Electrostatic Interactions Between Transmembrane Segments Mediate Folding of Shaker K$^+$ Channel Subunits

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ABSTRACT   In voltage-dependent Shaker K$^+$ channels, charged residues E293 in transmembrane segment S2 and R365, R368, and R371 in S4 contribute significantly to the gating charge movement that accompanies activation. Using an intragenic suppression strategy, we have now probed for structural interactions between transmembrane segments S2, S3, and S4 in Shaker channels. Charge reversal mutations of E283 in S2 and K374 in S4 disrupt maturation of the protein. Maturation was specifically and efficiently rescued by second-site charge reversal mutations, indicating that electrostatic interactions exist between E283 in S2 and R368 and R371 in S4, and between K374 in S4 and E293 in S2 and D316 in S3. Rescued subunits were incorporated into functional channels, demonstrating that a native structure was restored. Our data indicate that K374 interacts with E293 and D316 within the same subunit. These electrostatic interactions mediate the proper folding of the protein and are likely to persist in the native structure. Our results raise the possibility that the S4 segment is tilted relative to S2 and S3 in the voltage-sensing domain of Shaker channels. Such an arrangement might provide solvent access to voltage-sensing residues, which we find to be highly tolerant of mutations.

INTRODUCTION

Many voltage-dependent K$^+$, Na$^+$, and Ca^{2+} channels derive from a common evolutionary ancestor and activate by similar mechanisms (Sigworth, 1994). Depolarization of the membrane initiates conformational changes that result in the opening of an ion-selective pore. The ability of these channels to respond to voltage is conferred primarily by charged residues in the protein (Sigworth, 1994). In voltage-dependent Shaker K$^+$ channels, four conserved residues in transmembrane segments S2 and S4 make significant contributions to the voltage sensor. These are R365, R368, and R371, positively charged amino acids in the S4 segment, and E293, a negatively charged amino acid in S2 (Fig. 1) (Aggarwal and MacKinnon, 1996; Larsson et al., 1996; Mannuzzu et al., 1996; Seoh et al., 1996). S4 residues also undergo voltage-dependent conformational changes in Na$^+$ channels (Yang and Horn, 1995; Yang et al., 1996). Identification of these voltage-sensing residues is an important step toward understanding the molecular mechanism of voltage-dependent activation. A necessary next step will be to obtain information about the structure of the voltage sensor and the conformational changes it undergoes during activation.

The strategy of intragenic suppression has been used effectively to characterize protein structure. In intragenic suppression, the effects of a primary mutation that disrupts the function of a gene are suppressed by a second site

mutation in the same gene. To identify likely structural interactions, a primary mutation that prevents the proper folding or assembly of a protein can be rescued by specific second site mutations within the same gene. The second site mutation is thought to compensate for a structural interaction disrupted by the first mutation, restoring proper folding. Following this rationale, intragenic suppression has been used to identify positions that are in close proximity in a protein (di Rago et al., 1990; King et al., 1991; Lee et al., 1992; Hartzog and Cain, 1994; di Rago et al., 1995; Liu et al., 1995). This interpretation reflects the fact that many interactions that are important for proper folding and assembly persist in the native structure of a protein. A different category of suppressors acts by increasing overall protein stability. However, in this case the suppression lacks specificity (Shortle and Lin, 1985).

We have adapted the strategy of intragenic suppression to identify likely structural interactions in the voltage-sensing domain of Shaker K$^+$ channels (Papazian et al., 1995). Our approach involves monitoring the effect of mutations on Shaker protein biosynthesis and function. The Shaker protein is made initially as a core-glycosylated precursor in the endoplasmic reticulum (ER) (Santacruz-Toloza et al., 1994; Schulteis et al., 1995; Nagaya and Papazian, 1997). While in the ER, four pore-forming subunits assemble (Nagaya and Papazian, 1997). Subsequently, the precursor is efficiently transferred to the Golgi apparatus, where the oligosaccharide moieties are processed, generating the mature product (Schulteis et al., 1995; Nagaya and Papazian, 1997). Some site-directed mutations in the Shaker gene block maturation of the protein, trapping it in an immature state in the ER, thereby eliminating functional expression (Papazian et al., 1991; Perozo et al., 1994). We have proposed that these mutations prevent proper folding or oligomerization of channel subunits (Papazian et al., 1995), subjecting them
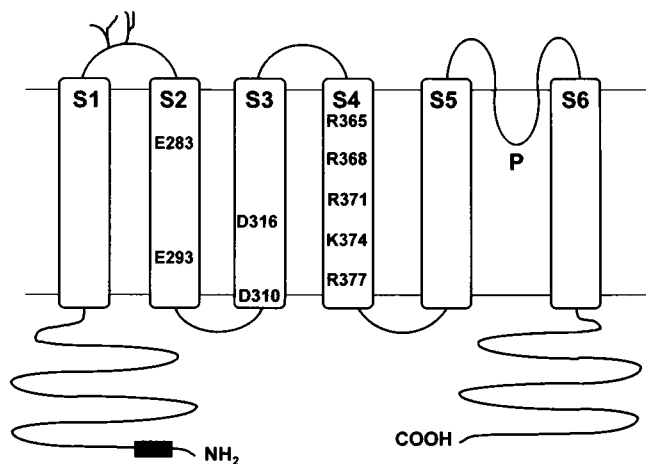
FIGURE 1 A model for the membrane topology of a Shaker K$^+$ channel subunit. Putative transmembrane segments S1-S6 and the P region, which contributes to the pore, are shown. The approximate locations of the ionizable amino acid residues analyzed in our experiments are labeled. In the extracellular S1-S2 loop of the Shaker protein, two asparagine residues (N259 and N263) are modified by N-linked glycosylation (Santacruz-Toloza et al., 1994). The intracellular N-terminal region contains an inactivation particle (*shaded box*) that is responsible for blocking the pore during N-type inactivation (Hoshi et al., 1990; Zagotta et al., 1990; Demo and Yellen, 1991). Many features of this model, including the transmembrane topology of the S4 segment, are supported by electrophysiological and biochemical data (Hoshi et al., 1990; MacKinnon and Yellen, 1990; Zagotta et al., 1990; Isacoff et al., 1991; Yellen et al., 1991; Santacruz-Toloza et al., 1994; Larsson et al., 1996; Manuzzu et al., 1996).

