Intersubunit Interaction between Amino- and Carboxyl-Terminal Cysteine Residues in Tetrameric Shaker K^+ Channels†

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Received May 6, 1996; Revised Manuscript Received July 1, 1996⊗

ABSTRACT: Shaker potassium (K^+) channels normally lack intrasubunit and intersubunit disulfide bonds. However, disulfide bonds are formed between Shaker subunits in intact cells exposed to oxidizing conditions. Upon electrophoresis under nonreducing conditions, intersubunit disulfide bond formation was detected by the presence of four high molecular weight adducts of Shaker protein. This result suggests that intracellular cysteine residues are in sufficiently close proximity in the native structure of the Shaker channel to form intersubunit disulfide bonds. To test this hypothesis, wild-type and mutant Shaker proteins were exposed to oxidizing conditions in intact cells. Intersubunit disulfide bond formation was eliminated upon serine substitution of either C96 in the amino terminal or C505 in the carboxyl terminal of the protein. In contrast, disulfide bond formation was not eliminated upon serine substitution of both C301 and C308 in the cytoplasmic loop between transmembrane segments S2 and S3. Exposure of Shaker-expressing cells to oxidizing conditions did not significantly alter the amplitude, kinetics, or voltage dependence of the Shaker current, demonstrating that the native tertiary and quaternary structures of the channel were maintained under oxidizing conditions. These results indicate that intersubunit disulfide bonds form between C96 and C505, providing evidence that the amino- and carboxyl-terminal regions of adjacent subunits are in proximity in the native structure of the channel. The disulfide-bonded adducts were found to represent a dimer, a trimer, and two forms of tetramer, one linear and one circular, containing one, two, three, or four disulfide bonds, respectively. These results provide a direct biochemical demonstration that Shaker K^+ channels contain four pore-forming subunits.

The Shaker potassium (K^+) channel belongs to a superfamily of voltage-dependent channels which control the excitability of nerve and muscle tissue (Hille, 1992). K^+ channels are multisubunit proteins (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). Functional diversity among K^+ channels arises from the existence of multiple types of subunits which may form homomultimeric or heteromultimeric channels (Covarrubias et al., 1991; Sheng et al., 1993; Wang et al., 1993). An amino-terminal region including approximately 100 amino acids, termed the NAB or T1 domain, regulates the subunit composition of K^+ channels (Li et al., 1992; Shen et al., 1993; Babila et al., 1994; Hopkins et al., 1994; Lee et al., 1994; Shen & Pfaffinger, 1995a; Tu et al., 1995; Xu et al., 1995; Yu et al., 1996). This domain is thought to mediate a homotypic interaction between the amino termini of the pore-forming subunits during assembly.

It was proposed that K^+ channels comprise four pore-forming subunits by analogy with Na^+ and Ca^{2+} channels, which contain one large pore-forming protein composed of four domains, each of which is structurally similar to one K^+ channel subunit (Noda et al., 1984; Tanabe et al., 1987; Tempel et al., 1987). To test the subunit stoichiometry of K^+ channels, MacKinnon (1991) coexpressed Shaker subunits with differing affinities for an isoform of charybdotoxin. The resulting data were best fit by assuming a tetrameric stoichiometry. Although elegant, these experiments were indirect and relied on two assumptions: first, that mutant and wild-type subunits with differing toxin sensitivities associated randomly, and second, that the toxin sensitivity of heteromultimeric channels was the same whether they contained one or more mutant subunits.

In an alternative approach, covalently-linked dimeric, trimeric, tetrameric, and pentameric constructs were analyzed electrophysiologically to demonstrate that functional channels contain an even number of subunits (Isacoff et al., 1990), most likely four (Liman et al., 1992). However, no biochemical evidence was presented to demonstrate that the covalently-linked constructs produced proteins of the expected sizes. Furthermore, it has been reported that the subunit composition of channels formed from covalently-linked subunits may diverge from that expected from the sequence of the construct (McCormack et al., 1992). More recently, electron microscopy of detergent-solubilized and purified Shaker protein and sucrose gradient centrifugation of *in vitro* translated and solubilized protein provided additional support for the idea that K^+ channels are tetramers (Shen et al., 1993; Li et al., 1994).

We have now exploited the reactivity of endogenous cysteine residues *in situ* to demonstrate directly and without

† Supported by grants from the W. M. Keck Foundation, the Pew Charitable Trusts, the Muscular Dystrophy Association, the Stein Oppenheimer Fund (UCLA School of Medicine), and the NIH (GM43459). C.T.S. is a predoctoral fellow of the Howard Hughes Medical Institute. N.N. is a postdoctoral fellow of the American Heart Association, Greater Los Angeles Affiliate.

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⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

S0006-2960(96)01083-5 CCC: $12.00

detergent solubilization that Shaker K^+ channels contain four pore-forming subunits. We have also shown that the cytoplasmic amino- and carboxyl-terminal domains of adjacent subunits interact closely in the native quaternary structure of the channel.

EXPERIMENTAL PROCEDURES

Molecular Biology. The Shaker B mutant constructs used in these experiments [C96S, C301S+C308S, C505S, N259Q+N263Q, and Δ6−46 (IR); single-letter amino acid code] have been described previously (Hoshi et al., 1990; Santacruz-Toloza et al., 1994b; Schulteis et al., 1995). A covalently-linked tetramer construct was the kind gift of Dr. Fred J. Sigworth, Yale University. This construct encodes four copies of an inactivation-removed Shaker 29-4 subunit connected by three 19 amino acid linkers in the vector pGEM-A (McCormack et al., 1992, 1993; Lin et al., 1994).

Except for the covalently-linked tetramer, the constructs were transferred into pBluescript II (Stratagene, La Jolla, CA) for *in vitro* transcription. For injection into *Xenopus laevis* oocytes, cRNA was transcribed using T7 RNA polymerase (Promega, Madison, WI) or the mMESSAGE mMACHINE RNA transcription kit (Ambion, Austin, TX).

