Depolarization Induces Intersubunit Cross-linking in a S4 Cysteine Mutant of the Shaker Potassium Channel*

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Voltage-gated potassium (Kv) channels are integral membrane proteins, composed of four subunits, each comprising six (S1–S6) transmembrane segments. S1–S4 comprise the voltage-sensing domain, and S5–S6 with the linker P-loop forms the ion conducting pore domain. During activation, S4 undergoes structural rearrangements that lead to the opening of the channel pore and ion conduction. To obtain details of these structural changes we have used the engineered disulfide bridge approach. For this we have introduced the L361C mutation at the extracellular end of S4 of the Shaker K channel and expressed the mutant channel in Xenopus oocytes. When exposed to mild oxidizing conditions (ambient oxygen or copper phenanthroline), Cys-361 formed an intersubunit disulfide bridge as revealed by the appearance of a dimeric band on Western blotting. As a consequence, the mutant channel suffered a significant loss in conductance (measured by two-electrode voltage clamp). Removal of native cysteines failed to prevent the disulfide formation, indicating that Cys-361 forms a disulfide with its counterpart in the neighboring subunit. The effect was voltage-dependent and occurred during channel activation after Cys-361 has been exposed to the extracellular phase. Although the disulfide bridge reduced the maximal conductance, it caused a hyperpolarizing shift in the conductance-voltage relationship and reduced the deactivation kinetics of the channel. The latter two effects suggest stabilization of the open state of the channel. In conclusion, we report that during activation the intersubunit distance between the N-terminal ends of the S4 segments of the L361C mutant Shaker K channel is reduced.

Voltage-gated potassium (Kv) channels are transmembrane proteins made up of two domains, a central pore domain and a surrounding voltage-sensing domain. The pore domain forms the water-filled, potassium ion-selective pore across the plasma membrane of the cell, whereas the voltage-sensing domain regulates the opening and closing of activation gates situated at the cytoplasmic end of the pore domain (1–4). The gates are closed at negative (resting) membrane potentials but open upon membrane depolarization to allow K⁺ ions to enter the pore. Due to the lack of three-dimensional structural data for any of the Kv channel proteins, it is not clear how the voltage sensor detects changes in membrane potential and transmits the signal to the activation gates.

Kv channels are made up of four subunits, each of which contains six transmembrane segments, named S1–S6. The S5–S6 and the “P-loop” connecting these segments form the central pore domain in Kv channels (2). The structure of the pore domain is thought to be similar to that of the bacterial potassium channel, KcsA, whose structure has been determined by x-ray diffraction (5–7). The remaining transmembrane segments, in particular the S2 to S4 segments, are thought to comprise the voltage-sensing domain of the channel (2, 9, 10). Of the four segments, S4 plays a pivotal role. When the membrane is depolarized, it moves out of the membrane, thereby carrying its charged residues (arginine and lysine), known as gating charges, across the membrane electric field (11–17). It is this movement that appears to trigger the opening of the activation gates.

Molecular modeling (10) and mutagenic (18–20) studies suggest that one face of the S4 segment is in direct contact with the pore domain, whereas the rest is surrounded by S1–S3, which seem to protect the charged S4 segment from the energetically unfavorable lipid environment by providing counter charges. Because of these extensive interactions that S4 appears to be engaged in, when S4 moves one would expect major changes in residue-residue contacts with the neighboring helices. Indirect evidence indicates that the S4 movement is accompanied by changes in the electrostatic interactions between its positive charges and the negatively charged residues present in the S2 and S3 segments (21). Defining the residue-residue contacts between S4 and the segments with which it is in contact and how they change during channel activation is critical for an appreciation of the molecular mechanism by which S4 is able to sense changes in membrane potential and transmit the signal to the pore domain.