to the quality control system of the ER, which recognizes and retains misfolded or unassembled proteins (Gething and Sambrook, 1992). This proposal is supported by the observation that such mutations can be rescued by introducing specific, complementary, second site mutations, which restore maturation and functional expression (Papazian et al., 1995).

Previously we presented evidence that K374 in the S4 segment interacts with E293 in S2 and D316 in S3 (Papazian et al., 1995). Using charge reversal mutations, we now demonstrate that these interactions are specific and electrostatic in nature. K374 interacts with E293 and D316 within the same subunit, rather than in adjacent subunits. In addition, E283 in S2 interacts electrostatically with R368 and R371 in S4. Our data indicate that establishment of electrostatic interactions contributes to the specificity and kinetics of the folding process. We also find that charged residues that contribute to the voltage sensor are highly mutable, consistent with some exposure to solvent, whereas charged residues that do not contribute to the voltage sensor are much less tolerant of mutation, consistent with an important structural role. The results suggest a model for the packing of the voltage-sensing domain in which the S4 segment is tilted at an angle relative to S2 and S3. This arrangement could provide a basis for solvent intrusion into the transmembrane region in the vicinity of the voltage-sensing residues, supporting models for the physical mechanism of activation in which the transmembrane electric field falls

across a section of the protein that is shorter than the thickness of the membrane bilayer (Goldstein, 1996; Seoh et al., 1996; Yang et al., 1996).

MATERIALS AND METHODS

Molecular biology

Mutations were generated by the polymerase chain reaction (PCR), using a three- or four-primer strategy (Horton et al., 1989; Landt et al., 1990). PCR products were digested with appropriate restriction enzymes and transferred into Bluescript (Stratagene, La Jolla, CA) subclones of the Shaker B wild type (Schwarz et al., 1988) or Shaker B-IR (Hoshi et al., 1990) cDNAs, as indicated. The sequences of transferred fragments were verified by dideoxy sequencing. Run-off transcripts of RNA were prepared using T7 RNA polymerase (Promega, Madison, WI) or the mMESSAGE mMACHINE kit (Ambion, Austin, TX) and injected into *Xenopus* oocytes as previously described (Timpe et al., 1988; Santacruz-Toloza et al., 1994).

Biochemistry and electrophysiology

For biochemical analysis of Shaker protein, oocytes were coinjected with RNA and 400 nCi per oocyte of in vitro translation grade [^{35}S]methionine (ICN) (Santacruz-Toloza et al., 1994; Papazian et al., 1995). Oocytes were harvested 44-48 h later unless otherwise noted. A crude membrane fraction was prepared, protein was solubilized, and Shaker protein was immunoprecipitated and subjected to denaturing electrophoresis as previously described (Santacruz-Toloza et al., 1994; Papazian et al., 1995). Gels were soaked in Fluoro-Hance (Research Products International, Mt. Prospect, IL) before drying and autoradiography. Autoradiograms were scanned and analyzed using a GS-700 scanning densitometer and Molecular Analyst v. 1.4 Software (BioRad, Hercules, CA). Relative amounts of mature and immature Shaker protein were quantified by peak integration analysis.

Channel activity was recorded in oocytes using a two-electrode voltage clamp (Warner Electronics, Hamden, CT) as previously described (Papazian et al., 1991; Santacruz-Toloza et al., 1994). Currents were recorded at room temperature in modified Barth's saline containing 1 mM KCl (Timpe et al., 1988). Leak currents were subtracted using the P/−4 protocol (Bezanilla and Armstrong, 1977).

Inactivation tagging

In inactivation-tagging experiments, subunits in an inactivating (wt) background were coexpressed with subunits in a noninactivating (IR) background at various ratios. To confirm the subunit ratios, the expression levels of Shaker-IR and K374E+E293K-wt subunits, which form active homotetramers, were titrated by current amplitude in each experiment. In coexpression experiments involving one inactive construct, RNAs were mixed in molar ratios. The concentration of RNA was determined by measuring absorption at 260 nm and confirmed by electrophoresis on denaturing agarose gels with an RNA ladder of known concentration (Gibco/BRL). Biochemical analysis indicated that comparable levels of total Shaker protein were produced after injecting equal amounts of RNA encoding Shaker-IR and the inactive single and double mutants, K374E-wt, K374E+D316K-wt, and E283R+R368E-wt (data not shown).

