# Conserved Cysteine Residues in the Shaker $K^+$ Channel Are Not Linked by a Disulfide Bond†

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ABSTRACT: Many voltage-activated $K^+$ channels contain two conserved cysteine residues in putative transmembrane segments S2 and S6. It has been proposed that these cysteines form an intrasubunit disulfide bond [Guy, H. R., & Conti, F. (1990) *Trends Neurosci. 13*, 201−206]. This proposal was tested using site-directed mutagenesis followed by electrophysiological and biochemical analysis of the Shaker B $K^+$ channel. Each Shaker B subunit contains seven cysteine residues, including the conserved residues C286 and C462 and a less conserved cysteine, C245. Each cysteine in the Shaker B protein can be mutated individually without eliminating functional activity, indicating that the protein does not contain a disulfide bond that is essential for protein folding or the assembly of active channels. To determine whether there is a nonessential disulfide bond, Shaker B protein was subjected to limited proteolysis. Fragments were analyzed by electrophoresis under reducing and nonreducing conditions followed by immunoblotting. The results indicate that the two conserved residues C286 and C462 do not form a disulfide bond with each other or with C245. In addition, the subunits are not linked by disulfide bonds. In HEK293T cells, Shaker B protein is first made as an incompletely glycosylated precursor that is converted to the fully glycosylated mature protein. Glycosylation occurs at two positions in the S1−S2 loop.

Voltage-dependent potassium ($K^+$) channels are integral membrane proteins that control the excitability of nerve and muscle. The cloning of genes encoding $K^+$ channels, including the Shaker channel of *Drosophila* (Chandy & Gutman, 1994), has led to structure/function analysis using the techniques of molecular biology, electrophysiology, and biochemistry. This combined approach has yielded valuable information about protein function and has provided indirect evidence about the topology of the channel subunit in the membrane (Figure 1; Miller, 1991; Chua et al., 1992; Santacruz-Toloza et al., 1994). $K^+$ channels are thought to be homo- or heterooligomers including four α-subunits that compose the pore (MacKinnon, 1991; Liman et al., 1992; Isacoff et al., 1990; Christie et al., 1990; Ruppersberg et al., 1990; Sheng et al., 1993; Wang et al., 1993; Li et al., 1994).

Despite this progress, little is known about the tertiary structure of $K^+$ channel proteins. High-resolution structural data are not yet available due to the difficulties in purifying and crystallizing a multisubunit membrane protein in its native conformation. Guy and colleagues, however, have proposed models for the structure of $K^+$ channels (Guy & Conti, 1990; Durell & Guy, 1992). In these models, one key element is the existence of an intrasubunit disulfide bond between conserved cysteine residues in putative transmembrane segments S2 and S6. Although disulfide bonds within transmembrane domains are not common, oxygen readily partitions into hydrophobic environments where it may generate an oxidizing condition (Altenbach et al., 1989). The proposed disulfide bond would provide an essential constraint on the packing arrangement of transmembrane segments. The

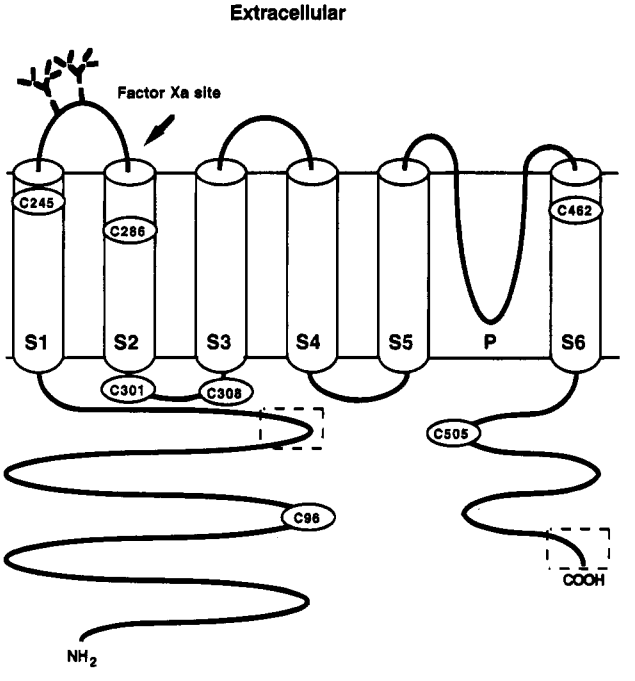


FIGURE 1: Putative membrane topology of the Shaker B protein with glycosylation sites and the engineered factor Xa site indicated in the S1−S2 extracellular loop. Amino- and carboxy-terminal antibody epitopes are boxed, and cysteine residues are circled in their approximate locations. An endogenous cryptic factor Xa protease site has been mapped to the carboxy terminus of the protein (not shown).

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determination of whether this bond exists is a critical test of the structural model.

The Shaker B $K^+$ channel subunit contains seven cysteines, including the conserved residues at positions 286 in S2 and 462 in S6 (Figure 1). In addition, a cysteine residue in S1 at position 245 is conserved between the Shaker (Kv1) and Shaw (Kv3) families (Chandy & Gutman, 1994). Biochemical analysis indicates that S1 is a transmembrane segment (Santacruz-Toloza et al., 1994). Therefore, cysteine 245 may interact in a disulfide bond with one of the conserved cysteines in putative transmembrane segments S2 or S6. The four remaining cysteine residues in the Shaker B protein are postulated to be cytoplasmic (Miller, 1991), where they would be subject to a reducing environment and would not be expected to form disulfide bonds.

Previously, it has been reported that the conserved cysteines in two voltage-dependent $K^+$ channel proteins can be mutated without eliminating channel function, as measured electrophysiologically after expression in *Xenopus* oocytes or human embryonic kidney cells (Boland et al., 1994; Zuhlke et al., 1994). Furthermore, substitution of all the cysteine residues in the Shaker B protein does not prevent folding or eliminate activity, although the level of functional expression is reduced (Boland et al., 1994). Thus, if the protein contains a disulfide bond, it is not essential for protein folding or the assembly of functional channels.

These results do not, however, directly address whether a disulfide bond exists in the wild-type Shaker B channel. Therefore, we determined biochemically whether cysteines located within the transmembrane segments form a disulfide bond. Heterologously expressed Shaker B protein was subjected to limited protease digestion, electrophoresis under reducing and nonreducing conditions, and immunoblot analysis. We show conclusively that no disulfide bonds link any of the three cysteine residues in the putative transmembrane domains of Shaker B protein. Thus, the tertiary structure of transmembrane segments in voltage-dependent $K^+$ channels need not be constrained as suggested by the model of Guy and Conti (1990).

A preliminary report of this work has appeared (Schulteis et al., 1994).