Toward this end, we set out to use the engineered disulfide bridge approach. This approach allows determination of the residue-residue contacts within the three-dimensional context of a protein (22) and also allows structural changes underlying the activation of a protein to be elucidated (23). The approach involves the introduction of pairs of cysteine residues at positions that are thought to lie in close proximity and then investigating which cysteine pairs can be induced to form a disulfide bridge. The formation of disulfide bridges will often impair or alter the course of further motions, thereby producing a change in the functional properties of the protein (23). In the absence of measurable functional changes, however, disulfides can be detected biochemically (22). Formation of a disulfide bridge is interpreted in terms of the residues being in close proximity. Any changes in the pattern of disulfide formation between the resting and activated states of the protein will reflect structural motions underlying the activation. The power of this
approach has been illustrated with a number of membrane proteins, including potassium channels (23, 24).

In the present study, we set out to identify which residues (from other segments) are in close proximity to Lys-361 of the Shaker potassium channel. We focused our attention on this residue because it occupies a critical position in the channel. It is located within the bilayer yet close to the extracellular boundary (11, 12, 14, 15). This means that when S4 moves out this residue is expected to sever all interactions with the neighboring membrane embedded segments. More importantly, its substitution with cysteine does not alter the net charge of S4, and hence would not be expected to disrupt electrostatic interactions that may be critical for the helical packing and normal functioning of the channel. Our results show that the mutant channels are susceptible to oxidation and that this oxidation occurs during depolarization of the membrane. Western blotting showed a dimeric Shaker protein band, indicating that oxidation leads to a disulfide bond between neighboring subunits. Removal of all the native cysteines failed to prevent the oxidation, which led us to suggest that the cysteine at position 361 forms an intersubunit disulfide with its counterpart from a neighboring subunit. Finally, we show that oxidation occurs at potentials where C-type inactivation is absent, indicating that the disulfide formation occurs during the activation of the mutant channel. Taken together, data presented here suggests that the N-terminal ends of the S4 segments move toward each other during the activation of the channel.

EXPERIMENTAL PROCEDURES

Molecular Biology—Amino acid substitutions were introduced into the inactivation ball (residues 6–46)–removed Shaker potassium channel (25), or into its cysteine-less version (C-less Shaker), by site directed mutagenesis. cRNA transcripts were made from HindIII-linearized plasmid (pKS-Bluescript) constructs containing the wild-type and mutant cDNA sequences using the MEGAGen kit (Ambion). All methods are as previously described (12).

Electrophysiology—Oocytes were isolated from Xenopus laevis and anesthetized by immersion in 0.2% 3-aminobenzoic acid ethyl ester (Sigma). The animals were then killed by cervical dislocation. Dumont stage V or VI oocytes were selected, defolliculated, and injected with 5–20 ng of cRNA. The oocytes were incubated at 19 °C in modified Barth’s solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, and 0.25 before and 1.08 m M-KCl (reduced) and incubated at 37 °C for 30 min. Proteins were separated by SDS-polyacrylamide gel (7%) electrophoresis and transferred on to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). Following transfer the membrane was soaked in 100% methanol for 15 s and allowed to dry for 30 min at 57 °C. The membrane was then incubated 1 h in blocking buffer containing 1% nonfat milk in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, followed by incubation in blocking buffer containing a 1:2500 dilution of the secondary antibody (horseradish peroxidase–conjugate of goat-anti rabbit antiserum, Bio-Rad). Protein was detected using the ECL plus chemiluminescence kit (Amersham Biosciences).

RESULTS

Effect of Copper (II) Phenanthroline on the Wild-type Shaker Potassium Channel—Oxidation of closely placed cysteine thiols to a disulfide bridge can be enhanced by using Cu (II) Phe as a catalyst (22). Because the Shaker potassium channel contains 28 cysteines (7 cysteines per subunit) that could potentially form disulfides, we have first examined the effect of Cu (II) Phe on the properties of the channel. For this, the channel was expressed in Xenopus oocytes, and the effect of perfusion of Cu (II) Phe on the properties of the channel was examined by two-electrode voltage clamp. Fig. 1 shows that the reagent has no effect on the current-voltage (I-V) relationship (Fig. 1C) or the activation (Fig. 1, B and D) and deactivation (Fig. 1E) kinetics of the channel. This suggests that under these experimental conditions, none of the native cysteines are close enough to undergo disulfide oxidation. An alternative explanation would be that any disulfides formed have no effect on the functional properties of the channel.