RESULTS

K374 in S4 interacts electrostatically with E293 in S2 and D316 in S3

We have previously shown that the mutation K374Q in the S4 segment eliminates Shaker protein maturation and activity (Papazian et al., 1991; Perozo et al., 1994). Maturation

and functional expression are efficiently restored in the double-mutant combinations K374Q+E293Q and K374Q+D316N, suggesting that in the native structure of the channel, K374 in S4 interacts electrostatically with E293 in S2 and D316 in S3 (Papazian et al., 1995). If the structural interactions between K374, E293, and D316 are indeed electrostatic in nature, then similar results might be expected when charge reversal mutations are used. To test this idea, the mutation K374E was made in a Shaker construct containing a deletion of amino acids 6 through 46 to remove N-type inactivation (IR) (Hoshi et al.,

1990), and expressed in *Xenopus* oocytes for biochemical and functional analysis.

The charge reversal mutation K374E eliminated both maturation (Fig. 2) and function (data not shown). To identify possible second site suppressors, K374E (in the IR background) was paired individually with complementary charge reversal mutations E283R and E293K in S2, and D310K and D316K in S3. After expression and metabolic labeling in *Xenopus* oocytes, maturation of the single and double mutants was analyzed electrophoretically on dena-
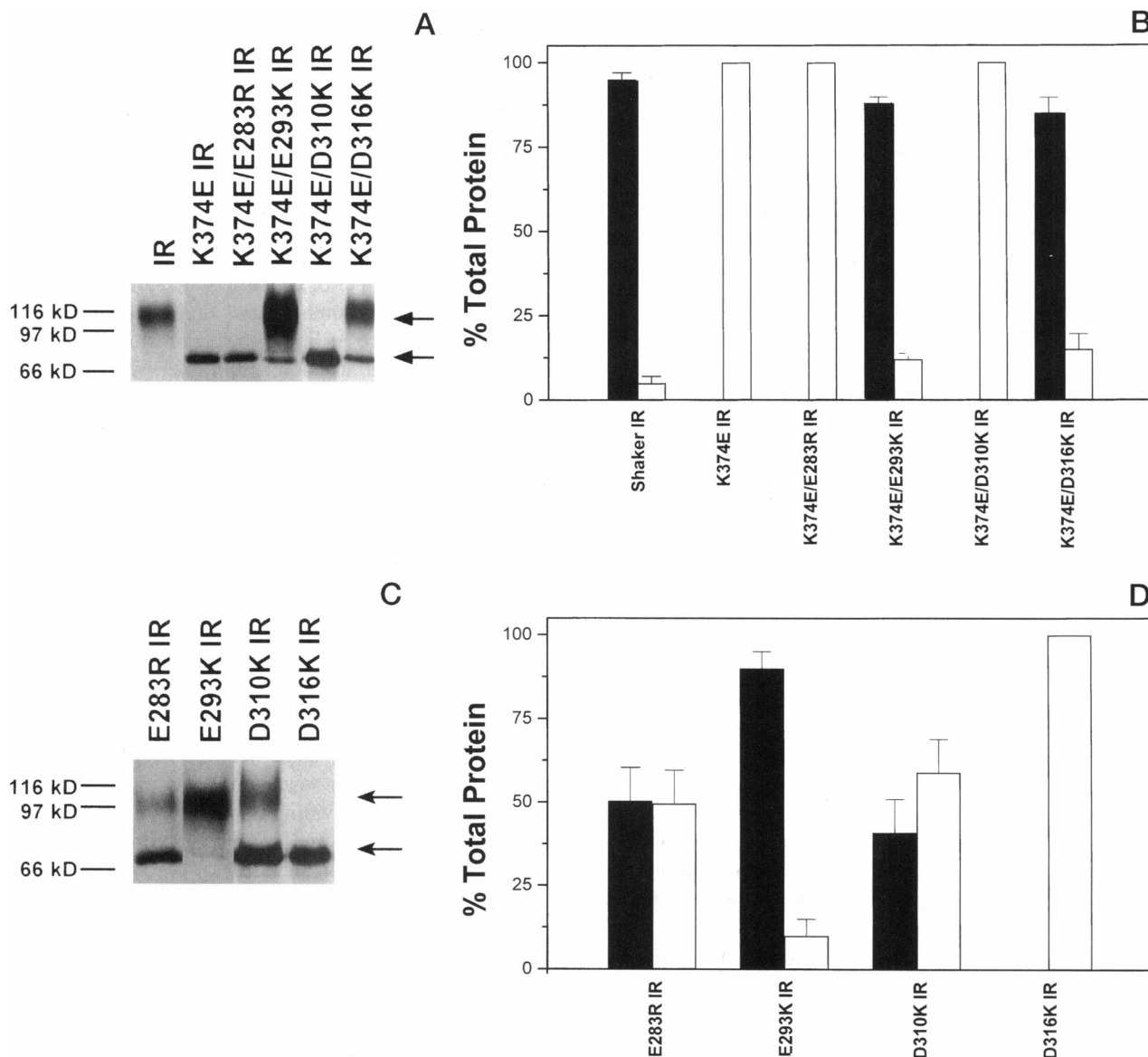