For transfection of mammalian cells, the Shaker B cDNA and mutant constructs were transferred into a plasmid vector, pcDNA1/AMP (Invitrogen, San Diego, CA), containing the cytomegalovirus promoter. Mock-transfections were performed with the pcDNA1/AMP vector without an insert. DNA used for transfections was purified on Qiagen columns (Qiagen, Chatsworth, CA).

Electrophysiology. *Xenopus laevis* oocytes were prepared and injected with 1−5 ng of Shaker B or mutant cRNA as described previously (Papazian et al., 1991). Whole cell currents were recorded at room temperature in modified Barth's saline (Timpe et al., 1988) or a modified phosphate-buffered saline solution (mPBS:[1] 89 mM NaCl, 2.7 mM KCl, 1.5 mM NaH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4) using a two-electrode voltage clamp (Warner Electronics, Hamden, CT). Data were acquired using an 80386 computer and pCLAMP v. 5.5.1 software (Axon Instruments, Foster City, CA). Oxidizing solutions were applied at a rate of approximately 1.5 mL/min using a gravity-driven superfusion system. In some experiments, oxidizing reagents were added directly to the bath from a stock solution.

Oxidation and Chemical Cross-Linking of Shaker Protein in Cell Culture. Human embryonic kidney cells constitutively expressing the SV-40 TAg (HEK293T, also known as tsa201; DuBridge et al., 1987) were the kind gift of Dr. R. B. DuBridge. Cells were grown and transfected as described previously (Schulteis et al., 1995).

Before oxidation of Shaker protein, transfected and mock-transfected HEK293T cells were washed in divalent-free PBS (Mediatech, Herndon, VA). Shaker protein was oxidized in intact cells by incubation in 1 mM iodine, 10 mM diamide, or various concentrations of H_2O_2 (Sigma, St. Louis, MO) for 10 min. Iodine was dissolved directly into divalent-free PBS and used within 1 h of preparation. Stock solutions of diamide (100 mM) and H_2O_2 (3%) were used to generate the final reaction conditions. The oxidation reactions were quenched by a 5 min incubation in 5 mM *N*-ethylmaleimide (NEM) (Sigma).

Alternatively, wild-type and mutant Shaker protein was cross-linked with the amino cross-linking reagent disuccinimidyl suberate (DSS) or the sulfhydryl cross-linking reagent bis(maleimidohexane) (BMH) (Pierce, Rockford, IL). DSS was added to solubilized protein from a 100 mM DSS stock dissolved in DMSO to achieve a final concentration of 500 μM DSS. The DSS reaction was quenched after 30 min by the addition of 1 M Tris base, pH 8.0, to a final concentration of 100 mM. BMH was added to intact cells from a stock solution of 10 mM BMH to achieve a final concentration of 500 μM. The BMH reaction was performed in solution buffered to pH 5.5 (155 mM NaCl, 5 mM KCl, 10 mM MES) to maintain reagent-specificity for sulfhydryl residues.

For immunoblot experiments, cells were collected by brief trituration and centrifugation. The cells were lysed in popping buffer (10 mM sodium phosphate, 1 mM EDTA, pH 7.0) as described previously (Klaiber et al., 1990; Santacruz-Toloza et al., 1994b). Shaker protein was solubilized in a 2% Lubrol-PX (w/v) buffer (75 mM KCl, 75 mM NaCl, 50 mM sodium phosphate, pH 7.2). These detergent conditions have been shown to solubilize wild-type Shaker protein in an active, assembled form (Santacruz-Toloza et al., 1994a). Both the popping and solubilization steps were performed in the presence of protease inhibitors as described previously (Schulteis et al., 1995). Following solubilization, Shaker protein was either immediately suspended in Laemmli sample buffer or concentrated via immunoprecipitation as described previously (Santacruz-Toloza et al., 1994b) using an antibody directed against the Shaker−β-galactosidase fusion protein (Schwarz et al., 1990). Protein was then subjected to electrophoresis and immunoblot analysis.

For metabolic labeling, cells were incubated for 30 min in methionine- and cysteine-free DMEM (Mediatech) and subsequently pulsed for 3 h with 200 μCi/mL ^{35}S-labeled methionine and cysteine (Trans^{35}S-Label, ICN, Irvine, CA). Cells were chased in complete, nonradioactive media for 3 h, collected, and lysed as described above. The Shaker protein was solubilized and immunoprecipitated as described above and subjected to electrophoresis and fluorography.

Oxidation of Shaker Protein in Xenopus Oocytes. Each oocyte was injected with 50−75 ng of cRNA and 500 nCi of ^{35}S-labeled methionine (*in vitro* translation grade; ICN). After a 36−48 h incubation at 18 °C, 60−80 oocytes were incubated for 10 min at room temperature in 3 mL of 1 mM iodine, 0.1% H_2O_2 (v/v; 29.3 mM), or 10 mM diamide dissolved in mPBS. The reaction was quenched by addition of 1500 μL of mPBS solution containing NEM and protease inhibitors to achieve a final concentration of 5 mM NEM and the concentrations of protease inhibitors described previously (Schulteis et al., 1995). The oocytes were incubated in the quenching solution for 10 min at room temperature and then subjected to homogenization, membrane isolation, protein solubilization, and immunoprecipitation as described previously (Santacruz-Toloza et al., 1994b), followed by electrophoresis under reducing and nonreducing conditions, and fluorography.