The L361C Mutant Shaker Channel Is Inhibited by Cu (II) Phe—Fig. 2 shows that the application of Cu (II) Phe to oocytes expressing the L361C mutant channel caused rapid inhibition (time constant, τ = 1.46 ± 0.16 min; n = 4) of currents. The inhibition could not be reversed by Cu (II) Phe removal alone (Ringer’s wash), but could be fully reversed with DTT (1 mM) (Fig. 2A), a reagent capable of reducing disulfide bridges to cysteines. The inhibition was incomplete, with about 30% of the currents remaining, when the inhibition reached a steady state. The residual currents displayed slowed activation kinetics (τact = 40 mV, before = 1.75 ± 0.1 ms; at 18.6–19.1 ms) (Fig. 2, D and E) and a negative shift (13 mV) in the current-voltage relationship (Fig. 2C). There was also a significant decrease in the effective gating valence (z values were 1.96 ± 0.25 before and 1.08 ± 0.07 after oxidation). In addition, the deactivation kinetics were dramatically reduced (Fig. 2F), with nearly 50% of the current remaining even after a 500 ms deactivating pulse at −110 mV. There was also some reduction in the rate of C-type inactivation (Fig. 2E) inactivating the upper 50% of the raising phase of the current data to an exponential function. Deactivation tail current traces (measured in 100K (100 mM KCl) Ringer’s solution) to a bi-exponential decay equation.

1 The abbreviations used are: DTT, dithiothreitol; C-less, cysteine-less; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; pCMBS, para-chloromercuribenzenesulphonate; I, current; V, voltage; Cu (II) Phe, copper (II) phenanthroline.
suggesting that the effect is not due to the binding of free Cu$^{2+}$

1:40 Cu (II) Phe) of the mutant channel current (Fig. 2).

Inhibition could be due to the binding of free copper (II) to

present in the reagent. To eliminate the possibility that the

imbalance is not due to channels that escaped oxidation but due
to channels modified by Cu (II) Phe.

The reagent used in the above experiment has 100 $\mu$M Cu$^{2+}$
and 300 $\mu$M phenanthroline (commonly used concentrations)
(22). At this metal ion to chelating agent ratio (1:3), a signifi-
cant amount of free, uncomplexed Cu$^{2+}$ would be expected to
be present in the reagent. To eliminate the possibility that the
inhibition could be due to the binding of free copper (II) to

cysteines, we have reduced the concentration of Cu (II) to 5 $\mu$M
and increased the concentration of phenanthroline to 200 $\mu$M.

The resulting reagent (ratio of Cu (II) to Phe 1:40), which would

contain a negligible amount of free copper, showed no signifi-
cant effect on the rate (1.46 ± 0.3 min with 1:3 Cu (II) Phe and
1.28 ± 0.18 min with 1:40 Cu (II) Phe) or the extent of inhibi-
tion (66.7 ± 4.0% with 1:3 Cu (II) Phe and 63.1 ± 4.4% with
1:40 Cu (II) Phe) of the mutant channel current (Fig. 2G), sug-

gesting that the effect is not due to the binding of free Cu$^{2+}$
to the cysteine at position 361. Free Cu$^{2+}$ also inhibits, but the
effect, unlike that produced by Cu (II) Phe, is fully reversed by

Ringer’s wash alone (data not shown). Taken together, these
data suggest that the observed inhibition is likely to be due to
the oxidation of cysteine thiols to disulfides.