FIGURE 2 Rescue of K374E maturation in double mutant combinations. (A) K374E-IR was paired individually with E283R, E293K, D310K, and D316K. After expression and metabolic labeling in *Xenopus* oocytes for 44–48 h, the protein products of Shaker-IR, K374E-IR, and the double mutant combinations were isolated by immunoprecipitation and subjected to electrophoresis and autoradiography. The arrows mark the positions of mature (*upper*) and immature (*lower*) forms of the protein. A representative experiment is shown. (B) For the constructs shown in A, the percentage of Shaker protein that was in the mature (*filled bars*) or immature (*open bars*) form was quantified by densitometry and is shown as mean ± SD, n = 4–8. (C) Results of a representative experiment depict the protein products of the single acidic charge reversal mutations, E283R-IR, E293K-IR, D310K-IR, and D316K-IR. (D) For the constructs shown in C, the percentage of Shaker protein that was in the mature (*filled bars*) or immature (*open bars*) form was quantified by densitometry and is shown as mean ± SD, n = 4–8.

turing gels. Maturation was assessed by monitoring the change in mobility resulting from the Golgi-specific processing of the core-glycosylated precursor (Nagaya and Papazian, 1997). Fig. 2 shows the results of a representative experiment (A and C) and quantified data obtained from four to eight experiments (B and D). The double-mutant combinations K374E+E293K-IR and K374E+D316K-IR restored maturation of the Shaker protein to near-control levels (Fig. 2, A and B). These results confirm the electrostatic nature of the interactions of K374 in S4 with E293 in S2 and D316 in S3.

In contrast, charge reversal mutations of residues E283 and D310 were unable to rescue K374E, despite the proximity of positions 283 and 293, and positions 310 and 316 in the primary structure (Fig. 2, A and B). These results demonstrate that suppression of the maturation defect of K374E by E293K and D316K is specific.

The individual acidic charge reversal mutations affected maturation of the Shaker protein to different degrees (Fig. 2, C and D). Only E293K-IR matured at near-control levels. E283R-IR and D310K-IR reduced the extent of maturation significantly, whereas D316K-IR eliminated maturation. It is worth noting that neither D316K nor K374E matured, but the double-mutant combination matured efficiently (Fig. 2).

E283 in S2 interacts electrostatically with R368 and R371 in S4

As shown in Fig. 2, E283R reduced the extent of maturation by 50%. Therefore, we used it as a primary mutation and paired it with S4 charge reversal mutations to extend the intragenic suppression strategy to other positions (Fig. 3 A). Two double-mutant combinations, E283R+R368E-IR and E283R+R371E-IR, restored maturation to the level of the control: more than 95% of the protein was in the mature form at 48 h postinjection. In contrast, combination with any other S4 charge reversal mutation made the maturation defect of E283R-IR worse (Fig. 3 A). These results indicate that the rescue of E283R maturation by R368E and R371E is specific, suggesting that E283 in S2 experiences strong electrostatic interactions with R368 and R371 in S4. In addition, these data demonstrate that the strategy of directed intragenic suppression does not require complete blockade of maturation, extending the potential usefulness of this approach.

Individually, S4 charge reversal mutations had different effects on maturation (Fig. 3 B). Only K374E-IR and R377E-IR eliminated maturation entirely, whereas R362E-IR, R365E-IR, and R368E-IR matured to the same extent as Shaker-IR protein. R371E-IR caused a small decrease in the extent of maturation (Fig. 3 B). Similar results have been obtained with S4 charge neutralization mutations. Of these, only K374Q and R377Q eliminate maturation (Papazian et al., 1995). Thus the native structure appears to be particularly sensitive to alterations at positions 374 and 377, whereas mutations of other basic residues in the S4 segment are well tolerated.

Mutations block maturation rather than changing its time course

To test whether the time course of maturation is altered in double-mutant subunits, we determined the extent of maturation at different times after injection of oocytes (Fig. 4). Fig. 4 A compares the time course of maturation of E283R+R365E-IR and E283R+R368E-IR. In E283R+R368E-IR, mature protein was present at the earliest time point (6 h postinjection) and was the predominant form of the protein at 18 h and all subsequent time points. In addition, the amount of mature protein continued to increase until at least 48 h postinjection, which likely indicates both the stability of the mature protein and its continuing synthesis. In contrast, the E283R+R365E-IR construct produced only immature protein at all time points. The amount of immature protein appeared to decline with time postinjection, which suggests an increased degradation rate of protein retained in the ER. It should be noted that the time course data do not directly indicate the kinetics of maturation, but instead reflect continuing synthesis, maturation, and degradation of the Shaker protein.

Results for a number of constructs are summarized in Fig. 4 B. Double-mutant combinations that restored maturation followed a time course similar to that of Shaker-IR. Mature protein was detected as soon as 6 h after injection. At later times, the mature form constituted at least 80% of the total Shaker protein. In contrast, nonsuppressed mutant combinations showed little or no detectable mature protein between 6 and 60 h after injection. Later times were not examined, because of declining survival of oocytes and increasing degradation of the Shaker protein. These data indicate that maturation in these mutants is effectively blocked.

The mature form of the protein incorporates into active channels

In previous experiments using neutralization mutations, restoration of maturation was accompanied by the recovery of functional activity, providing compelling evidence that the mature form of the protein folds and assembles into a native channel structure (Papazian et al., 1995). Using charge reversal mutations, restoration of maturation might not be accompanied by recovery of functional activity, depending on the role of the original charged residues in the mechanism of voltage-dependent activation.