Electrophoresis, Immunoblot Analysis, and Fluorography. Immunoprecipitated protein resuspended in Laemmli sample buffer including either 10% 2-mercaptoethanol (reducing conditions) or 16 mM iodoacetamide (nonreducing conditions) was subjected to electrophoresis on denaturing 5%

[1] Abbreviations: BMH, bis(maleimidohexane); DMEM, Dulbecco's modification of Eagle's medium; DSS, disuccinimidyl suberate; IR, inactivation-removed; MES, 2-(*N*-morpholino)ethanesulfonic acid; mPBS, modified phosphate buffered saline; NEM, *N*-ethylmaleimide.

polyacrylamide gels or 5−20% gradient gels with 3% or 4% polyacrylamide stacking gels. For immunoblots, protein was transferred to nitrocellulose and probed with an antipeptide antibody as described previously (Santacruz-Toloza et al., 1994b). The primary antiserum (kind gift of Dr. Lily Jan) recognized the carboxyl-terminal 14 amino acids in the Shaker protein (Schulteis et al., 1995). For fluorography, gels were immersed in Fluoro-Hance (Research Products International, Mt. Prospect, IL) for 20 min prior to drying and exposure to film. Apparent molecular weights were calculated based on the migration of broad molecular weight standards (Bio-Rad, Hercules, CA) and heavy molecular weight standards (Pharmacia, Piscataway, NJ).

Immunoblots of gels were scanned and analyzed using a Model GS-700 scanning densitometer and Molecular Analyst Software v. 1.4 (Bio-Rad). The mean optical densities of the Shaker monomer and adduct bands were measured by densitometry using a constant volume.

RESULTS

Cysteine Residues in the Amino and Carboxyl Termini of Adjacent Subunits Form Disulfide Bonds under Oxidizing Conditions. Shaker protein heterologously expressed in a human embryonic kidney cell line (HEK293T) and in *Xenopus* oocytes migrates as two bands on SDS−polyacrylamide gels. These bands correspond to an immature, core-glycosylated precursor and the mature product, in which the oligosaccharides have been modified to the complex type in the Golgi apparatus (Nagaya & Papazian, 1995; Schulteis et al., 1995). In both expression systems, glycosylation occurs at two asparagine residues (N259 and N263) in the extracellular loop connecting the S1 and S2 transmembrane segments (Figure 1B) (Santacruz-Toloza et al., 1994b; Schulteis et al., 1995). However, a Shaker subunit in which both glycosylation sites have been mutated, N259Q+N263Q, forms active, cell-surface channels despite the lack of glycosylation (Santacruz-Toloza et al., 1994b).

We have previously demonstrated that native Shaker K^+ channels expressed in HEK293T cells lack both intersubunit and intrasubunit disulfide bonds (Schulteis et al., 1995). Intersubunit disulfide bonds form, however, if cells are lysed without protecting free sulfhydryl groups. The N259Q+N263Q mutant, which produces unglycosylated Shaker protein, was expressed in HEK293T cells. Cells were lysed in the absence of reducing or alkylating agents, thus exposing intracellular cysteine residues, normally in the reducing environment of the cytoplasm, to the oxidizing extracellular environment. Figure 1A shows an immunoblot of Shaker protein which has been subjected to SDS−polyacrylamide gel electrophoresis under reducing (lane 1) or nonreducing conditions (lane 2). In addition to the monomer band (arrow A, 72 kDa), four high molecular mass adducts were detected under nonreducing conditions (lane 2): a band migrating at 170 kDa (arrow B), a band of low intensity at 230 kDa (arrow C), and two bands of higher intensity at about 265 and 320 kDa (arrows D and E, respectively). These adducts were not present under reducing conditions (lane 1), demonstrating that the adducts were generated by disulfide bond formation.

Similar adducts were detected after exposing intact, Shaker-expressing HEK293T cells to oxidizing conditions (see Figure 2A). Based on these observations, we postulated that two or more intracellular cysteine residues are close enough in the native channel structure to form intersubunit disulfide bonds upon exposure to oxidizing conditions. Of the seven cysteine residues in the Shaker protein, four are predicted to be intracellular (Figure 1B) (Miller, 1991). These are located in the amino terminus (C96), in the intracellular loop linking S2 and S3 (C301 and C308), and in the carboxyl terminus (C505) (Tempel et al., 1987). To determine whether any of these residues were responsible for disulfide bond formation under oxidizing conditions, the cysteines were replaced with serine either singly (C96S, C505S) or in combination (C301S+C308S).

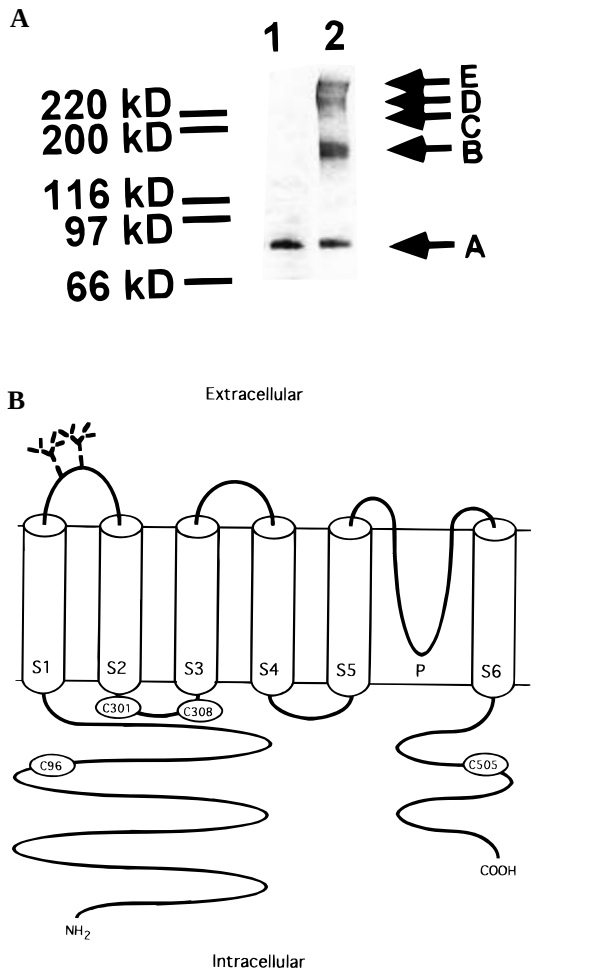

FIGURE 1: (A) High molecular mass adducts of Shaker protein are formed after cells are lysed without protecting free sulfhydryl groups. The Shaker mutant N259Q+N263Q, which produces unglycosylated protein that forms active, cell surface channels, was expressed in HEK293T cells. Cells were lysed, and protein was subjected to electrophoresis on a gradient gel under reducing (lane 1) or nonreducing (lane 2) conditions, followed by immunoblot analysis. Arrow A indicates the monomer band (72 kDa), whereas arrows B (170 kDa), C (230 kDa), D (265 kDa), and E (320 kDa) indicate high molecular mass adducts of Shaker protein seen only under nonreducing conditions. (B) Model for the membrane topology of the Shaker subunit. The approximate locations of intracellular cysteine residues are circled. Sites in the S1−S2 loop that are normally modified by N-linked glycosylation are indicated.