L361C Mutant Shaker Channels Are Oxidized by the Ambient Oxygen—Data in Fig. 2 showed that Cu (II) Phe produces very rapid oxidation of the mutant channel. We wondered if the ambient oxygen itself is adequate to induce oxidation. To test this, we have incubated the injected oocytes in ND-96 medium without DTT (we routinely included 50 $\mu$M DTT in our medi-
um). These oocytes expressed low currents and displayed slow activation kinetics with a time constant (20.8 ± 1.2 ms) (Fig. 3, C, and D) that is similar to the Cu (II) Phe oxidized mutant channel (18.6 ± 1.9 ms) (Fig. 2, D and E). Application of DTT caused a rapid increase in steady-state currents (Fig. 3A), which was accompanied by an increase in the rate of activation ($\tau_g = 5.9 ± 2.8$ ms) (Fig. 3, C and D). We found that when the oocytes were incubated in ND-96 lacking DTT currents at the end of DTT application were routinely higher than the currents seen at the beginning of the recording, although there were
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Disulfide Bridges Are Formed between the Cysteine at Position 361 of Neighboring Subunits—To identify the cysteine with which the cysteine at position 361 forms the intersubunit disulfide bridge, we have introduced a single cysteine at position 361 of a Shaker mutant channel from which all native cysteines have been removed (referred to as C-less Shaker) (26). The idea was to subsequently introduce cysteines at the native positions and examine the effect of Cu (II) Phe. However, when we tested for the effect, Cu (II) Phe caused inhibition of currents through this mutant channel (Fig. 5, A and B). Moreover, oocytes (expressing the Cys-361 C-less Shaker channels) incubated in DTT minus medium elicited currents that increased with the application of DTT (Fig. 5D), indicating that ambient oxygen can also inhibit the mutant channel currents.

The ability of DTT to reverse the effects of ambient oxygen and Cu (II) Phe suggests that, as in the wild-type channel, cysteines (at 361) in the C-less background also undergo oxidation to disulfides. The low levels of expression of C-less Shaker channels in oocytes prevented us from confirming this by Western blotting; thus, we were unable to completely rule out the possibility that oxidation might lead to products other than disulfides. As an alternative, we have tested the ability of Tris/2-carboxyethylphosphine hydrochloride (TCEP) to reverse the effect of Cu (II) Phe. The mechanism of action of TCEP is different from that of DTT, and TCEP, unlike DTT, is highly specific for reducing disulfide bridges (27). Fig. 5 (E and F) shows that TCEP fully reversed the effect of Cu (II) Phe, confirming the fact that disulfide formation is the cause of inhibition by Cu (II) Phe. Because this mutant channel contains no cysteines other than those at position 361, we conclude that Cys-361 of one subunit forms a disulfide bridge with its counterpart in the neighboring subunit.

Intersubunit Disulfide Formation Is Voltage-dependent—We next investigated the voltage-dependence of current inhibition by Cu (II) Phe for the L361C mutant channel (Fig. 6). In these experiments, oocytes were held at various potentials (−120 mV to −20 mV) for 200 s while superfusing the oocyte with the

![Fig. 3. Oxidation of L361C mutant channel by the ambient oxygen.](http://www.jbc.org/)}
were treated with Cu (II) Phe and 1 mM TCEP. Traces corresponding to the time points labeled 1–5. 

**E** superfusion (5 min) of cysteine at position 361 into the extracellular medium. The slope factor (RT/zF) was 3.08, absolute temperature, and z the effective charge that has moved across the potential for half-maximal inhibition, R the gas constant, T the temperature, and z the effective charge that has moved across the membrane. The slope factor (RT/zF) was 3.08 ± 0.16 mV and V0.5 was −77 ± 0.17 mV. Also shown are the data obtained for voltage-dependence of exposure (3) of cysteine at position 361 into the extracellular phase, measured using pCMBS as a probe. 100 μM pCMBS was applied for 200 s at the indicated voltage using voltage protocols identical to those used for cross-linking and the data fitted as above. The measured slope factor and V0.5 values for the exposure of cysteine at −80 mV. Percent loss in current was estimated by comparing with the current from control recordings. The data were fitted to Boltzmann relationship (n = 4) for L361C is also shown (A). Also shown is the voltage-dependence of C-type inactivation for this mutant (B). Channels were held at the indicated potentials for 200 s, and the current remaining was measured by pulsing to +40 mV from −80 mV. Percent loss in current was estimated by comparing with the current from control recordings. The data were fitted to Boltzmann function, from which V0.5 and k values for inactivation were calculated, respectively, as 13.65 ± 1.44 mV and 9.8 ± 1.26 mV.