To determine whether double-mutant combinations that restore maturation formed active channels, the constructs were analyzed by using a two-electrode voltage clamp. Two double-mutant combinations, E283R+R371E-IR and K374E+E293K-IR, formed active, homotetrameric Shaker channels (Fig. 5 A). Thus the mature proteins formed by E283R+R371E-IR and K374E+E293K-IR fold and assemble properly. As is evident in Fig. 5, the double-mutant combination E283R+R371E-IR exhibited altered gating properties, which result from a shift in the voltage dependence

FIGURE 3 Rescue of E283R maturation in double mutant combinations. (A) E283R-IR was paired individually with S4 charge reversal mutations for biochemical analysis. The percentage of Shaker protein that was in the mature (*filled bars*) or immature (*open bars*) form was quantified by densitometry and is shown as mean ± SD, $n = 4-8$. (B) The single S4 charge reversal mutants were expressed for biochemical analysis. The percentage of Shaker protein that was in the mature (*filled bars*) or immature (*open bars*) form was quantified by densitometry and is shown as mean ± SD, $n = 4-8$.
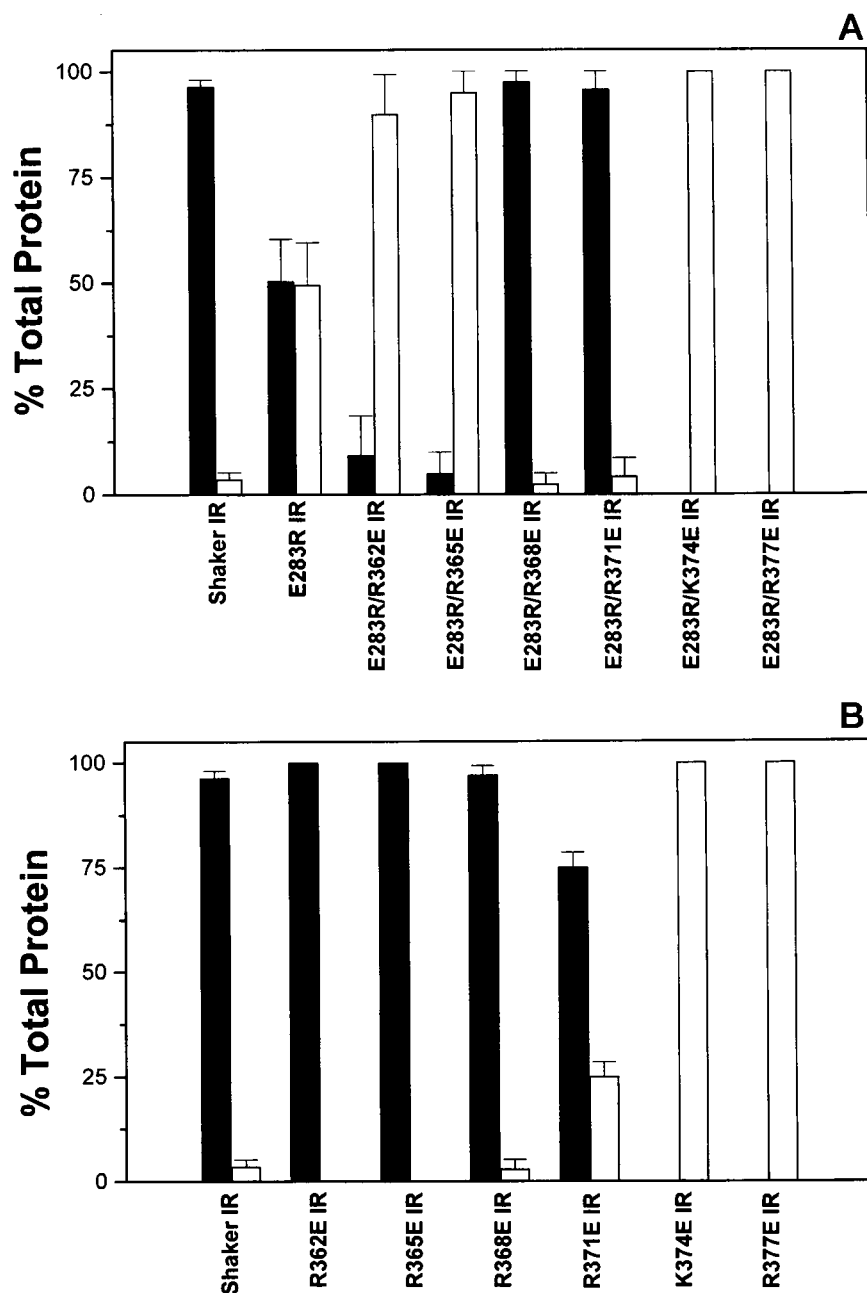
of activation to hyperpolarized potentials (S. K. Tiwari-Woodruff and D. M. Papazian, unpublished observations).

