher oxidation products may be formed in the wild-type and single-cysteine mutant channels but do not affect the current amplitude or gating (Fig. 1). Furthermore, both catalytic reactions are effectively irreversible under these oxidized experimental conditions. Application of Cu(phe)3 to the KC/WC mutant eliminates the current, presumably by disulfide bond formation, and is reversible under reducing conditions. Therefore the rate of formation of higher oxidation products is slowed compared with the rate of disulfide bond formation. The rate constants of the disulfide formation (*k*disulfide) were determined by fitting a single-component reaction scheme to the observed time course of the Cu(phe)3 inhibition of the current (see legend of Fig. 2) (Bénitah et al., 1997a). Figure 2 shows a representative disulfide reaction time course for the KC/WC channels, including a nonlinear fit illustrating good agreement between the single-component reaction scheme and the data.

**Occupancy of a particular inactivated state facilitates disulfide formation**

During depolarization, voltage-gated ion channels assume nonconducting inactivated states, which may be characterized as “fast” or “slow,” depending on the rate at which the channels recover from inactivation when subsequently repolarized (Rudy, 1978). Short depolarization favors fast inactivation, whereas slow-inactivated conformations develop with longer depolarizations. Although fast inactivation involves internal structures exposed to the cytoplasm [the N terminus in K channels (Hoshi et al., 1990) and the III–IV linker in Na channels (Stühmer et al., 1989)], slow inactivation seems to involve residues in the outer pore. In Shaker potassium channels (Li et al., 1996), a slow (C-type) inactivation process has been linked to dynamic rearrangement of residues in the P-segment. Furthermore, recent studies in α Na+ channels (Balser et al., 1996) have shown that cysteine substitution of a single P-segment tryptophan (W402C) reduces slow inactivation. Given the spatial proximity between W402 and both K1237 and W1531 (Bénitah et al., 1997a), we examined the relationship between slow inactivation and K1237C–W1531C disulfide bond formation. Our previous work (Bénitah et al., 1997a) showed that the effect of Cu(phe)3 on disulfide formation is not overtly voltage dependent; however, these experiments were not designed to discriminate among particular inactivated states. Figure 3 illustrates that the time course of Cu(phe)3 inhibition depends on the duration of depolarization. Macroscopic currents from oocytes expressing KC/WC channels were monitored during a 0.05 Hz pulse train with depolarizations from −100 to −40 mV before and after addition of 100 μM Cu(phe)3 to the bath solution. Although with 50 or 5000 msec pulse duration complete inhibition requires >9 min, with 200 msec pulses the Cu(phe)3 effect is nearly complete within 5 min.

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trains of depolarizing pulses ranging from 3 msec to 5 sec. In all cases, the pulse frequency (0.05 Hz) was slow enough to allow complete recovery from inactivation between depolarizations (data not shown). Disulfide bond formation hastened as the pulse lengthened, reaching a peak value at 200 msec (Figs. 3, 4). When even longer pulses were applied, $k_{ss}$ slowed to a rate nearly the same as that in the briefest (3 msec) pulse. These results indicate that pulse durations of intermediate length enhance the rate of disulfide formation, suggesting that occupancy of an “intermediate” inactivated state may facilitate the catalytic effect of Cu(phe)$_3$.

When the $\alpha$ and $\beta_1$ subunits of $\mu 1$ channels are coexpressed in Xenopus oocytes and subjected to 1 sec depolarizations, inactivation is characterized by a minimum of three readily distinguishable kinetic components (Nuss et al., 1995). These include an initial rapid component ($t_{fast}$) with a recovery time constant of ~1 msec, which exhibits marked sensitivity to mutations in the III–IV linker (West et al., 1992; Lawrence et al., 1996), and two slower components ($t_{intermediate}$ and $t_{slow}$) with recovery time constants of ~100 msec and 1 sec, which are sensitive to domain I, P-segment mutations (Balser et al., 1996; Kambouris et al., 1998). Although a number of gating schemes are consistent with these kinetic data, the following model provides a convenient framework for discussing the possible interaction between fast ($I_F$), intermediate ($I_M$), and slow-inactivated ($I_S$) states and disulfide bond formation: Closed $\leftrightarrow$ Open $\leftrightarrow I_F \leftrightarrow I_M \leftrightarrow I_S$.