## MATERIALS AND METHODS

*Molecular Biology.* The Shaker B cDNA in pBluescript II (Stratagene, La Jolla, CA) was subjected to oligonucleotide-directed mutagenesis on a single-stranded template using the $dut^-$ $ung^-$ genetic selection (Kunkel, 1985) or on a double-stranded template using PCR methods (Landt et al., 1990). The mutated regions were transferred into the wild-type Shaker B backbone using unique restriction sites, and the mutations were confirmed by dideoxy sequencing of the transferred regions. Each of the cysteine residues in Shaker B (positions 96, 245, 286, 301, 308, 462, and 505) was individually mutated to serine. A factor Xa protease site was engineered into the Shaker B sequence by the substitutions E269G and D270R, creating the sequence IEGR (single-letter amino acid codes), which is the consensus recognition amino acid sequence for factor Xa protease (Carter, 1990). This construct has been termed FX270. The double glycosylation mutation (N259Q/N263Q) has been described previously (Santacruz-Toloza et al., 1994).

For injection into *Xenopus laevis* oocytes, cRNA was transcribed using T7 RNA polymerase (Promega, Madison, WI). For transfection of mammalian cells, the Shaker B cDNA and mutant constructs were transferred into a plasmid containing the cytomegalovirus promoter, pcDNA1/AMP (Invitrogen, San Diego, CA). DNA used for transfections was purified on Qiagen columns (Qiagen Inc., Chatsworth, CA).

*Electrophysiology.* One to five nanograms of wild-type or mutant Shaker B cRNA was injected into oocytes as described previously (Papazian et al., 1991; Timpe et al., 1988a,b; Santacruz-Toloza et al., 1994). Whole cell currents were recorded in modified Barth's saline (Timpe et al., 1988a) at room temperature using a two-electrode voltage clamp (Warner Electronics, Hamden, CT). Activation−voltage and steady-state inactivation−voltage relationships were determined as previously described (Timpe et al., 1988b; Papazian et al., 1991). Data acquisition and analysis were performed using an 80386 computer and pClamp v. 5.5.1 software (Axon Instruments, Foster City, CA).

*Tissue Culture and Metabolic Labeling of Shaker B Protein.* Human embryonic kidney cells constitutively expressing the SV-40 TAg, which allows plasmid replication using the SV-40 origin (HEK293T, formerly known as tsA201; DuBridge et al., 1987), were the kind gift of Dr. R. B. DuBridge. Cells were grown in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 20 units/mL penicillin, and 20 mg/mL streptomycin (Gibco-BRL, Grand Island, NY). Cells were transfected using conventional calcium phosphate techniques (Ausubel et al., 1991).

For pulse−chase experiments, transfected and untransfected cells at 24 or 36 h posttransfection were incubated for 30 min in methionine- and cysteine-free DMEM (Mediatech) and subsequently pulsed for 1 h with 200 $\mu$Ci/mL $^{35}$S-labeled methionine and cysteine (Tran$^{35}$S-Label, ICN Biomedicals, Costa Mesa, CA). Cells were chased in complete medium and collected at 30 min intervals by trituration and brief centrifugation. The cell pellet was resuspended and incubated for 5 min on ice in lysis buffer (1% Triton X-100 (w/v), 150 mM NaCl, 10 mM EDTA, and 20 mM Tris-HCl, pH 8.0) plus the following protease inhibitors (Sigma, St. Louis, MO, or Boehringer Mannheim, Indianapolis, IN): 0.5 mM phenylmethanesulfonyl fluoride, 50 $\mu$g/mL antipain, 25 $\mu$g/mL (4-amidinophenyl)methanesulfonyl fluoride, 40 $\mu$g/mL bestatin, 2 $\mu$g/mL aprotinin, 0.5 $\mu$g/mL leupeptin, 0.7 $\mu$g/mL pepstatin, and 0.1 mM EDTA. The lysed cells were centrifuged briefly to pellet cell nuclei, and Shaker B protein in the supernatant was concentrated via immunoprecipitation as described previously (Santacruz-Toloza et al., 1994) using an antibody directed against a Shaker−$\beta$-galactosidase fusion protein (Schwarz et al., 1990). Protein was then subjected to electrophoresis and autoradiography.

*Protease Digestion of Shaker B Protein.* Trypsin and chymotrypsin proteolysis was performed on intact cells to limit digestion of the protein. After transfection, cells were incubated in complete medium for 48 h. Untransfected and transfected cells were washed twice in Dulbecco's phosphate-buffered saline (Mediatech) and incubated in trypsin (0.05% trypsin, 0.53 mM EDTA·4Na in HBSS, Gibco-BRL) for 2 min at room temperature. Chymotrypsin digestion was performed for 10 or 15 min at room temperature in 1 mg/mL chymotrypsin (Calbiochem, La Jolla, CA) dissolved in PBS. Phenylmethanesulfonyl fluoride (PMSF) was added

to a final concentration of 2 mM to stop the reactions. After protease digestion, cells were resuspended by trituration and pelleted by brief centrifugation. The cell pellets were resuspended in Laemmli sample buffer containing either 10% 2-mercaptoethanol (reducing conditions) or 16 mM iodoacetamide (nonreducing conditions). The samples were then heated to 100 °C for 3 min and subjected to electrophoresis.

For factor Xa protease digestion, Shaker B protein was first immunoprecipitated and deglycosylated. Transfected and untransfected cells were washed in cold PBS, resuspended by trituration in the presence of 20 mM *N*-ethylmaleimide to prevent spurious oxidation of free sulfhydryls, and pelleted by brief centrifugation. The cells were resuspended and lysed in the presence of protease inhibitors, as described earlier. After extensive washing, the beads were resuspended in 50 $\mu$L of reaction buffer (100 mM NaCl, 1 mM $CaCl_2$, and 50 mM Tris-HCl, pH 8.0) and incubated in factor Xa protease (0.02 mg/mL, Boehringer Mannheim) and *N*-glycanase (0.5−0.75 units, Genzyme) for 2−8 h. In some cases, deglycosylation was performed in the presence of protease inhibitors, followed by extensive washing of the beads and factor Xa digestion. The reaction was stopped with the addition of reducing or nonreducing Laemmli sample buffer, and the resulting proteolytic fragments were analyzed by electrophoresis and immunoblotting.

*Electrophoresis and Immunoblot Analysis.* Crude cell homogenates and immunoprecipitated protein were subjected to electrophoresis on denaturing 7.5%, 10%, or 5−20% polyacrylamide gels with 4% polyacrylamide stacking gels. Protein was transferred to nitrocellulose and probed with antipeptide antibodies as described previously (Santacruz-Toloza et al., 1994). The primary antisera (kind gift of L. Y. Jan and Y. N. Jan) were prepared by Berkeley Antibody Company (Richmond, CA) against a synthetic peptide conjugated to keyhole limpet hemocyanin as described previously (Schwarz et al., 1990). The anti-amino-terminal antibody is directed against the peptide $NH_2$-CKFREDEGFIKEEERPLPDN-COOH (single-letter amino acid codes), which corresponds to amino acids 189−207 in the Shaker B sequence plus a cysteine for conjugation to the carrier protein. The carboxy-terminal antibody is directed against the peptide $NH_2$-CRHNNAMAVSIETDV-COOH, which corresponds to amino acids 603−616 of the Shaker A alternative splice variant and to amino acids 643−656 of Shaker B, with the exception of a substitution of leucine for methionine in the Shaker B sequence plus a cysteine for conjugation. Molecular weight of the proteolytic fragments was estimated on the basis of the migration of broad range molecular weight standards (Bio-Rad, Hercules, CA). Theoretical sizes of proteolytic fragments were determined using the Genetics Computer Group sequence analysis software package version 7 (Madison, WI).