In contrast, E283R+R368E and K374E+D316K matured but did not form functional homotetrameric channels (data not shown). This result could indicate a defect in folding or assembly. Alternatively, these proteins may fold and assemble properly but produce channels that are unable to open. For instance, the mutations might shift activation to depolarized potentials beyond the range of our voltage clamp apparatus. To determine whether E283R+R368E and K374E+D316K subunits are capable of folding properly and incorporating into active channels, we applied an inactivation-tagging strategy (MacKinnon et al., 1993). Fast inactivation of wild-type Shaker channels is mediated by an amino-terminal inactivation domain (Fig. 1) (Hoshi et al., 1990; Zagotta et al., 1990). During fast, or N-type, inactivation, this domain blocks the internal mouth of the pore (Zagotta et al., 1990; Demo and Yellen, 1991). Mutations or deletions in the N-terminal domain remove inactivation (Hoshi et al., 1990; see Shaker-IR, Fig. 5 A). Only one inactivation particle is needed to inactivate the channel, despite the presence of four in the wild-type channel (MacKinnon et al., 1993). Therefore, the N-terminal inactivation domain can be used to tag a subunit and signal its incorporation with noninactivating (IR) subunits into active channels (MacKinnon et al., 1993). Upon coexpression of wild-type (wt) inactivating subunits and noninactivating (IR) subunits at a 1:1 ratio, an inactivating component of
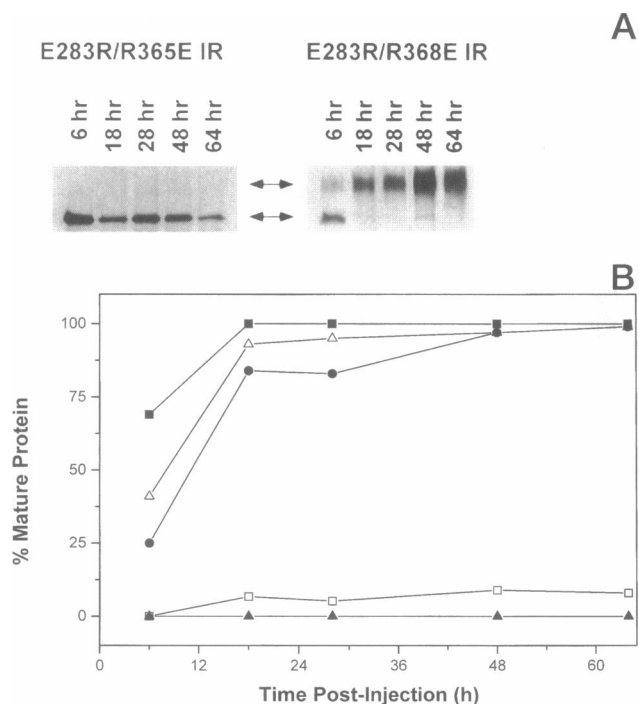
FIGURE 4   Time course of maturation of Shaker-IR and double mutant constructs. (A) The protein products of E283R+R365E-IR and E283R+R368E-IR were analyzed at 6, 18, 28, 48, and 64 h postinjection. Representative experiments are shown. (B) The percentage of protein in the mature form was quantified and compared at various times postinjection for Shaker-IR (■), E283R+R362E-IR (□), E283R+R365E-IR (▲), E283R+R368E-IR (△), and E283R+R371E-IR (●).

current larger than 50% of the peak current is readily detected, indicating inactivation of heterotetramers containing IR and wt subunits (Fig. 5 B).

We coinjected RNA for K374E+D316K-wt subunits, in the wild-type (inactivating) background, with Shaker-IR RNA in a 1:1 ratio. A prominent component of inactivating current was observed (Fig. 5 B). Because K374E+D316K subunits do not form functional homotetramers, the inactivating current must have arisen from the incorporation of K374E+D316K-wt subunits into heteromultimeric channels with IR subunits. Therefore, the K374E+D316K subunit can properly fold and assemble into active channels.

Upon coexpression of E283R+R368E-wt and IR subunits, a significant inactivating component of current was also observed, indicating that E283R+R368E subunits can incorporate into active channels (Fig. 5 B). In this case, the rate and extent of inactivation were reduced. Because inactivation is slower and less complete when fewer inactivation-tagged subunits are incorporated into the channel (MacKinnon et al., 1993), it is possible that the incorporation of more than one E283R+R368E subunit prevents channel opening. Alternatively, a shift in the voltage dependence of activation could account for the altered inactivation properties (Papazian et al., 1991).

The inactivating component of current seen in coinjection experiments was not due to free-floating inactivation parti-

cles cleaved from wt subunits. Upon coexpression of Shaker-IR and an excess of a truncated Shaker protein containing the complete N-terminal region through the S1 segment, no inactivation was observed (data not shown).

Electrostatic interactions occur within one subunit

Because Shaker channels contain four subunits (MacKinnon, 1991; Li et al., 1994; Schulteis et al., 1996), electrostatic interactions between charged residues in different transmembrane segments could occur between residues in the same subunit, stabilizing the tertiary structure of the channel, or between residues on different subunits, stabilizing the quaternary structure of the channel.