To examine the hypothesis that the rate of disulfide formation is associated with entry into a particular inactivated state with intermediate kinetics ($I_M$ in the scheme above), we studied the rate of inactivation of the KC/WC mutant using a paired-pulse voltage-clamp protocol (Fig. 5, top). Channels were depolarized for incremental periods, and fractional recovery was assessed.
rate parameters \(k\) to the data: 1

describing the single-component reaction scheme (see Results) was fitted of

disulfide formation in the outer pore is facilitated by occupancy diac (hH1) Na channels revealed that raising \([\text{Na}^+]_o\) inhibitions (\(\Delta S\)). Furthermore, the time constant of entry into the \(I_{M}\) state is consistent with the pulse duration that induced the most rapid rate of disulfide formation (200 msec) (Fig. 4). Much shorter pulses would populate only \(I_F\), whereas longer pulses begin to recruit an appreciable fraction of \(I_O\). These findings suggest that disulfide formation in the outer pore is facilitated by occupancy of \(I_M\). Furthermore, the biphasic nature of \(k_{ss}\) suggests that channels occupying fast-inactivated states (\(I_F\)) are less likely to form disulfides, as are the states favored by even longer depolarizations (\(I_S\)).

**[Na\(^+\)]_o modulates the rate of disulfide formation**

Although flux generally increases as a function of permeant ion concentration, an unanticipated inhibition of open probability by a reduction in external Na\(^+\) was described recently (Townsend et al., 1997). Both whole-cell and single-channel recordings of cardiac (hH1) Na channels revealed that raising [Na\(^+\)]_o inhibits the rate of slow inactivation at depolarized membrane potentials, as if binding of the alkali metal cation in the pore inhibits closure of the slow inactivation gate. We thus examined the time course of Cu(phen)$_3$-catalyzed disulfide bond formation in oocytes bathed in different extracellular Na\(^+\) concentrations. Figure 6A plots the time course of current inhibition by Cu(phen)$_3$ in representative oocytes bathed in 48, 96, or 140 mM Na\(^+\) solutions. KC/WC current is inhibited slowly and completely within 10 min in 96 mM [Na\(^-\)]_o (open circles). Raising [Na\(^-\)]_o, (open diamonds) slowed this time course, whereas decreasing [Na\(^-\)]_o, (open triangles) accelerated the inhibitory effect of the Cu(phen)$_3$. The time courses were analyzed as described previously to determine the rate constants of the redox reaction (Fig. 2) (Bénitah et al., 1997a). The rate constants of disulfide bond formation (\(k_{ss}\)) changed notably as a function of the permeant ion concentration. Increasing [Na\(^+\)]_o (and therefore ion occupancy and relative conductance) slowed the kinetics of disulfide bond formation (Table 1). Although it remains possible that increasing Na\(^+\) occupancy in the pore had a direct and unanticipated inhibitory effect on disulfide formation, the decrease in \(k_{ss}\) with increasing ionic conductance is consistent with the inhibitory effect of [Na\(^-\)]_o on the rate of slow inactivation (Fig. 6B) (Townsend and Horn, 1997). Alteration in the rate of [Na\(^-\)]_o-induced occupancy of slow inactivated states is not unique to the KC/WC mutant. Entry into the \(I_M\) state of the wild-type channel is accelerated in low [Na\(^-\)]_o (Fig. 6B), suggesting that the KC/WC double mutant does not dramatically alter the outer pore structure. Thus, the observations of dependence on [Na\(^-\)]_o, and on depolarizing pulse duration may share a common basis in the fractional occupancy of particular inactivated states (e.g., \(I_{M}\)).
Redox state modulates a slow-inactivated state transition

The experiments discussed above examine the influence of gating on pore mobility. To examine the converse issue, the influence of mobility on gating, we used another double-cysteine mutant, **Y401C**1**E758C (YC/EC). YC/EC spontaneously forms a functionally evident disulfide bond (Bénitah et al., 1996) under oxidizing conditions, whereas a reducing environment breaks the covalent bond, reestablishing free motion in the outer pore. The unique characteristics of this double-cysteine mutation allowed us to examine the effect of a partially immobilized outer pore structure on the development of slow inactivation. Figure 7 shows the

**Figure 4.** Cu(phe)3 catalyzed disulfide bond formation in the K1237C+W1531C double mutant has a biphasic dependence on pulse duration. The disulfide formation rates (kss) plotted as a function of the pulse duration for oocytes expressing the K1237C+W1531C double mutant. The Na currents were elicited by depolarizing pulses at a rate of 0.05 Hz and durations ranging from 3 msec to 5 sec. We detect no cumulative of inactivation at this slow stimulation frequency for even the longest (5 sec) depolarizations. Currents were measured before and after addition of 100 μM Cu(phe)3, and the reaction rate constants were determined as described for Figure 2. The plotted values (means ± SEM) represent measurements from at least four different oocytes for each data point. *, p < 0.02; **, p < 0.002.