**C-type inactivation**—During depolarization the Δ(6–46) Shaker channel undergoes fast activation followed by slow C-type inactivation (2, 28–33). There is evidence that S4 undergoes distinct conformational changes during both these steps (29, 30, 34). Thus the observed disulfide bridge formation can occur during either of these steps. To distinguish between these two possibilities, we have followed C-type inactivation of L361C as a function of membrane voltage. As can be seen from reagent. This was followed by repeated pulsing to +40 mV (from a holding potential of −80 mV) to record the currents. Fig. 6 (A and B) shows that inhibition of currents through L361C was highly voltage-dependent and occurred with a V0.5 (voltage at which 50% of the channels were inhibited) value of −77 mV and a slope factor of 3.08 ± 0.16 mV. The data suggest that it is during depolarization that S4 segments move close enough toward each other to result in a disulfide between the cysteines at position 361. It may be noted that the voltage-dependence is not due to the effect of the electric field on the intersubunit disulfide formation between the L361C subunits. Currents were recorded from Xenopus oocytes expressing L361C by repeated pulsing (200 ms duration) to +40 mV from a holding potential of −80 mV. Oocytes were then held at the indicated holding potentials for 200 s while 100 μM Cu (II) Phen was superfused. Following this, current recordings were resumed (the voltage protocol is shown at the top of each plot) until a steady-state inhibition was obtained. DTT (1 mM) was then perfused to reverse the inhibition. Representative recordings are shown for each voltage. B, intersubunit disulfide formation between the L361C subunits is voltage-dependent and accompanies the outward movement of S4. Percentage of inhibition was calculated from the extent of inhibition (data from A) at each holding potential and the maximal inhibition (recorded after resumption of depolarising test pulses) and plotted as a function of voltage at which the reagent was applied. Each point (•) represents mean ± S.E. (n = 3–4). Because the inhibition increases during channel activation (i.e. depends on the conformational state of the channel), the data were fitted to the Boltzmann function, percent inhibition = 100 [1 + exp{−2F (Vm − V0.5)/zF}]−1, where V0.5 is the holding potential, Vm, the potential for half-maximal inhibition, R the gas constant, T the absolute temperature, and z the effective charge that has moved across the membrane. The slope factor (RT/zF) was 3.08 ± 0.16 mV and V0.5 was −77 ± 0.17 mV. Also shown are the data obtained for voltage-dependence of exposure (3) of cysteine at position 361 into the extracellular phase, measured using pCMBS as a probe. 100 μM pCMBS was applied for 200 s at the indicated voltage using voltage protocols identical to those used for cross-linking and the data fitted as above. The measured slope factor and V0.5 values for the exposure of cysteine at −80 mV. Percent loss in current was estimated by comparing with the current from control recordings. The data were fitted to Boltzmann function, from which V0.5 and k values for inactivation were calculated, respectively, as 13.65 ± 1.44 mV and 9.8 ± 1.26 mV.

**Disulfide Formation Occurs during Activation Rather than C-type Inactivation**—During depolarization the Δ(6–46) Shaker channel undergoes fast activation followed by slow C-type inactivation (2, 28–33). There is evidence that S4 undergoes distinct conformational changes during both these steps (29, 30, 34). Thus the observed disulfide bridge formation can occur during either of these steps. To distinguish between these two possibilities, we have followed C-type inactivation of L361C as a function of membrane voltage. As can be seen from...
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**FIG. 7. Effect of Cu (II) Phe on C-type-inactivated L361C channels.** A, time course data for C-type-inactivated L361C mutant channels. Oocytes expressing mutant channels were held at a holding potential of −80 mV and stepped to +40 mV for a duration of 500 ms every 10 s to obtain steady-state currents; this was followed by a 35 s long pulse to +40 mV to maximally inactivate L361C channels (pulse numbered 3). The cells were then held at 0 mV to prevent recovery from C-type inactivation and 100 μM Cu (II) Phe applied for 3 min followed by perfusion of Ringer's solution for 3 min (to wash away the reagent). The holding potential was then returned to −80 mV, and current measurements were resumed while perfusing the reagents indicated over the horizontal bars. B, current traces taken from time points indicated in A.

Fig. 6B, C-type inactivation occurs at much more (−90 mV) positive potentials (V_{0.5} = 13.6 ± 1.4 mV) than disulfide cross-linking (V_{0.5} = −77 ± 0.17 mV), suggesting that oxidation to disulfides must have occurred during activation.