We used the inactivation-tagging strategy to determine whether E293K must be on the same or an adjacent subunit to rescue K374E. E293K-IR makes mature protein and forms functional channels, whereas K374E-IR eliminates both maturation and function (Fig. 2; data not shown). The double-mutant combination K374E+E293K-IR restores maturation and function (Figs. 2 and 5). Upon coinjection of RNAs for E293K-IR and K374E-wt at a 9:1 ratio, no inactivating component of current was observed (Fig. 6 A, top). In contrast, when IR and the double-mutant K374E+E293K-wt were coexpressed at a 9:1 ratio, a prominent component of inactivating current was seen (Fig. 6 A, middle). In this experiment, an excess of IR subunits was used to limit the number of K374E+E293K-wt homotetramers, which are functional (Fig. 5 A). If the IR and double-mutant subunits are produced in the expected ratio and associate randomly, only 0.01% of the channels in the coinjection experiment would be double-mutant homotetramers. In contrast, if heterotetramers between IR and K374E+E293K-wt subunits are formed after coexpression at a 9:1 ratio, 34% of the channels would be expected to inactivate because of the presence of one or more inactivation-tagged subunits. Upon coexpression of IR and K374E+E293K-wt subunits at a 9:1 ratio, the inactivating component was approximately 31% of the peak current, measured at +80 mV (Fig. 6 A). As a control, the same amount of double-mutant RNA was injected in the absence of IR RNA (Fig. 6 A, bottom). The amplitude of the inactivating component was too small to account for the inactivation detected after coexpression with IR (compare middle and bottom panels). These results indicate that the inactivating component seen upon coexpression of the double mutant and Shaker-IR is primarily due to coassembled heterotetramers of K374E+E293K-wt and IR subunits, rather than separate populations of double-mutant and IR homotetramers.

Coexpression of Shaker-IR and K374E+E293K-wt subunits at various ratios resulted in an inactivating component of current (Fig. 6 B, filled squares) consistent with that predicted by assuming a random association of IR and double mutant subunits (Fig. 6 B, solid line). In contrast, in most experiments, coinjection of E293K-IR and K374E-wt subunits did not result in any detectable inactivating compo-
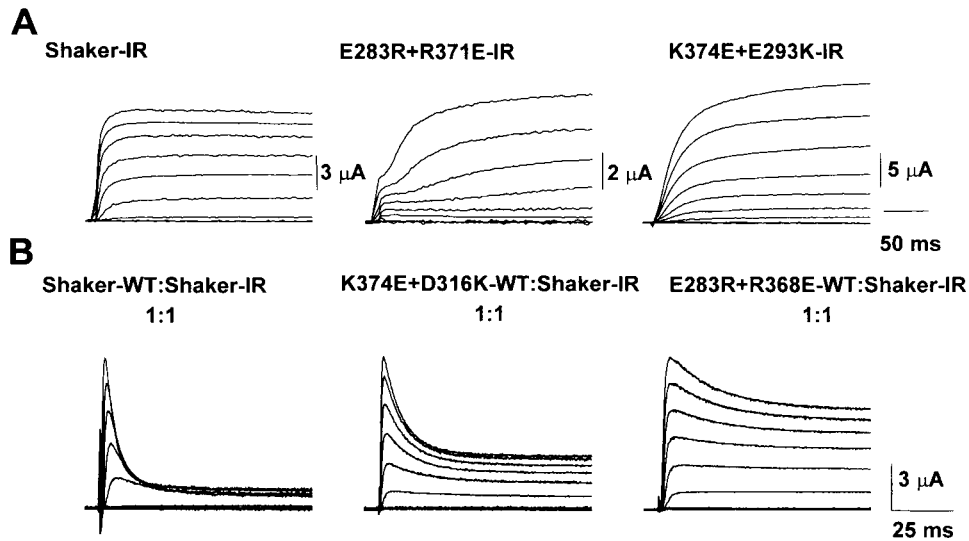
FIGURE 5   Incorporation of rescued subunits into functional channels. (A) Shaker-IR, E283R+R371E-IR, and K374E+E293K-IR were expressed individually in *Xenopus* oocytes for voltage clamp analysis. Currents, recorded between 24 and 48 h after RNA injection, were evoked by stepping from a holding potential of −100 mV to potentials ranging from +60 to −80 mV in −20-mV increments for 250 ms. (B) Shaker-wt, K374E+D316K-wt, and E283R+R368E-wt were individually coexpressed at a 1:1 ratio with Shaker-IR in oocytes. Incorporation of double mutant subunits into active channels was detected by the presence of an inactivating component of current. Currents were evoked by stepping from a holding potential of −80 mV to potentials ranging from −60 to +80 mV in 20-mV increments for 100 ms.

nent of current (Fig. 6 B, *open circles*). In one experiment, a small amount of inactivation was seen, but this was much less than that predicted by random association of subunits.

In Fig. 6, A and B, the inactivating component was observed after coexpression of Shaker-IR and K374E+E293K-wt, but not after coexpression of E293K-IR and K374E-wt at comparable current amplitudes. Therefore, the inactivating component of current seen upon coexpression of Shaker-IR and K374E+E293K-wt subunits cannot be attributed to potassium accumulation in a restricted extracellular space.

Our results demonstrate that E293K must be on the same subunit to rescue K374E. Its presence on a separate subunit is not sufficient. This suggests that the electrostatic interaction between K374 and E293 occurs within one subunit.

We also investigated whether D316K must be on the same subunit to rescue K374E. In this case, rescue could be assessed by measuring maturation, because each mutation alone prevents maturation (Fig. 2). Separate RNAs encoding K374E and D316K were coinjected into oocytes. In contrast to the double-mutant combination, K374E+D316K, coexpression of K374E and D316K on separate subunits failed to restore maturation (Fig. 6 C). Therefore, K374E and D316K must be in the same subunit to restore maturation.