**Figure 5.** Rate of development of intermediate and slow-inactivation for the double-mutant K1237C+W1531C. As shown in the voltage-clamp protocol (top), the duration of the depolarizing prepulse (−40 mV) was varied, and the extent of recovery after 20 msec at −100 mV was assessed using a 50 msec test pulse to −40 mV. The 20 msec recovery interval removed the most rapidly recovering inactivation component (Iₐ) from consideration. Plotted is the fractional recovery from inactivation as a function of the prepulse duration. Data were collected from seven oocytes. The dotted line shows a nonlinear fit to the mean data using the function: \( y = A_1 \exp(-t/t_1) + A_2 \exp(-t/t_2) \). The least squares error was minimized when \( A_1 = 0.2 \), \( t_1 = 66 \) msec, \( A_2 = 0.8 \), and \( t_2 = 5212 \) msec.

**Figure 6.** External Na⁺ concentration modulates the rate of inactivation and disulfide bond formation. A. Representative examples of the time course of the inhibition of K1237C+W1531C by 100 mM Cu(phe)3 obtained from oocytes bathed in either (○) 48 mM [Na⁺]o, (●) 96 mM [Na⁺]o, or (□) 140 mM [Na⁺]o. Sorbitol was used as a substitute for Na⁺ to maintain constant osmolarity. The currents were elicited by repetitive 50 msec pulses from −100 to −35 mV (0.5 Hz). Plotted are the whole-cell Na current amplitudes normalized to the value measured before the application of Cu(phe)3 (100 μM). Summary data for kss as a function of extracellular [Na⁺]o are given in Table 1. B. Rates of the development of inactivation of wild-type m1 channels coexpressed with the β1 subunit. The protocol is identical to that shown in Figure 5 for the KC/WC mutant. Reducing the [Na⁺]o enhances the rate of development of slow inactivation. In 96 mM [Na⁺]o (●) there is little development of intermediate inactivation (< 3%). In contrast, in 10 mM [Na⁺]o (○) a 1 sec prepulse inactivates 35% of the current.

**Figure 7.** Redox state modulates a slow-inactivated state transition.

The experiments discussed above examine the influence of gating on pore mobility. To examine the converse issue, the influence of mobility on gating, we used another double-cysteine mutant, YC/EC. YC/EC spontaneously forms a functionally evident disulfide bond (Bénitah et al., 1996) under oxidizing conditions, whereas a reducing environment breaks the covalent bond, reestablishing free motion in the outer pore. The unique characteristics of this double-cysteine mutation allowed us to examine the effect of a partially immobilized outer pore structure on the development of slow inactivation. Figure 7 shows the
time dependence of recovery from inactivation of YC/EC. Oocytes were held at −100 mV, and a conditioning pulse was applied for either 50 msec (Fig. 7A) or 1 sec (Fig. 7B). The conditioning pulse was followed by recovery at −100 mV for intervals varying from 10 msec to 1 sec, and the Na⁺ current was measured in response to a subsequent 50 msec test depolarization. Cumulative inactivation between experiments was excluded by using a long (30 sec) recovery interval at −100 mV after each test pulse, and by confirming that there was no variation in the Na current elicited by successive conditioning pulses. Oocytes were sequentially bathed in normal oxidized solution (left, solid symbols) and a reducing environment (right, open symbols). Exposure to DTT increased the magnitude of the Na current, presumably by breaking the disulfide bond (Benitah et al., 1996). In addition, after the longer depolarizing prepulse (Fig. 7B), DTT significantly slowed the rate of recovery from inactivation. Figure 7C plots the fractional recovery of the peak Na⁺ current amplitude against the recovery interval at −100 mV. As shown previously for wild-type μ1 channels (Nuss et al., 1996), YC/EC recovery from inactivation exhibited three readily separable kinetic components. The recovery rates (τ) and the relative amplitudes of the three components as a function of the external environment and the conditioning pulse length are summarized in Table 2. After the brief conditioning pulses (50 msec), <20% of the Na channels entered slow-inactivated states (Table 2), and the redox environment had no significant effect on the relative amplitudes or time constants of the three components of recovery. However, with longer conditioning pulses (1 sec), >60% of the channels entered slowly recovering inactivated states (I_M, I_s). Exposure to a reducing environment speeded recovery by decreasing the amplitude of the intermediate component (Table 2). The time constant of recovery from this intermediate component of inactivation was also increased (Table 2), although the extent to which this influenced the overall recovery profile was small. These data suggest that a reducing environment raised the energy barrier controlling entry to, and exit from, a slow-inactivated state with intermediate kinetics similar to that facilitating K1237C–W1531C disulfide bond formation (Figs. 3, 4). Our findings are consistent with the notion that “freezing” the outer pore with a disulfide bond facilitates both entry into and egress from an inactivated state that possesses intermediate recovery kinetics (I_M).