## RESULTS

*Electrophysiological Analysis in Xenopus Oocytes of Individual Cysteine to Serine Mutations.* Each of the seven cysteine residues in the Shaker B subunit was individually mutated to serine. After expression in *Xenopus* oocytes, each mutant produced a transient outward $K^+$ current similar to that produced by wild-type Shaker B protein (Figure 2A). With the exception of C286S, the mutations produced only small shifts in the voltage dependence of activation and

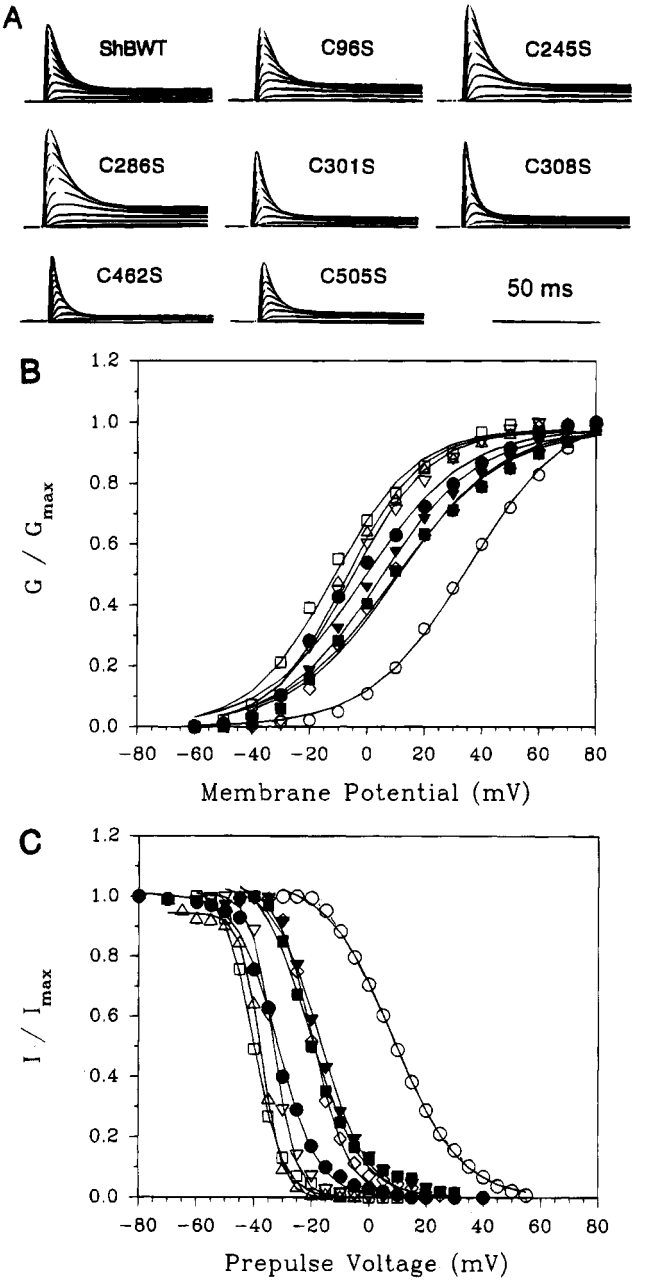


FIGURE 2: (A) Expression of cysteine mutants in *Xenopus* oocytes results in currents similar to those of wild-type Shaker B protein when recorded with a two-electrode voltage clamp. Representative current amplitudes at +60 mV are as follows (in $\mu$A): Shaker B wild-type, 7.8; C96S, 7.3; C245S, 19.0; C286S, 13.0; C301S, 7.6; C308S, 8.6; C462S, 6.7; and C505S, 6.0. (B) Conductance−voltage curves showing the voltage dependence of activation of cysteine mutants and wild-type Shaker B. Midpoint and slope of the conductance−voltage curve for each mutant are as follows (in mV ± SD and mV ± SD): (●) ShBWT, −2.0 ± 3.3 and 16 ± 1.6; (■) C96S, 6.6 ± 4.3 and 18 ± 1.9; (◇) C245S, 10.2 ± 0.9 and 18 ± 1.4; (○) C286S, 35.6 ± 3.2 and 17.2 ± 0.9; (▽) C301S, −5.3 ± 2.1 and 13.7 ± 0.5; (□) C308S, −11.7 ± 3.0 and 14.0 ± 0.4; (△) C462S, −7.4 ± 0.6 and 12.8 ± 0.1; and (▼) C505S, 4.4 ± 3.3 and 16.8 ± 1.2. (C) Current−voltage curves showing the voltage dependence of prepulse inactivation of cysteine mutants and wild-type Shaker B. Midpoint and slope of the current−prepulse voltage curve for each mutant are as follows (in mV ± SD and mV ± SD): (●) ShBWT, −33.5 ± 6.8 and 7.5 ± 1.5; (■) C96S, −20.2 ± 0.3 and 8.6 ± 1.7; (◇) C245S, −19.5 ± 1.7 and 6.1 ± 0.3; (○) C286S, 7.2 ± 2.7 and 11.7 ± 0.65; (▽) C301S, −32.4 ± 1.1 and 4.1 ± 0.3; (□) C308S, −40.0 ± 3.5 and 4.4 ± 0.24; (△) C462S, −37.5 ± 0.65 and 3.7 ± 0.4; and (▼) C505S, −16.9 ± 1.3 and 7.7 ± 0.4.

prepulse inactivation (Figure 2B,C). Such shifts are likely to represent differences in the relative stabilities of the closed and open conformations of the channel protein caused by substituting the relatively hydrophobic amino acid cysteine with the more hydrophilic serine residue. C286S had the largest effect, shifting both the activation and prepulse inactivation curves approximately 35 mV in the depolarized direction, which indicates a decrease in the stability of the open conformation relative to the closed states of the channel.

These results demonstrate that if a disulfide bond exists in the Shaker B protein, it is not essential for the proper folding, assembly, or activity of the channel. Similar results have been reported by others using a non-inactivating Shaker B mutant channel or Kv2.1, a rat brain $K^+$ channel (Boland et al., 1994; Zuhlke et al., 1994). However, mutagenesis and electrophysiological analysis do not address directly whether the protein contains a disulfide bond that is not essential for protein folding or function. To answer this question, biochemical experiments are required.

*Biochemical Characterization of Shaker B Protein Expressed in Mammalian Cells.* For biochemical analysis, wild-type or mutant channel protein was expressed in HEK293T cells transiently transfected with a plasmid encoding the Shaker B sequence under the control of a cytomegalovirus promoter. Metabolically labeled wild-type Shaker B protein was detected as two bands on an autoradiogram: a heavy band of approximately 105 kDa and a light band of approximately 79 kDa (Figure 3A). A pulse−chase experiment showed that the lighter band is converted to the heavier band over a chase period of about 2.5 h (Figure 3A).