To provide further evidence, we have allowed the channels to inactivate maximally (−80% current loss) by using a long pulse to +40 mV and then applied Cu (II) Phe for 2 min while holding the cells at 0 mV to prevent recovery from inactivation (Fig. 7). Washing with Ringer's solution reversed over 40% of the lost current. Because the Cu (II) Phe effect is not reversible by Ringer's solution, this reversal must represent recovery from inactivation. It also suggests that the inactivated channels were unaffected by Cu (II) Phe. Subsequent application of DTT produced further recovery from inhibition (−20%), which might represent channels that have not undergone inactivation during the long inactivation pulse, and hence are susceptible to the Cu (II) Phe effect. Following maximal reversal of currents, when Cu (II) Phe was re-applied it produced the normal rapid inhibition (reversible by DTT), the magnitude (−55%) of which is larger than that (20%) produced by the reagent when applied to channels during the long inactivating pulse. These data argue that cross-linking of adjacent cysteines at position 361 occurs during activation rather than during C-type inactivation.

Intersubunit Disulfide Bond Formation Occurs after S4 Begins to Move Out of the Membrane Electric Field—We also investigated whether the intersubunit disulfide formation occurs before or after S4 begins to move out of the membrane electric field. For this, we studied the movement of cysteine at position 361 out of the membrane bilayer, as a function of voltage, using pCMBS. pCMBS, a membrane-impermeable cysteine reagent, like the water-soluble methanethiosulphonate reagents (11, 13), reacts with S4 cysteines only when they move out of the membrane bilayer, thereby reporting the outward movement of an S4 residue (12). The data (Fig. 6B) show that the outward movement of the cysteine at position 361 begins at more negative potentials (by −30 mV) compared with the disulfide cross-linking, suggesting that the exposure of S4 to the extracellular phase may precede cross-linking.

**DISCUSSION**

Previous studies (11, 12, 14, 15) have shown that in response to membrane depolarization the S4 segment of the Shaker potassium channel moves out of the transmembrane field by exposing over 7 residues to the extracellular phase. It is believed that this movement is accompanied by changes in its interaction with the other segments of the channel that ultimately lead to the activation of the channel. To investigate this, we have used the engineered disulfide method (22, 23), an approach that has been successfully used to reveal changes in residue-residue interactions during the activation of channels (23) and receptors (35). Our data reveal that during activation the intersubunit distance between the N-terminal ends of S4 decreases such that cysteines engineered at position 361 form an intersubunit disulfide.

Cysteines Substituted at Position 361 of S4 Form Intersubunit Disulfides—When the Shaker channel containing a cysteine at position 361 was expressed in *Xenopus* oocytes and exposed to ambient oxygen or to the mild oxidising agent Cu (II) Phe, there was a reduction in the current flowing through the channel (Fig. 2). This was due to the formation of a disulfide bridge as the effect was reversed by DTT (Figs. 2 and 3) and TCEP (Fig. 5), and a dimeric band (Fig. 4) was detected when the oxidized L361C channel protein was subjected to Western blotting. The latter finding also indicates that the disulfide was formed between cysteines from neighboring subunits, rather than from within a subunit, of the channel. In an attempt to identify the cysteine with which Cys-361 forms the disulfide bridge, we have first examined the effect of Cu (II) Phe on the Shaker channel containing cysteines at position 361 but depleted of all native cysteines (Fig. 5). Rather unexpectedly, this mutant was also inhibited by Cu (II) Phe. The most plausible interpretation of this finding is that the cysteine at position 361 forms an intersubunit disulfide with its counterpart in a neighboring subunit.