Intact HEK293T cells expressing wild-type or mutant Shaker protein were exposed to 1 mM iodine, a membrane-permeable oxidizing reagent. The reaction was quenched prior to cell lysis by the addition of *N*-ethylmaleimide (NEM) to a final concentration of 5 mM. The results of iodine oxidation of wild-type Shaker protein are shown in Figure 2A (lanes 2). As expected for Shaker protein retaining both glycosylation sites, two monomer bands were evident: the diffuse, mature form containing complex oligosaccharides

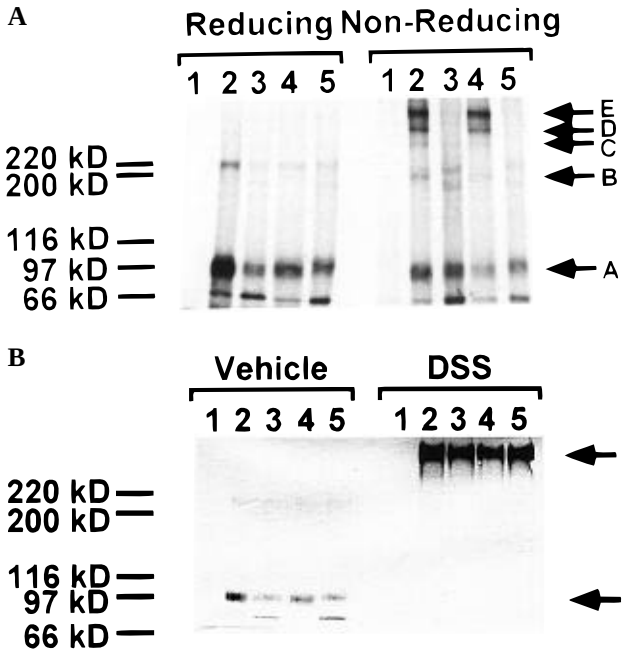

FIGURE 2: (A) Disulfide bonds between Shaker subunits are formed in intact HEK293T cells exposed to iodine. Following metabolic labeling, intact, transfected cells were exposed to 1 mM iodine, followed by 5 mM NEM. Shaker protein was immunoprecipitated and subjected to electrophoresis under reducing (10% 2-mercaptoethanol) or nonreducing (16 mM iodoacetamide) conditions. An autoradiogram is shown. Lanes 1, mock-transfected control; lanes 2, wild-type; lanes 3, C96S; lanes 4, C301S+C308S; lanes 5, C505S. Arrows indicate: A, monomeric mature protein (104 ± 6 kDa); and adducts B, 197 ± 10 kDa; C, 254 ± 18 kDa; D, 287 ± 22 kDa; and E, 349 ± 30 kDa. Apparent molecular masses are given as mean ± SD, $n = 9$ or 10. The two bands running above and below adduct B under reducing and nonreducing conditions are likely to represent noncovalent dimers of Shaker protein. (B) Cross-linking with the amino-specific reagent DSS demonstrates that the cysteine mutants are capable of multimer formation. An immunoblot of Shaker protein cross-linked with DSS or treated with 5% DMSO (vehicle) is shown. Lane assignments are the same as in panel A. The upper arrow indicates the position of the one adduct (310 ± 0 kDa, $n = 2$) seen upon DSS treatment; the lower arrow indicates the monomeric band.

(arrow A; 104 ± 6 kDa) as well as the core-glycosylated immature form (84 ± 6 kDa) (Nagaya & Papazian, 1995; Schulteis et al., 1995). Under nonreducing conditions, four high molecular mass adducts were observed: a band at 197 ± 10 kDa (arrow B), a band of low intensity at 254 ± 18 kDa (arrow C), and two higher intensity bands at 287 ± 22 kDa (arrow D) and 349 ± 30 kDa (arrow E; values given as mean ± SD, $n = 9$ or 10). No larger adducts were detected. The adducts of wild-type protein were not detected under reducing conditions. Due to the presence of glycosylation, the apparent molecular masses of the wild-type adducts were larger than those of the N259Q+N263Q adducts (see Figure 1A).

Serine substitution of either C96 in the amino terminus or C505 in the carboxyl terminus eliminated formation of the high molecular mass adducts (Figure 2A, lanes 3 and 5). In contrast, mutation of both C301 and C308 to serine did not eliminate disulfide bond formation between Shaker subunits (Figure 2A, lanes 4). These results suggest that intersubunit disulfide bonds form between C96 in the amino terminus and C505 in the carboxyl terminus of adjacent subunits.

Electrophysiological analysis in *Xenopus laevis* oocytes demonstrated that the mutants C96S, C505S (Schulteis et al., 1995), and C301S+C308S (data not shown) form channels with functional properties similar to those of the wild-type channel. Because functional activity is a key indicator of structural integrity, these results demonstrate that the C96S and C505S proteins fold and assemble properly. Therefore, the failure of C96S and C505S to form intersubunit disulfide bonds under oxidizing conditions cannot be attributed to an assembly defect in the mutants.