Upon treatment with *N*-glycanase, both bands collapsed to a single band of 74 kDa (Figure 3B). Thus, both the precursor and product forms of the protein are glycosylated. This suggests that the precursor may be a core-glycosylated, endoplasmic reticulum form of the protein, whereas the mature product has been further glycosylated in the Golgi apparatus (Abeijon & Hirschberg, 1992; Rowling & Freedman, 1993).

Expression of a double mutant in which the two potential glycosylation sites in the S1−S2 loop have been altered (N259Q/N263Q) resulted in a protein that comigrated with the deglycosylated protein (Figure 3B). This indicates that Shaker B protein expressed in mammalian cells is glycosylated in the S1−S2 loop at either N259, N263, or both sites. Partial deglycosylation of wild-type Shaker B protein with *N*-glycanase resulted in three bands visible on an immunoblot (Figure 3C). The heaviest and lightest bands corresponded to the fully glycosylated and fully deglycosylated forms of the protein. A band of intermediate size (~97 kDa) was also seen, consistent with the removal of one of two N-linked carbohydrate chains from the S1−S2 loop. These data suggest that Shaker B protein expressed in HEK293T cells is glycosylated at both N259 and N263, as was previously found in *Xenopus* oocytes (Santacruz-Toloza et al., 1994).

*Trypsin and Chymotrypsin Digestion of Wild-Type Shaker B Protein.* On immunoblots, the migration of heavy and light Shaker B protein bands was unaltered under nonreducing conditions (Figure 4A, lane 4), indicating that there are no disulfide bonds between subunits. In expression systems, functional $K^+$ channels are thought to be tetramers of α-subunits (MacKinnon, 1991; Liman et al., 1992). These

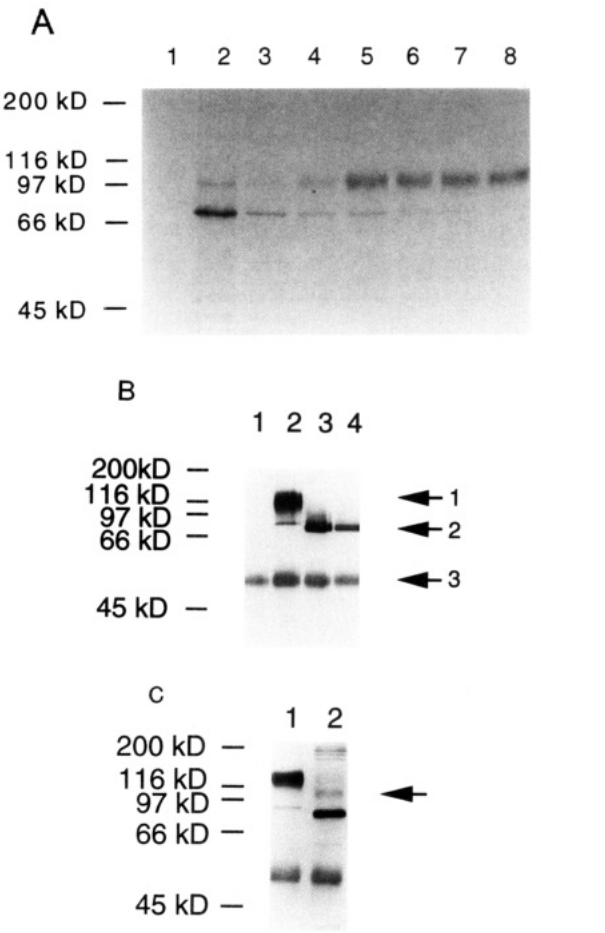


FIGURE 3: (A) Autoradiogram of SDS−PAGE with Shaker B protein metabolically labeled with [$^{35}$S]methionine and cysteine, collected after a 1 h pulse (lane 2) and at 30 min intervals thereafter (lanes 3−8). During the chase, Shaker B protein is converted from an immature precursor (~79 kDa) to a mature product (~105 kDa). Lane 1: untransfected control. (B) Immunoblot of SDS−PAGE with immunoprecipitated Shaker B protein, probed with a carboxy-terminal antipeptide antibody: lane 1, untransfected control; lane 2, wild-type protein (arrow 1 indicates the heavy form of the protein with the light form running below it); lane 3, wild-type protein treated with *N*-glycanase to remove N-linked sugars (both heavy and light bands collapse to a single band of ~74 kDa (arrow 2); lane 4, mutant N259Q/N263Q migrates identically to enzymatically deglycosylated protein (arrow 3 indicates heavy chain of IgG). (C) Immunoblot of partially deglycosylated Shaker B protein: lane 1, wild-type protein; lane 2, wild-type protein treated for a short duration with *N*-glycanase. Arrow indicates a partially glycosylated band running at approximately 97 kDa, consistent with the removal of one of two N-linked carbohydrate moieties. Other bands are as indicated in part B.

results indicate that subunit assembly occurs via noncovalent interactions.

To determine biochemically whether cysteines located within the transmembrane segments form an intrasubunit disulfide bond, Shaker B protein was digested with various proteases to separate cysteine residues on different proteolytic fragments. The resulting fragments were subjected to electrophoresis and immunoblot analysis. Possible disulfide bonds between cysteines in separate proteolytic fragments were evaluated by comparing the migration of the fragments under reducing and nonreducing conditions.

The Shaker B protein contains multiple sites for trypsin and chymotrypsin digestion. To limit proteolysis, Shaker B protein was digested by these enzymes in intact cells. These conditions generated partial digestion with a major