**DISCUSSION**

Previous site-directed mutagenesis studies (Balser et al., 1996; Kambouris et al., 1998) suggest that slow kinetic components of Na⁺ channel inactivation involve conformational changes in the outer pore. W402C, a domain I P-segment substitution, markedly enhanced recovery by reducing the likelihood that depolarized μ1 channels would occupy slowly recovering inactivated states. Notably, μ1 channels are capable of entering two inactivated states with kinetics slower than the duration of the I_s transient; these include both intermediate and slow kinetic components with recovery time constants at −100 mV on the order of ~100 msec and 1 sec, respectively (Nuss et al., 1995, 1996). Similar to the W402 P-segment mutations (Kambouris et al., 1998), the findings presented here suggest that disulfide formation in the outer pore of the KC/WC mutant is primarily sensitive to the inactivated state with intermediate kinetics (I_M). It is possible that the mutations themselves alter channel conformation or movement in a way that is not representative of the wild-type μ1 channel. We observed no change in the gating of the W1531C component of the double mutant (Balser et al., 1996). The K1237C component alters both permeation (Favre et al., 1996) and the development of ultra-slow inactivation (Todt et al., 1997) but not the rate of occupancy of I_M (data not shown). The biphasic dependence of the rate of disulfide formation (k_s) (Figs. 3, 4) on pulse duration suggests that when either I_P or I_s is occupied, the rate of disulfide formation is slowed compared with that when I_M is the predominant inactivated state. One possible interpretation, illustrated in Figure 8, is that the spatial relationship between the 1237 and 1531 cysteines is optimized by occupancy of the I_M state. Consistent with this hypothesis, the rate of disulfide formation decreases when the rate of entry into slow-inactivated states is modified by elevating the external [Na⁺] (Fig. 6, Table 2) (Townsend et al., 1997). Admittedly, the sensitivity of particular slow inactivated states (e.g., I_M vs I_s) to alkali metal cations has not been characterized, and it remains possible that the inhibitory effect of external Na⁺ on disulfide formation results from a direct competition between the covalent reaction and Na⁺ binding in the pore. However, the converse experiment using a spontaneously forming disulfide (YC/EC) (Fig. 7) showed that freezing the pore with a disulfide selectively facilitated entry into, and recovery from, an inactivated state with intermediate gating kinetics (Table 2). Hence, our results suggest that greater flexibility decreases the occupancy of a particular inactivated state with intermediate kinetics and furthermore that occupancy of this inactivated state facilitates KC/WC disulfide formation. These findings are consistent with the general notion of microscopic reversibility: inactivation gating influences motion in the outer pore, and vice versa.

These data have several implications for the structure of the Na channel pore. In the recently published KCsA channel structure (Doyle et al., 1998), the tryptophan in the selectivity region makes important contacts with other aromatic residues facing away from the pore, forming an aromatic cuff around the selectivity filter. The structural data are consistent with the finding that some mutations at this tryptophan [e.g., W434F (Perozo et al., 1993)] eliminate ionic flux by “locking” the K channel in an inactivated state (Yang et al., 1997). This is distinct from the phenotype of the Na channel with mutations of a comparable ring of tryptophans (Tomaselli et al., 1995; Balser et al., 1996). The Na channel pore is not compromised by single mutations of the tryptophans in any of the domains. In domains I, III, and IV, mutagenesis data suggest that this side chain points into the pore, consistent with the findings of Lu and Miller (1995), who found that single tryptophan to cysteine mutations in the K channel were accessible to block by silver. In domain II the permeation phenotype of the Na channel mutant (W756C) is identical to that of the wild-type channel (Perez-Garcia et al., 1996). The tryptophans in the K channel are part of the pore helix on the amino terminal side of the selectivity filter. By sequence alignment with the K channel, this ring of tryptophans in the Na channel is predicted to be in the filter loop on the C-terminal side.