Furthermore, assembly of the cysteine mutants was detected by chemical cross-linking. Shaker protein, solubilized under conditions that maintain the native, active structure (Santacruz-Toloza et al., 1994a), was treated with the amino cross-linking reagent disuccinimidyl suberate (DSS). This reaction resulted in the formation of a single high molecular mass adduct in both wild-type and mutant channels (Figure 2B), indicating that the cysteine mutants were able to assemble. Cross-linking in intact cells with the sulfhydryl-specific cross-linking reagent bis(maleimidohexane) (BMH) produced high molecular mass adducts in wild-type and C301S+C308S protein, but not in C96S or C505S protein (data not shown). These results demonstrate that C96 and C505 are required for intersubunit cross-linking by BMH.

In Figure 2A, two bands (179 ± 7 and 209 ± 7 kDa, $n = 8$) migrating just above and below adduct B were detected in both wild-type and mutant constructs under reducing and nonreducing conditions. These bands cannot be due to disulfide bond formation, because they persisted under reducing conditions (Figure 2A), and they were present in cells treated with NEM but not exposed to oxidizing conditions (data not shown). In addition, these bands persisted despite treatment with higher concentrations of 2-mercaptoethanol or other reducing reagents such as dithiothreitol (data not shown). These bands may correspond to noncovalent dimers of the mature and immature forms of the protein. Noncovalent oligomers of membrane proteins are often detected on SDS−polyacrylamide gels (Soulié et al., 1996).

Intersubunit Disulfide Bonds between C96 and C505 Also Form in Xenopus Oocytes Exposed to Oxidizing Conditions. Shaker-IR channels, which contain a deletion of amino acids 6−46 to remove N-type inactivation, were expressed and metabolically-labeled in *Xenopus* oocytes. Protein was oxidized in intact oocytes with 0.1% (29.3 mM) H_2O_2 or 10 mM diamide for 10 min (Figure 3A). As in the mammalian expression system, high molecular mass adducts were detected only under nonreducing conditions. Treatment with 10 mM diamide resulted in the formation of all four high mass adducts present under iodine oxidation conditions (Figure 3A, lanes 2). However, H_2O_2, which at 0.1% is a milder oxidizing agent, resulted in the formation of only three of the four adducts (Figure 3A, lanes 1). It is worth noting that the deletion of the amino-terminal residues 6−46 in the IR channel did not prevent adduct formation, demonstrating that N-type inactivation need not be present for formation of intersubunit disulfide bonds between C96 and C505.

The Shaker mutant C96S failed to form high molecular mass adducts in oocytes (data not shown), verifying that the site of disulfide bond formation is identical in *Xenopus* oocytes and HEK293T cells.

Formation of Disulfide Bonds between C96 and C505 upon Exposure to Oxidizing Conditions Occurs in the Native Protein Conformation. To determine whether the oxidizing

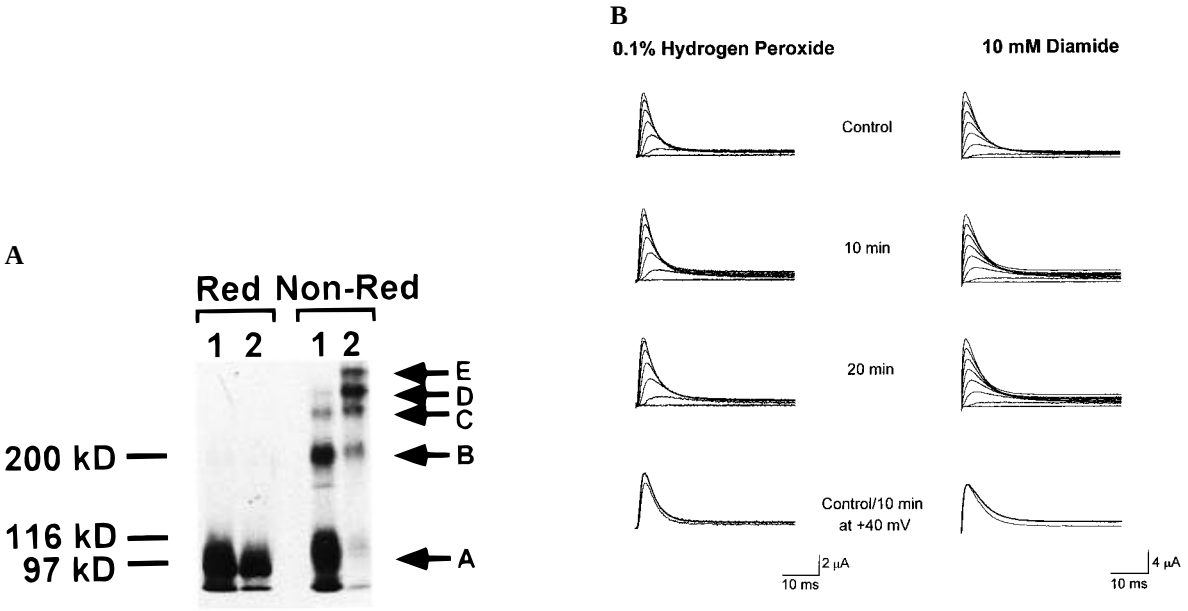

FIGURE 3: (A) Disulfide-bonded adducts of Shaker-IR protein are formed in *Xenopus* oocytes exposed to oxidizing conditions. In intact oocytes, metabolically-labeled Shaker protein was subjected to oxidation with 0.1% H_2O_2 (lanes 1) or 10 mM diamide (lanes 2), immunoprecipitation, and electrophoresis under reducing or nonreducing conditions. Arrow A indicates the position of the Shaker-IR monomer (104 kDa). Adducts observed under nonreducing conditions are indicated with arrows B (200 kDa), C (265 kDa), D (300 kDa), and E (350 kDa). (B) Wild-type Shaker channels are functionally active under oxidizing conditions in *Xenopus* oocytes. Whole cell currents were elicited at room temperature with a two-electrode voltage clamp. The membrane potential was stepped from a holding potential of −80 mV to potentials between −60 and +80 mV in 20 mV increments for 100 ms. Control records were obtained before addition of the indicated oxidizing reagents. Subsequently, perfusion of the oxidation reagent was initiated, and currents were recorded 10 and 20 min later. Representative sets of traces are shown (H_2O_2, $n = 4$; diamide, $n = 5$). At the bottom, records obtained at +40 mV before (control, thin lines) and after 10 min of perfusion with oxidant (10 min, thick lines) have been superimposed without scaling for comparison of current amplitudes and kinetics. Peak current amplitudes at +40 mV were: H_2O_2 control, 4.3 μA; 10 min, 5.2 μA; 20 min, 5.1 μA; diamide control, 7.5 μA; 10 min, 7.7 μA; 20 min, 7.6 μA.