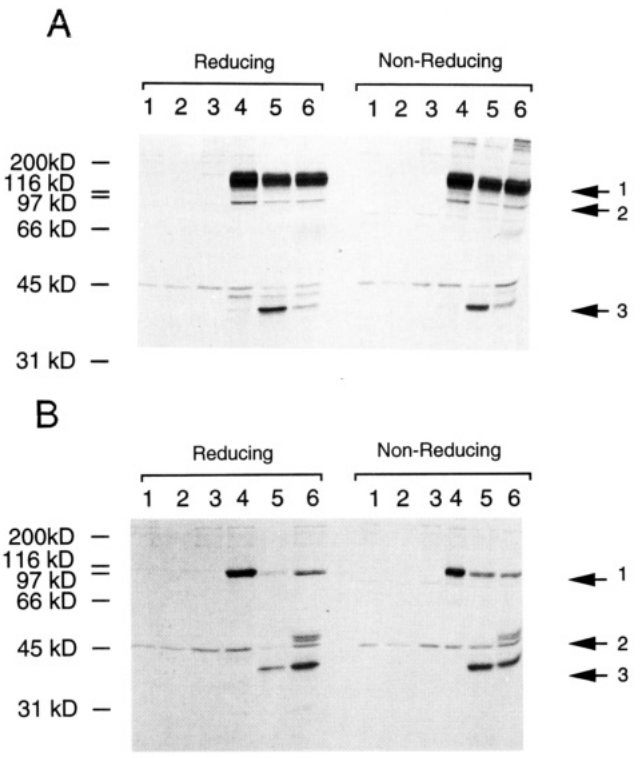


FIGURE 4: (A) Immunoblot of a crude cell homogenate of HEK293T cells expressing wild-type Shaker B protein probed with an antibody against the 14 carboxy-terminal amino acids of Shaker B: lanes 1−3, untransfected control; lanes 4−6, wild-type Shaker B protein. Protein in lanes 2 and 5 was cut with trypsin, and protein in lanes 3 and 6 was cut with chymotrypsin. Arrows 1 and 2 indicate mature and immature uncut Shaker B protein, respectively. Arrow 3 indicates the 36 kDa carboxy-terminal fragment produced by trypsin and chymotrypsin treatment. Note that the migration of fragments is identical under both reducing and nonreducing conditions. A band migrating at approximately 41 kDa is apparent in both transfected and untransfected lanes. This represents an endogenous cellular protein that is recognized by the C-terminal antibody. It is not seen in samples immunoprecipitated with the Shaker−$\beta$−galactosidase antibody (compare with Figure 3B). An additional proteolytic fragment appears in both cut and uncut lanes, migrating between the 36 kDa fragment and the background band. (B) Immunoblot of a crude cell homogenate of HEK293T cells expressing the Shaker B protein double glycosylation mutant probed with an antibody against the 14 carboxy-terminal amino acids of Shaker B. Lane assignments are the same as in part A. Arrow 1 indicates uncut Shaker B protein. Arrow 2 indicates the triplet of carboxy-terminal fragments produced by chymotrypsin digestion. Arrow 3 indicates the 36 kDa carboxy-terminal fragment produced by both trypsin and chymotrypsin treatment. Note that the migration of fragments is identical under both reducing and nonreducing conditions. The nonspecific background band is visible at 41 kDa.

proteolytic fragment of approximately 36 ± 1 kDa ($n = 8$) that was recognized by an antibody directed against the 14 carboxy-terminal amino acids of the protein (Figure 4A, lanes 5 and 6, arrow 3). This fragment was generated reproducibly in eight separate experiments despite differences in the amount of protease digestion. It was not seen in untransfected control lanes. On the basis of its immunoreactivity with the carboxy-terminal antibody and its estimated molecular weight, the fragment extended from the carboxy terminus to a region past the S6 hydrophobic segment, probably to the putative extracellular loop between S3 and S4. This loop contains several potential sites for trypsin (K) and chymotrypsin (L, V, and M) digestion. Proteolysis in this loop is expected to produce carboxy-terminal fragments ranging in size from approximately 33 to 36 kDa. The addition of soybean trypsin inhibitor did not block chymotrypsin generation of this fragment (data not shown), indicating that digestion by chymotrypsin was not due to trypsin contamination.

The 36 kDa proteolytic fragment contained C462 and C505, separating these residues from cysteines in the amino-terminal region, including the cysteines in S1 and S2. The proteolytic fragment produced by trypsin and chymotrypsin digestion migrated identically under reducing and nonreducing conditions, indicating that no disulfide bond links cysteines 462 or 505 with cysteines in the amino-terminal region of the protein, including the conserved cysteine in S2 at position 286 and the partially conserved cysteine at 245.

In addition, proteolytic fragments of approximately 40 kDa (Figure 4A, lanes 4−6) and 60 kDa (Figure 4A, lane 6) were detected by the carboxy-terminal antibody. These fragments, which were not detected in untransfected control lanes (Figure 4A, lanes 1−3), migrated identically under reducing and nonreducing conditions. The 40 kDa fragment was probably generated by endogenous proteases because it was present in the uncut protein sample (Figure 4A, lane 4). Given its size and reactivity with the carboxy-terminal antibody, this fragment may have been generated by digestion in the S2−S3 loop, a region of the protein that is predicted to be intracellular (Figure 1). The 60 kDa fragment, generated by chymotrypsin, migrated as a broad, heterogeneous band. Its estimated molecular weight and microheterogeneity are consistent with proteolysis in a region of the protein amino-terminal to the glycosylation sites in the S1−S2 loop.

An antibody directed against an amino-terminal epitope detected proteolytic fragments that also ran identically under reducing and nonreducing conditions (data not shown). The estimated molecular weight and heterogeneous appearance of these fragments were consistent with the presence of the heavily glycosylated S1−S2 loop; thus, they may represent all or part of the amino-terminal fragment resulting from trypsin and chymotrypsin digestion. These results indicate that large fragments of the amino-terminal region of the protein remain after protease digestion.

*Trypsin and Chymotrypsin Digestion of Unglycosylated Protein.* Shaker B protein containing the double mutant, N259Q/N263Q, which produces functional but unglycosylated protein (Figure 3B; Santacruz-Toloza et al., 1994), was expressed and subjected to proteolysis in intact HEK293T cells. Trypsin digestion generated the same proteolytic fragment as did digestion of wild type Shaker B protein (37 ± 3 kDa, $n = 4$; Figure 4B, lane 5). No additional trypsin sites were revealed by removing the carbohydrate moieties. The efficiency of tryptic digestion of the protein increased upon removal of the sugars, however, suggesting that the enzyme had better access to the protein.

After chymotrypsin digestion of the unglycosylated protein, several additional chymotrypsin sites were revealed, resulting in a triplet of proteolytic fragments that ranged in size from 45 to 52 kDa (Figure 4B, lane 6). The appearance of these fragments was highly reproducible; however, the heaviest band of this triplet is often difficult to distinguish. Their sizes are consistent with digestion in the putative S1−S2 loop or in the S1 transmembrane segment, the sequences of which contain several potential chymotrypsin cleavage sites. This would be consistent with the observation that