### Table I. Effect of Na⁺ concentration on the rate of disulfide bond catalysis by Cu (Phe), on the double-mutant K1237C+W1531C (mean ± SD)

<table>
<thead>
<tr>
<th>[Na⁺]₀ (mM)</th>
<th>k_s (molecule⁻¹·sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>15.25 ± 6.61</td>
</tr>
<tr>
<td>96</td>
<td>7.58 ± 6.19</td>
</tr>
<tr>
<td>140</td>
<td>7.59 ± 1.94</td>
</tr>
</tbody>
</table>

n = 3 (n = 11) (n = 6)
of the filter residues. It appears that the pore tryptophans do not have analogous roles in the Na and K channels.

The combination of paired cysteine mutagenesis and disulfide trapping is a powerful technique for indirect structural study of the channel pore and the role of motion of the external vestibule in channel gating. Several limitations of the technique should be enumerated. First, it is unclear whether the backbone or side chains of the substituted residues are exhibiting flexibility and permitting trapping. Structural models of the Na channel (Guy and Durell, 1995) and KcsA (Doyle et al., 1998) suggest that the residues (or analogous residues in the K channel) are sufficiently separated that simple side chain movement would not place the cysteins close enough to form a disulfide. Second, it is possible that disulfide trapping captures the channel in an infrequently occupied and unphysiological state. However, the enhancement of the rate of disulfide formation during occupancy of \( I_{M} \), a state normally occupied by the channel, argues against trapping in a completely unnatural configuration. Finally, as noted previously, the mutations themselves may alter the flexibility and movement in the vestibule.

These data support the concept that analogous to C-type inactivation (Hoshi et al., 1991; Liu et al., 1996) in K channels, inactivation involving structural rearrangement in the outer pore occurs in the Na channel. It is possible that both intermediate inactivation (\( I_{M} \)) (Kambouris et al., 1998) and ultraslow inactivation (\( I_{S} \)) (Todt et al., 1997) involve structural changes in the outer pore, but only occupancy of \( I_{M} \) influences the rate of disulfide bond formation between paired cysteine mutants exam-
Table 2. The effect of redox environment on Y401C+E758C recovery from inactivation (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DTT</th>
<th>Control</th>
<th>DTT</th>
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<tbody>
<tr>
<td>( \tau_1 ) (msec)</td>
<td>2.5 ± 0.9</td>
<td>2.3 ± 0.4 (NS)</td>
<td>4.4 ± 1.1</td>
<td>4.9 ± 4.0 (NS)</td>
</tr>
<tr>
<td>( A_1 )</td>
<td>0.82 ± 0.13</td>
<td>0.79 ± 0.09 (NS)</td>
<td>0.42 ± 0.18</td>
<td>0.45 ± 0.12 (NS)</td>
</tr>
<tr>
<td>( \tau_2 ) (msec)</td>
<td>43.7 ± 24.1</td>
<td>36.2 ± 18.1 (NS)</td>
<td>58.6 ± 37.8</td>
<td>123.1 ± 82.8 (p &lt; 0.05)</td>
</tr>
<tr>
<td>( A_2 )</td>
<td>0.13 ± 0.10</td>
<td>0.13 ± 0.07 (NS)</td>
<td>0.30 ± 0.09</td>
<td>0.22 ± 0.04 (p &lt; 0.05)</td>
</tr>
<tr>
<td>( \tau_3 ) (msec)</td>
<td>728 ± 742</td>
<td>907 ± 640 (NS)</td>
<td>4301 ± 2426</td>
<td>5852 ± 5930 (NS)</td>
</tr>
<tr>
<td>( A_3 )</td>
<td>0.05 ± 0.07</td>
<td>0.08 ± 0.09 (NS)</td>
<td>0.28 ± 0.10</td>
<td>0.33 ± 0.12 (NS)</td>
</tr>
</tbody>
</table>

**Figure 8.** Occupancy of an intermediate inactivated state facilitates disulfide formation in the outer pore. The diagram illustrates sequential occupancy of three distinct inactivated states (\( I_1 \), \( I_2 \), and \( I_3 \)) as the length of the depolarization is extended. Time constants for entry into \( I_3 \) and \( I_2 \) are based on the biexponential fit to the data in Figure 5, and the time constant for development of fast inactivation (\( I_1 \)) was estimated from the literature (Nuss et al., 1995). The scheme suggests that when the \( I_3 \) state is occupied, the 1237 and 1531 cysteines are spatially optimized in the outer pore for disulfide formation.

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