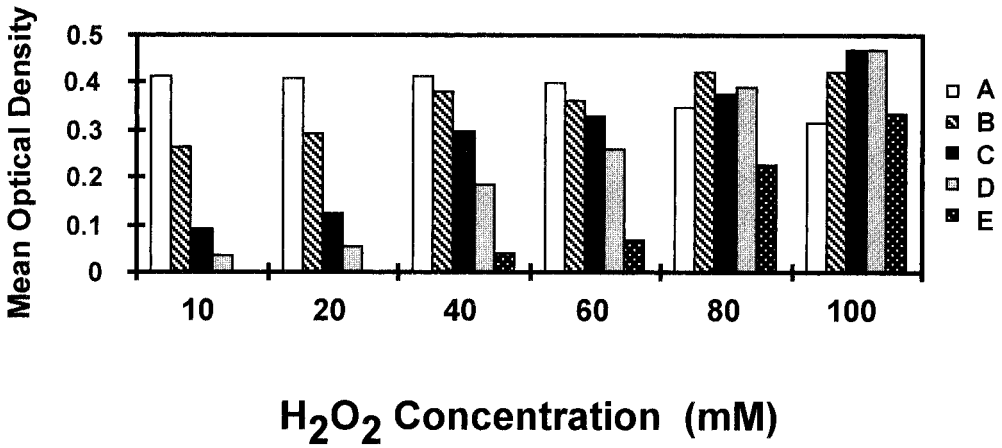

FIGURE 4: Increasing concentrations of H_2O_2 lead to the progressive formation of disulfide-bonded adducts of Shaker protein in intact HEK293T cells. Intact, transfected cells were treated for 10 min with 10, 20, 40, 60, 80, or 100 mM H_2O_2. Shaker protein was subjected to electrophoresis under nonreducing conditions and immunoblot analysis. The mean optical densities of the monomer band, A, and the high molecular mass adducts, B−E (see Figures 1A, 2A, and 3A), were measured by densitometry using a constant volume. Results of a representative experiment are shown ($n = 3$).

conditions used in these experiments disrupt the native tertiary or quaternary structure of the channel, wild-type Shaker channels were expressed in oocytes and assayed electrophysiologically under oxidizing conditions identical to those which generated disulfide-bonded adducts of metabolically-labeled protein (Figure 3). Functional activity was maintained after exposing the oocytes to oxidizing reagents for 20 min, whereas adducts were detected biochemically after only 10 min. No changes in the voltage-dependence of activation or the kinetics of inactivation were observed upon treatment with the oxidizing reagents (Figure 3B; data not shown). Furthermore, there was no significant change in the peak current amplitude. These data demonstrate that disulfide bonds between C96 and C505 generated under oxidizing conditions form in the native conformation of the Shaker channel.

Adducts of Shaker Protein Are Formed Sequentially. H_2O_2 is a less efficient oxidizing agent than iodine or diamide. To determine the order of formation of the disulfide-bonded adducts of Shaker protein, intact, transfected HEK293T cells were treated for 10 min with 10, 20, 40, 60, 80, or 100 mM H_2O_2. Figure 4 shows a histogram of the results, with the adducts designated B−E as in Figures 1A, 2A, and 3A. A significant amount of adduct B was detected after treatment with 10 mM H_2O_2. With increasing concentrations of H_2O_2, the amounts of the larger adducts increased sequentially. The largest adduct E was detected prominently after treatment with 80 or 100 mM H_2O_2. As with iodine and diamide

oxidation, adduct E accumulated after extensive reaction. No larger adducts were detected.

These data demonstrate that adducts form in the order from lightest to heaviest. Because no adducts larger than E were detected even after extensive reaction, we conclude that adduct E is the final product of intersubunit disulfide formation. Assuming that multimeric K^+ channels are radially symmetric, the final product is expected to be circular, regardless of the number of subunits. Because they have markedly different hydrodynamic properties, it is unlikely that the final circular adduct and the penultimate linear adduct will comigrate on SDS gels.

Our results suggest that adduct E represents a circular tetramer, generated by the formation of four intersubunit disulfide bonds. Adduct B forms first, at low H_2O_2 concentration, suggesting that it represents a dimer containing one disulfide bond, formed between C96 in one subunit and C505 in an adjacent subunit. The formation of two or three such disulfide bonds would produce trimers or tetramers, respectively. Depending on the number of subunits in the channel, formation of four disulfide bonds between adjacent subunits would produce either a linear pentamer or a circular tetramer. In our experiments, a fifth adduct, which might represent a circular pentamer, was never detected. Therefore, we conclude that adduct E is likely to represent a circular tetramer.

The Largest Adduct of Shaker Protein Comigrates with a Covalently-Linked Shaker Tetramer Subjected to Oxidizing Conditions. To test the conclusion that adduct E represents a circular tetramer, we used a covalent tetramer construct as a molecular mass marker. The covalent tetramer consists of 4 identical, inactivation-removed Shaker 29-4 subunits joined by linkers 19 amino acids in length (McCormack et al., 1992, 1993; Lin et al., 1994). Each of the four domains in this construct contains cysteine residues equivalent to C96 and C505. The predicted molecular masses of the covalent tetramer and the sum of four Shaker B-IR subunits are equivalent, approximately 280 kDa.