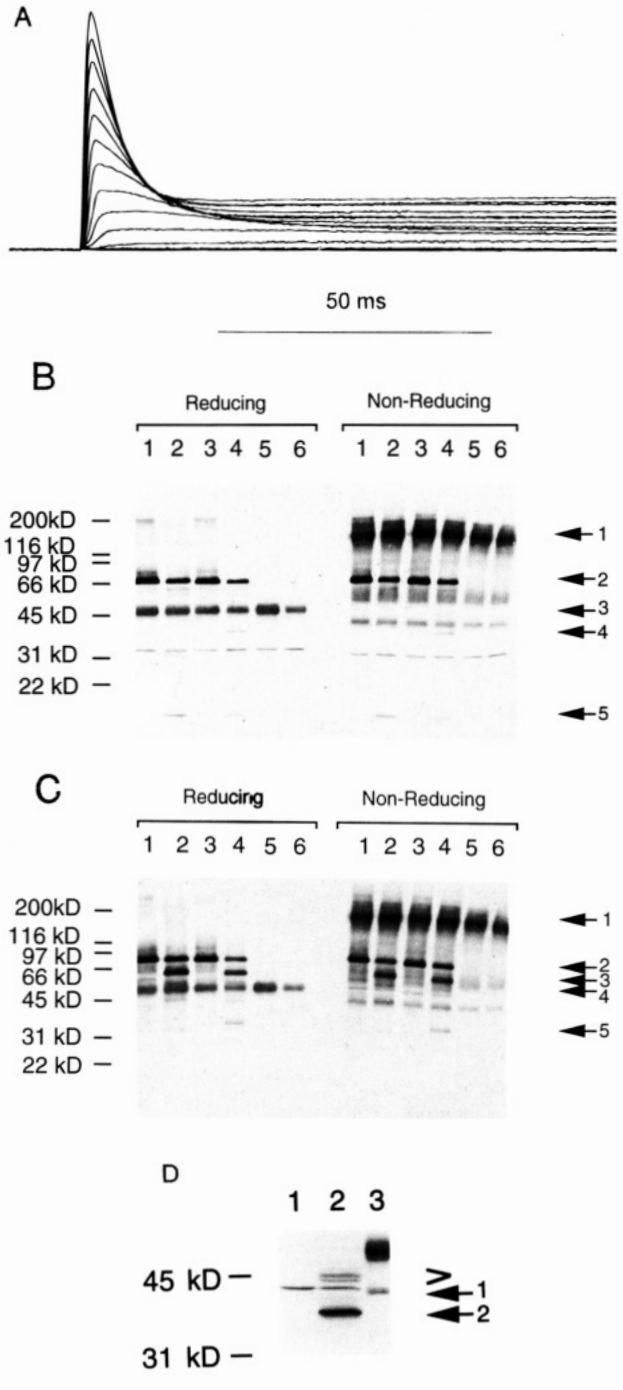


FIGURE 5: (A) Two-electrode voltage clamp recording of the FX270 mutant. Representative current at +60 mV is 16.8 $\mu$A. Current kinetics are similar to that of the wild-type Shaker B protein (see Figure 2A). (B) Immunoblot of wild-type and FX270 Shaker B proteins probed with the carboxy-terminal antibody: lanes 1 and 2, wild-type protein; lanes 3 and 4, FX270; lanes 5 and 6, untransfected controls. All samples were treated with *N*-glycanase, while protein in even-numbered lanes was digested with factor Xa. Arrow 2 indicates uncut, deglycosylated Shaker B protein. Arrow 4 indicates the 41 kDa carboxy-terminal fragment produced by factor Xa digestion of FX270 after amino acid 270. Note that this fragment migrates identically under both reducing and nonreducing conditions. Arrow 5 indicates a carboxy-terminal fragment produced by digestion at a cryptic factor Xa site endogenous to wild-type and FX270 Shaker B proteins. Arrows 1 and 3 indicate IgG under nonreducing and reducing conditions, respectively. (C) Immunoblot of wild-type and FX270 Shaker B proteins probed with an amino-terminal antibody. Lane assignments are the same as in part B. Arrow 2 indicates uncut, deglycosylated Shaker B protein. Arrow 5 indicates the 35 kDa amino-terminal fragment produced by factor Xa digestion of FX270 after amino acid 270. Note that this fragment migrates identically under both reducing and nonreducing conditions. Arrow 3 indicates an amino-terminal fragment produced by digestion at a cryptic factor Xa site endogenous to wild-type and FX270 Shaker B proteins. Arrows 1 and 4 indicate IgG under nonreducing and reducing conditions, respectively. (D) Immunoblot probed with a carboxy-terminal antibody. Immunoprecipitated FX270 protein cut with factor Xa (lane 3; see also Figure 5B, lane 4) and crude cell homogenate of HEK293T cells expressing the double glycosylation mutant cut with chymotrypsin (lane 2; see also Figure 4B, lane 6) run under reducing conditions on the same gel. Arrow 1 indicates the fragment produced by factor Xa digestion. The bracket delineates the triplet of carboxy-terminal fragments produced by chymotrypsin digestion. Arrow 2 indicates the 36 kDa carboxy-terminal fragment produced by chymotrypsin digestion. IgG is apparent in lane 3 running at approximately 50 kDa. Immunoprecipitation of the sample in lane 3 has eliminated the background band visible in lanes 1 and 2. Lane 1 is a crude cell homogenate of untransfected HEK293T cells treated with chymotrypsin.

these sites are accessible only when the S1−S2 loop is not glycosylated.

The triplet of chymotryptic fragments migrated identically under reducing and nonreducing conditions. Cutting in the S1−S2 loop would effectively separate the cysteine residues at positions 245 and 286 in the S1 and S2 regions, respectively. These results suggest that there is no disulfide bond linking cysteines 245 and 286 or 245 and 462. However, because these fragments could have been generated by digestion in the S1 segment, it is possible that C245 and C286 have not been separated onto different fragments by chymotrypsin digestion.

*Factor Xa Protease Digestion of Wild-Type and Mutant Shaker B Proteins.* To digest Shaker B protein at a defined site in the S1−S2 loop, a site-specific protease, factor Xa, was used to cut the Shaker B protein at an engineered recognition site (sequence IEGR). Factor Xa cuts after the R in the recognition sequence, which corresponds to amino acid 270 in the S1−S2 loop in the FX270 construct. The mutant construct was expressed in *Xenopus* oocytes and tested electrophysiologically (Figure 5A). It produced currents similar to the wild-type channel, indicating that the structure of the protein was not altered significantly by the mutation introducing the factor Xa site. Functional channels from the FX270 construct have also been recorded in transfected HEK293T cells (data not shown).

To increase the efficiency of digestion at the engineered protease site, protein from transfected cells was immunoprecipitated and then deglycosylated with *N*-glycanase concurrently with factor Xa digestion. The presence of *N*-glycanase was critical because glycosylation of the protein at positions N259 and N263 limited protease access to sites in the S1−S2 loop, severely decreasing the efficiency of digestion.

Digestion of the FX270 protein with factor Xa resulted in the production of a 41 ± 2 kDa band ($n = 10$) recognized by the carboxy-terminal antibody (Figure 5B, lane 4, arrow 4), whereas an amino-terminal antibody recognized a 37 ± 3 kDa fragment ($n = 3$; Figure 5C, lane 4, arrow 5). These fragments were not detected after factor Xa treatment of wild-type deglycosylated protein (Figure 5B,C, lane 2) or in samples from untransfected cells (Figure 5B,C, lane 6). The sizes of these fragments are consistent with the digestion of FX270 protein at the engineered factor Xa site, which would be expected to produce carboxy- and amino-terminal frag-

ments of 43 and 32 kDa, respectively. Both the amino- and carboxy-terminal fragments produced by factor Xa digestion migrated identically under reducing and nonreducing conditions. This proteolysis unambiguously separates C245 from C286 and C462 on different protein fragments and demonstrates the absence of a disulfide bond linking these cysteine residues.

Wild-type Shaker B and FX270 proteins were cleaved by factor Xa at an endogenous, cryptic recognition site located in the carboxy-terminal region, generating a fragment of approximately 17 kDa detected by the carboxy-terminal antibody (Figure 5B, arrow 5). Short digestions were used in these experiments, producing inefficient cutting at this cryptic site. It was therefore feasible to detect the unique band derived from the FX270 construct with the carboxy-terminal antibody.