The Intersubunit Disulfide Formation Is Voltage-dependent and Seems to Occur after the Cysteine at Position 361 Is Exposed to the Extracellular Phase—We found that the intersubunit disulfide formation between the cysteines at position 361 does not occur at −80 mV, where the channels are in their closed state. Upon depolarization, however, they undergo rapid oxidation to disulfides (Fig. 6). These data suggest that in the closed state of the channel the 361 cysteines were not close to one another, but during depolarization, when the channel begins to open (and may also begin to inactivate), they move close enough to undergo disulfide oxidation. Previous studies (11, 12, 14, 15) have shown that the cysteine at position 361 can be fully exposed to the extracellular phase at −90 mV (also see Fig. 6B). This means that the cysteines from the neighboring subunits move toward each other's proximity after the residues have been exposed to the extracellular phase, i.e. after S4s have, at least partially, moved out of the membrane electric field. Con-
sistent with this suggestion, we found that cross-linking reduces the effective gating charge (z) from 1.96 ± 0.25 to 1.08 ± 0.07.

Disulfide Formation Occurs during Activation Rather than C-type Inactivation—Depolarization of the membrane has two effects on the Shaker channel (N-type inactivation-removed), a fast activation followed by a slow C-type inactivation (2). Previous studies (2, 28–33) have shown that the structural changes associated with the activation motion of S4 are distinct from those occurring during the inactivation process. The motions observed in this study are more likely to occur during activation rather than during inactivation. The reasons are as follows: (i) C-type inactivation for L361C begins to occur at more positive potentials (~90 mV) than disulfide cross-linking (Fig. 6B); (ii) cross-linking reduces rather than stabilizing C-type inactivation in the L361C mutant channel (Fig. 2E); (iii) and finally, and more importantly, application of Cu (II) Phe to L361C channels that had already undergone maximal C-type inactivation does not cause irreversible inhibition of channel currents (Fig. 7). Thus we conclude that the movement of cysteine residues at position 361 into each other’s proximity occurs during the activation of the channel rather than during C-type inactivation.

CONCLUSIONS

Our data suggesting that during activation the intersubunit distance between positions 361 is reduced are obtained from the L361C mutant channel. It therefore raises the critical question: does this occur in the native channel? Could the effect be due to structural change imparted by the mutation? In the absence of direct structural data, this is a very difficult question to address. However, the facts that residues at position 361 are not conserved among K+ channels and that substitution of cysteine at this position does not affect the net charge of S4 and, more importantly, the functional properties of the channel (11, 12), suggest that any structural change induced by the mutation is likely to be subtle rather than substantial. Thus, we are tempted to suggest that the depolarization-induced motions may reduce the intersubunit distance between positions 361 of S4 in the native channel.

The engineered disulfide approach gives accurate information on the distance between a pair of residues. The actual distance between the ε-carbons of disulfide-linked cysteines is 5.6 ± 0.6 Å. This means that in the cross-linked state, the N-terminal ends of S4 are much closer than could be predicted from the current models of structure (see below). Although these results are quite unexpected, we could not dismiss the disulfide formation as some kind of nonspecific (functionally irrelevant) effect, because the effect was seen only during the voltage-dependent activation of the channel. More interestingly, the cross-linked channels are functional, with properties (slow closure of the channel and a hyperpolarizing shift in the conductance-voltage relationship (see Fig. 2)) that reflect stabilization of a conformational state from which the channel appears to open more readily (due to a shift in equilibrium from the closed to open state). It is possible that the cross-linked species may represent an intermediate conformational state, because the cross-linked channel opens with less gating charge movement (Z ~ 1.08 ± 0.07 compared with 1.96 ± 0.25 for the uncross-linked mutant channel (Fig. 2)).

As mentioned above, the finding that the N-terminal ends of S4s can be readily cross-linked with an engineered disulfide was quite unexpected. This is because according to the current models (10, 18–20, 36), the four S4s surround the central pore domain (which has an estimated diameter of ~50 Å at the extracellular end) with a tetrameric symmetry. As such, they are expected to be at a substantial distance from each other, making it difficult to conceive motions that would bring the N-terminal ends together without disrupting the tetrameric symmetry. However, if we view the channel as a symmetric dimer of dimers, as has been proposed previously for K+ channels (37) and the cyclic nucleotide gated channels (38, 39), and consider the recent report that in SKCa channels gating occurs through a dimerization of the intracellular regulatory domains (8, 40), it would be possible to imagine S4s from neighboring subunits moving closer to each other. Structural data and further protein chemistry experiments are required to test this possibility and propose models of S4 motion.

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