The covalent tetramer construct and Shaker-IR were expressed and metabolically-labeled in *Xenopus* oocytes. Intact oocytes were oxidized with 1 mM iodine, and the reaction was quenched with NEM. Shaker protein was immunoprecipitated and subjected to electrophoresis under reducing or nonreducing conditions. Under reducing conditions, a prominent band representing the mature form of the covalent tetramer (305 ± 7 kDa, $n = 2$) was detected (Figure 5). Some immature protein was also observed (258 ± 4 kDa, $n = 2$). The mature form of the Shaker tetramer comigrated with the third adduct (D) formed by oxidation of Shaker-IR monomers (lane 2, nonreducing conditions), indicating that adduct D corresponds to a linear tetramer (4x). After electrophoresis of the covalent tetramer under nonreducing conditions, a new band of apparent molecular mass 350 ± 2 kDa ($n = 2$) was observed. This band was not detected under reducing conditions. It is therefore likely to represent a circular form of the covalent tetramer, generated by disulfide bond formation between the first and last domains of the construct. This band comigrated with the largest adduct (E) formed by oxidation of Shaker-IR monomers, supporting the conclusion that adduct E is a circular tetramer (4xc). Combined with the result that adducts larger than E were not detected after extensive oxidation, these data provide strong and direct biochemical evidence that the Shaker K^+ channel is indeed formed from four pore-forming subunits.

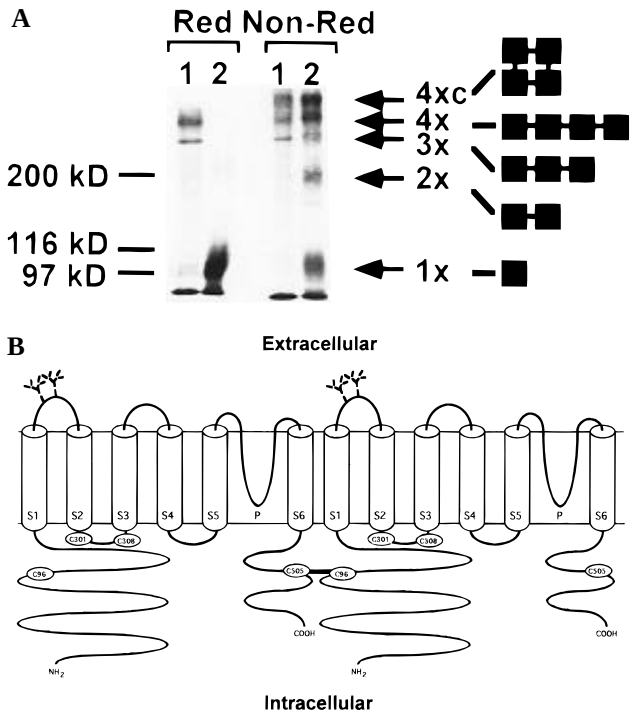

FIGURE 5: (A) High molecular mass adducts produced by iodine oxidation of Shaker-IR comigrate with protein produced by a covalent tetramer construct. A covalent tetramer (lanes 1) and Shaker-IR (lanes 2) were metabolically-labeled in *Xenopus* oocytes, oxidized with iodine, immunoprecipitated, and subjected to electrophoresis under reducing and nonreducing conditions. An autoradiogram is shown. Under reducing conditions, the mature form of the covalent tetramer comigrates with the third of four adducts (D) formed by oxidation of Shaker IR, identifying adduct D as a linear tetramer (arrow 4x). Under nonreducing conditions, an oxidized form of the covalent tetramer comigrates with adduct E, the largest of the oxidation products of Shaker IR, identifying adduct E as a circular tetramer (4xc). The mature form of Shaker-IR and adducts B and C are labeled as monomer (1x), dimer (2x), and trimer (3x), respectively. At the right, box diagrams illustrate the predicted structure of each band. Boxes represent individual Shaker subunits, connected by lines representing the appropriate disulfide bonds. (B) Cartoon illustrates the structure of an oxidized Shaker dimer, with an intersubunit disulfide bond indicated between C96 in the amino terminal and C505 in the carboxyl terminal of adjacent subunits.

DISCUSSION

Shaker Channels Are Tetramers. Exploiting the reactivity of two endogenous cysteine residues, C96 and C505, we have provided direct biochemical evidence that Shaker K^+ channels contain four pore-forming subunits. By exposing intact cells to oxidizing conditions, disulfide bonds were generated between adjacent subunits, resulting in the formation of dimers, trimers, linear tetramers, and circular tetramers of Shaker protein. Serine substitution of an amino-terminal (C96) or a carboxyl-terminal (C505) cysteine residue eliminated the oxidation reaction. Formation of disulfide-bonded adducts occurred *in situ* under conditions that preserved the functional activity of the channel, and did not require detergent solubilization. Thus, the reaction between C96 and C505 can be catalyzed in the native structure of the Shaker channel.

Disulfide Bonds Form between C96 and C505 in Adjacent Subunits. Our results demonstrate that oxidizing conditions

lead to the formation of intersubunit disulfide bonds between residues C96 and C505 (Figure 5B). C96 does not react with C96 in another subunit, nor does C505 react with C505 in another subunit. Not only is this possibility unlikely in a channel with 4-fold symmetry, it is incompatible with our data. If C96 and C505 reacted only with the homologous residue in another subunit, four reactive cysteines would remain in the mutants C96S or C505S, and disulfide-bonded dimers would be expected, in contrast to our results. The fact that disulfide bond formation was eliminated upon serine substitution of either C96 or C505 precludes the possibility that either residue reacts with the homologous residue in an adjacent subunit.