To determine whether chymotrypsin cut the unglycosylated Shaker B protein on the amino- or carboxy-terminal side of residue 270, the migration of the carboxy-terminal factor Xa fragment from FX270 and the chymotrypsin fragments of unglycosylated Shaker B protein were compared side by side under reducing conditions (Figure 5D). The factor Xa fragment (lane 3, arrow 1) was approximately 4 ± 1 kDa larger than the 36 kDa chymotrypsin fragment ($n = 11$; lane 2, arrow 2), indicating that chymotrypsin cuts approximately 29−44 amino acids carboxy-terminal of position 270. This would be in the S3 transmembrane domain, separating C462 from C286. The FX270 fragment is about 2 ± 1 kDa smaller than the smallest of the triplet of chymotryptic fragments ($n = 10$; Figure 5D, lane 2, bracket), consistent with chymotrypsin digestion approximately 12−25 amino acids amino-terminal of position 270. This would be in the S1−S2 loop or near the extracellular surface of S1, separating cysteines 245 and 286. These results support the previous assignments of the trypsin and chymotrypsin cleavage sites.

The presence of the immunoglobulin in the immunoprecipitated protein samples digested with factor Xa protease verified the reducing and nonreducing conditions used in these experiments (Figure 5B,C). The immunoglobulin migrated at approximately 52 kDa under reducing conditions (Figure 5B, arrow 3; Figure 5C, arrow 4), consistent with the size of individual heavy chains, while under nonreducing conditions the immunoglobulin migrated at 150−200 kDa (Figure 5B and 5C, arrow 1), a size consistent with an intact antibody structure, including two light and two heavy chains linked by disulfide bonds (Harlow & Lane, 1988).

## DISCUSSION

*Biochemical Characteristics of Shaker B Protein in HEK293T Cells.* Shaker B protein produced in transiently transfected HEK293T cells is made initially as an immature, partially glycosylated precursor that is converted within 2.5 h to the mature, fully glycosylated protein. Carbohydrate is attached to two asparagines, N259 and N263, in the S1−S2 loop. The maturation of the protein suggests that the precursor represents core-glycosylated protein from the endoplasmic reticulum, whereas the mature protein represents fully glycosylated protein that has been further processed in the Golgi apparatus (Abeijon & Hirschberg, 1992; Rowling & Freedman, 1993). The time course of conversion of the precursor to the final product is consistent with that demonstrated for the rat brain $Na^+$ channel (Schmidt & Catterall, 1986). We have previously shown that, in amphibian oocytes, Shaker B protein is made in two forms that are remarkably similar to those made in mammalian tissue culture cells (Santacruz-Toloza et al., 1994). Glycosylation occurs at the same sites in these different expression systems. Although pulse−chase experiments have not been feasible in *Xenopus* oocytes, it is reasonable to assume that the two forms of the protein made in that system share a precursor/product relationship similar to what we have now demonstrated in HEK293T cells. In both systems, complete glycosylation results in a large change in the mobility of the protein on gels. This may result from the attachment of a large amount of carbohydrate or from anomalous migration due to changes in hydrodynamic properties of the protein (Leach et al., 1980; Santacruz-Toloza et al., 1994).

*Shaker B Protein Lacks Disulfide Bonds.* We used biochemical techniques to test the structural models of Guy and Conti (1990) and Durell and Guy (1992). These models proposed the existence of a disulfide bond linking the conserved cysteine residues in the S2 and S6 transmembrane domains (positions C286 and C462 in Shaker B). This bond is a key element of the model since it constrains the positions of S2 and S6 and limits the number of possible arrangements for the transmembrane segments. Proteolytic digestion of Shaker B protein with trypsin or chymotrypsin separated the conserved S2 and S6 cysteine residues on different proteolytic fragments. Subsequent electrophoresis and immunoblot analysis of the fragments under reducing and nonreducing conditions demonstrated that the proposed intrasubunit disulfide bond does not exist. In addition, digestion with chymotrypsin and factor Xa protease showed that the less well-conserved cysteine residue in S1 (C245) does not form a disulfide bond with conserved cysteines in S2 (C286) or S6 (C462). Thus, structural models in which S2 and S6 are not adjacent cannot be excluded. However, the results do not preclude the possibility that cysteine residues in transmembrane domains S1, S2, or S6 are adjacent, but not involved in disulfide bonds, or that the distance between the cysteines is greater than that required for disulfide bond formation. The absence of disulfide bonds between the cysteine residues in the putative transmembrane domains is consistent with the relatively minor alterations in protein activity produced by mutation of these residues to serine or to other amino acid residues (Figure 2; Boland et al., 1994; Zuhlke et al., 1994). We have also demonstrated that Shaker B subunits are not linked by a disulfide bond.

In our experiments, immunoreactivity of proteolytic fragments with an antipeptide antibody recognizing the last 14 amino acids of the Shaker B carboxyterminus made it feasible to estimate the site of proteolysis. All fragments recognized by this antibody must include the carboxy terminus of the protein. The major tryptic/chymotryptic fragment had an estimated molecular mass of 36 kDa, indicating that the most likely site of proteolysis was in the S3−S4 loop. This would separate the S2 and S6 transmembrane segments.

Factor Xa proteolysis of the FX270 construct can be specifically and unambiguously assigned to the S1−S2 loop at position 270. Comparison of the factor Xa carboxy-terminal fragment with those produced by chymotrypsin digestion of unglycosylated Shaker B protein confirmed the assignment of the sites for protease digestion by trypsin and chymotrypsin.

A possible criticism of this approach for mapping disulfide bonds is that a small proteolytic fragment linked via a disulfide bond may be too small to change the migration pattern of a larger fragment. In the case of trypsin digestion, the smallest estimated proteolytic fragments including C245 or C286 would be 2.9 and 3.7 kDa, respectively, assuming that trypsin cuts at the nearest lysine or arginine residues and has access to these sites in intact cells. These fragments would produce a detectable shift in migration of the 36 kDa fragment under the SDS−PAGE conditions utilized in these experiments. A similar argument may not be made for the relatively less selective protease chymotrypsin, but the similarity of the fragments produced from these two digestions suggests that both enzymes are cutting in similar positions. It is extremely unlikely, however, that trypsin or chymotrypsin cleaved at every possible site in our experiments. Intact protein remained after the digestion, and large proteolytic fragments were detected from both the amino and carboxyl regions of the protein.

No conclusions can be drawn from the present results about whether C301 and C308 participate in disulfide bonds, although these residues are located in a putative intracellular loop (Miller, 1991). In this location, these residues would be in a reducing environment. Similarly, C96 and C505 are located in intracellular regions of the protein (Hoshi et al., 1990; Isacoff et al., 1990, 1991; Tempel et al., 1987; Zagotta et al., 1990). It is therefore unlikely that these residues are involved in disulfide bonds. This assumption is further supported by the lack of change in channel function upon substitution of these residues (Figure 2; Boland et al., 1994).