In the Native Channel Structure, the Amino and Carboxyl Termini of Adjacent Subunits Are in Close Proximity. The unique reactivity of C96 and C505 under conditions that maintain the functional activity of the channel provides evidence that the amino and carboxyl termini of adjacent subunits are in proximity within the native structure of the Shaker K^+ channel. For sulfhydryl groups to react, the β carbons of the cysteine residues must be located within 3.4−4.6 Å of each other (Careaga & Falke, 1992). In flexible proteins, however, disulfide bonds may form between residues that are normally separated by as much as 15 Å, trapping the protein in a relatively rare conformation (Falke & Koshland, 1987; Careaga & Falke, 1992). Our results indicate that C96 and C505 are close neighbors in the native structure or that the amino- and carboxyl-terminal regions of adjacent subunits are flexible enough to bring these residues into close proximity. Even in the latter case, the amino- and carboxyl-terminal regions of adjacent subunits must interact closely to account for our data. Furthermore, if disulfide bond formation trapped the channel in a rare conformation, some functional effects of oxidation on the voltage dependence, kinetics, or amplitude of the current might be expected, in contrast to our results. Previously, indirect evidence has been provided that the amino- and carboxyl-terminal regions of Kv2.1 interact (VanDongen et al., 1990).

An amino-terminal region, which has been called the NAB or T1 domain, has been implicated in subunit recognition and association in Shaker and related K^+ channels (Li et al., 1992; Shen et al., 1993; Babila et al., 1994; Lee et al., 1994; Shen & Pfaffinger, 1995a; Tu et al., 1995; Xu et al., 1995; Yu et al., 1996). This domain, which includes approximately 100 amino acids of the amino terminus, extends from about R98 to K198 in the Shaker subunit, and is therefore close in the primary structure to C96 (Shen & Pfaffinger, 1995a; Xu et al., 1995). In fact, residues corresponding to S104 and F108 in the Shaker protein appear to be critical for subunit assembly (Shen & Pfaffinger, 1995b). A polypeptide derived from this amino-terminal domain self-associates *in vitro* (Li et al., 1992; Shen et al., 1993) and has a strong dominant negative effect on the expression of normal subunits *in vivo* (Li et al., 1992; Babila et al., 1994). This region of the protein is involved in limiting heteromultimer formation among pore-forming subunits to the members of a subfamily of closely-related proteins (Li et al., 1992; Shen & Pfaffinger, 1995a; Xu et al., 1995; Yu et al., 1996). These results suggest that this domain mediates a homotypic interaction between the amino termini of the four pore-forming subunits during channel assembly.

Thus, the amino-terminal region of the Shaker protein from C96 to K198 appears to be capable of more than one type of structural interaction. This domain is thought to self-associate during channel assembly, and, according to our results, it interacts closely with the carboxyl termini of adjacent subunits in the native quaternary structure of the channel. These interactions may not be mutually exclusive if they occur on different faces of the amino terminal domain. Alternatively, the homotypic self-association of amino termini may be transient, occurring early in channel biogenesis, whereas the proximity between the amino and carboxyl termini of adjacent subunits may be established later in the assembly process, as it persists in the native structure. Further experiments will be required to determine whether homotypic interactions between the amino-terminal domains (residues 98−198) of adjacent subunits occur in the native quaternary structure of K^+ channels.

In some cell types, K^+ channels may contain cytoplasmic β subunits in addition to four pore-forming (α) subunits (Parcej & Dolly, 1989; Trimmer, 1991; Rhodes et al., 1995). The stoichiometry of α:β interactions has been estimated to be 1:1 in K^+ channel protein purified from bovine brain (Parcej & Dolly, 1989; Parcej et al., 1992). Such β subunits may regulate the stability, cell surface expression, or functional properties of the channel (Rettig et al., 1994; Chouinard et al., 1995; Nakahira et al., 1996). A region of the amino-terminal domain, corresponding to amino acids 178−187 in the Shaker protein, is critical for the association of α and β subunits (Sewing et al., 1996; Yu et al., 1996). Therefore, the interaction of α and β subunits represents a third potential association of the domain including residues 96−198 in Shaker channels. Endogenous β subunits have not been detected in *Xenopus* oocytes or in HEK293T cells (Uebele et al., 1996). It is not known whether the reaction between C96 and C505 can be catalyzed in the presence of β subunits.

Disulfide-Bonded Adducts Correspond to Dimers, Trimers, Linear Tetramers, and Circular Tetramers. Identification of the specific site of subunit cross-linking provides a distinct advantage in identifying the disulfide-bonded adducts produced by oxidation. A single disulfide bond would generate a dimer, two bonds a trimer, and so forth. The apparent molecular weights of the adducts should correspond to integral multiples of that of the monomer. Our results are reasonably consistent with this expectation.

After more extensive cross-linking with the amino-specific reagent DSS, an adduct band that migrated between the linear and circular tetramers generated by iodine oxidation was detected. Given the multiple reaction sites for DSS in Shaker protein, this adduct is likely to contain multiple cross-links, which probably account for its migration as a single band.

Because membrane and soluble proteins have different hydrodynamic properties, soluble molecular weight markers may not provide an accurate estimate of the molecular mass of a hydrophobic protein. A more appropriate molecular weight marker is the covalent tetramer protein. Not only does this protein have the same predicted molecular weight as that of a Shaker-IR tetramer, but more importantly, it should possess nearly identical hydrodynamic properties. The reduced tetramer protein comigrates with the adduct designated as the linear tetramer, while the oxidized tetramer comigrates with the designated circular tetramer, strongly supporting these adduct assignments.

Our results demonstrate that the Shaker K^+ channel is formed from four pore-forming subunits. This conclusion is consistent with results obtained from indirect electrophysi-

ological experiments (MacKinnon, 1991; Liman et al., 1992). In addition, we have demonstrated that the amino and carboxyl termini of adjacent subunits are in proximity. Similar techniques may be applied to pairs of engineered cysteine residues as a means of mapping tertiary and quaternary protein structure.

ACKNOWLEDGMENT

We thank Dr. Fred J. Sigworth for the covalent tetramer cDNA, Drs. Pamela Conley and Robert B. DuBridge for the HEK293T cells, and Dr. Lily Y. Jan for the Shaker antibodies used in these experiments. We are grateful to Allan F. Mock for expert technical assistance, members of the Papazian laboratory for their comments on the manuscript, and Dr. Ernest M. Wright for support.

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BI961083S