A remaining question is why the cysteine residues in S2 and S6 are so highly conserved in the absence of a major role in determining protein stability via a disulfide interaction. Mutation of C286 to serine decreases the relative stability of the open state, as indicated by a shift in the midpoint of the conductance−voltage curve (Figure 2B). A similar result was obtained using the rat brain $K^+$ channel Kv2.1 (Zuhlke et al., 1994). The conserved cysteine residue in S6 has been shown to play a role in slow inactivation (Boland et al., 1994; Zuhlke et al., 1994). In addition, the conserved cysteines may be modified covalently by fatty acylation. Posttranslational modification of proteins by lipid attachment to cysteine residues via thioester linkages has been demonstrated in cell surface proteins such as myelin-associated glycoprotein (Pedraza et al., 1990), viral glycoproteins (Schmidt & Schlesinger, 1980), G-protein-coupled receptors (Papac et al., 1992), and $Na^+$ channel α-subunits (Schmidt & Catterall, 1987). Although no individual cysteine residue is critical to Shaker B $K^+$ channel assembly and function, the cysteine-less mutant is expressed less efficiently than control constructs (Boland et al., 1994). This fact may be partly explained by the lack of acylation at one or more cysteine residues.

The facts that none of the seven cysteine residues in the Shaker B protein are critical for protein function, that a disulfide bond is absent, and that a functional cysteine-less construct exits (Boland et al., 1994) set the stage for the use of cysteine substitution mutagenesis and sulfhydryl chemistry to probe the secondary and tertiary structure of the Shaker B subunit. Such approaches have been used successfully in prokaryotic proteins such as the chemosensory receptors or lactose permease of *Escherichia coli* (Falke & Koshland, 1987; Jung et al., 1993).

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## REFERENCES

Abeijon, C., & Hirschberg, C. B. (1992) *Trends In Biochem. Sci.*, 32−36.
Altenbach, C., Flitsch, S. L., Khorana, H. G., & Hubbell, W. L. (1989) *Biochemistry 28*, 7806−7812.
Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1991) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York.
Boland, L. M., Jurman, M. E., & Yellen, G. (1994) *Biophys. J. 66*, 694−699.
Carter, P. (1990) in *Protein Purification from Molecular Mechanisms to Large Scale Processes* (Ladisch, M. R., Willson, R. C., Painton, C. C., & Builder, S. E., Eds.) pp 181−193, American Chemical Society, Washington, D.C.
Chandy, K. G., & Gutman, G. A. (1994) in *Ligand- and Voltage-Gated Ion Channels* (North, R. A., Ed.) pp 1−71, CRC Press, Boca Raton, FL.
Christie, M. J., North, R. A., Osborne, P. B., Douglass, J., & Adelman, J. P. (1990) *Neuron 2*, 405−411.
Chua, K., Tytgat, J., Liman, E., & Hess, P. (1992) *Biophys. J. 61*, A289.
DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P.-M., Miller, J. H., & Calos, M. P. (1987) *Mol. Cell. Biol. 7*, 379−387.
Durell, S. R., & Guy, H. R. (1992) *Biophys. J. 62*, 238−250.
Falke, J. J., & Koshland, D. E. J. (1987) *Science 237*, 1596−1600.
Guy, H. R., & Conti, F. (1990) *Trends Neurosci. 13*, 201−206.
Harlow, E., & Lane, D. (1988) *Antibodies*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Hoshi, T., Zagotta, W. N., & Aldrich, R. W. (1990) *Science 250*, 533−538.
Isacoff, E. Y., Jan, Y. N., & Jan, L. Y. (1990) *Nature 345*, 530−534.
Isacoff, E. Y., Jan, Y. N., & Jan, L. Y. (1991) *Nature 353*, 86−90.
Jung, K., Jung, H., Wu, J., Prive, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273−12278.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Landt, O., Grunert, H.-P., & Hahn, U. (1990) *Gene 96*, 125−128.
Leach, B. S., Collawn, J. F. J., & Fish, W. W. (1980) *Biochemistry 19*, 5734−5741.
Li, M., Unwin, N., Stauffer, K. A., Jan, Y. N., & Jan, L. Y. (1994) *Curr. Biol. 4*, 110−115.
Liman, E. R., Tytgat, J., Hess, P. (1992) *Neuron 9*, 861−871.
MacKinnon, R. (1991) *Nature 350*, 232−235.
Miller, C. (1991): *Science 252*, 1092−1096.
Papac, D. I., Thornburg, K. R., Bullesbach, E. E., Crouch, R. K., & Knapp, D. R. (1992) *J. Biol. Chem. 267*, 16889−16894.
Papazian, D. M., Timpe, L. C., Jan, Y. N., & Jan, L. Y. (1991) *Nature 349*, 305−310.
Pedraza, L., Owens, G. C., Green, L. A. D., & Salzer, J. L. (1990) *J. Cell Biol. 111*, 2651−2661.
Rowling, P. J. E., & Freedman, R. B. (1993) in *Endoplasmic Reticulum* (Borgese, N., & Harris, J. R., Eds.) pp 41−80, Plenum Press, New York.
Ruppersberg, J. P., Schroter, K. H., Sakmann, B., Stocker, M., Sewing, S., & Pongs, O. (1990) *Nature 345*, 535−537.
Santacruz-Toloza, L., Huang, Y., John, S. A., & Papazian, D. M. (1994) *Biochemistry 33*, 5607−5613.
Schmidt, M. F. G., & Schlesinger, M. J. (1980) *J. Biol. Chem. 255*, 3334−3339.
Schmidt, J. W., & Catterall, W. A. (1986) *Cell 46*, 437−445.
Schmidt, J. W., & Catterall, W. A. (1987) *J. Biol. Chem. 262*, 13713−13723.

Schulteis, C. T., John, S. A., Huang, Y., & Papazian, D. M. (1994) *Biophys. J. 66*, A107.
Schwarz, T. L., Papazian, D. M., Carretto, R. C., Jan, Y. N., & Jan, L. Y. (1990) *Neuron 2*, 119−127.
Sheng, M., Liao, Y. J., Jan, Y. N., & Jan, L. Y. (1993) *Nature 365*, 72−75.
Tempel, B. L., Papazian, D. M., Schwarz, T. L., Jan, Y. N., & Jan, L. Y. (1987) *Science 237*, 770−775.
Timpe, L. C., Jan, Y. N., & Jan, L. Y. (1988a) *Neuron 1*, 659−667.
Timpe, L. C., Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, Y. N., & Jan, L. Y. (1988b) *Nature 331*, 143−145.
Wang, H., Kinkel, D. D., Martin, T. M., Schwartskroin, P. A., & Tempel, B. L. (1993) *Nature 365*, 75−85.
Zagotta, W. N., Hoshi, T., & Aldrich, R. W. (1990) *Science 250*, 568−571.
Zuhlke, R. D., Zhang, H.-J., & Joho, R. H. (1994) *Recept. Channels 2*, 237−248.

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