DISCUSSION

Establishment of electrostatic interactions between transmembrane segments mediates the folding of Shaker subunits

We have used a strategy of directed intragenic suppression to infer the existence of strong electrostatic interactions

between transmembrane charged residues in Shaker K^+ channels. Our results indicate that the establishment of specific electrostatic interactions between transmembrane segments S2, S3, and S4 is required for the proper folding of Shaker subunits. Charge reversal mutations that reduce or prevent maturation of the Shaker protein were rescued by combination with specific second site suppressor mutations. Restoration of maturation reestablished a native structure, as demonstrated by the incorporation of rescued subunits into functional channels. Our results indicate that electrostatic interactions exist between E283 in S2 and R368 and R371 in S4, and between K374 in S4 and E293 in S2 and D316 in S3 (Fig. 7 A). Furthermore, we have demonstrated that the suppressor mutation E293K must be present in the same subunit to rescue K374E efficiently. Similarly, D316K and K374E must be in the same subunit to restore maturation. These results are consistent with the idea that interactions between K374, E293, and D316 affect the tertiary rather than the quaternary structure of the channel.

The rescue of the charge reversal mutations E283R and K374E by opposite charge reversals indicates that the inferred interactions are electrostatic, consisting of charge-charge interactions or polarized hydrogen bonds. The specific rescue of K374E by E293K or D316K but not by E283R is compatible with previous results obtained using charge neutralization mutations. Like K374E, K374Q eliminates maturation and function; these defects are efficiently and specifically suppressed by E293Q or D316N but not by E283Q (Papazian et al., 1995). In rescued constructs containing two polar amino acids, neutral hydrogen bonds would presumably replace the more polarized interactions that existed between the original ionizable residues.
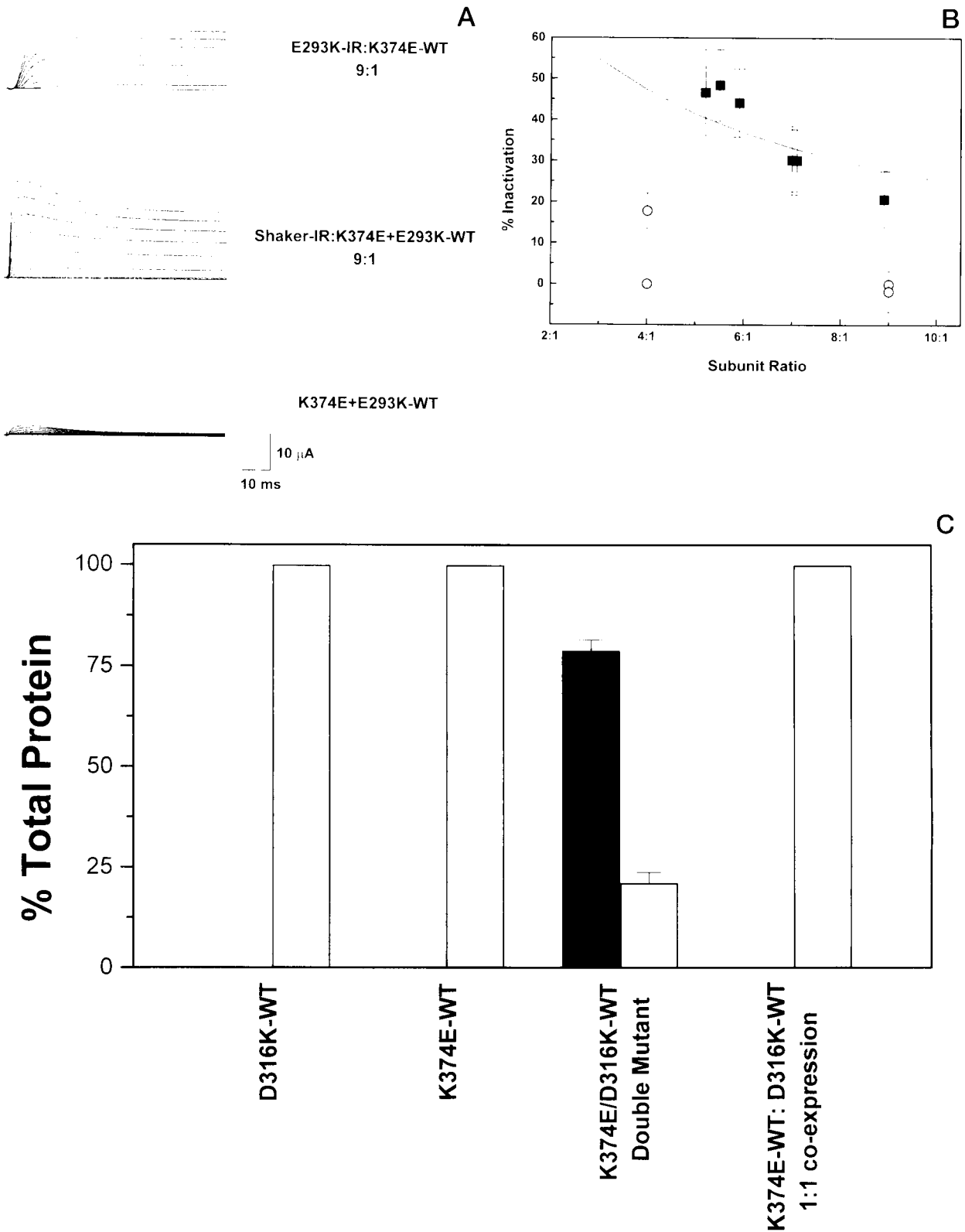
FIGURE 6   Rescue of K374E by mutations in the same subunit. (A) *Top*: E293K-IR and K374E-wt RNAs were expressed at a 9:1 ratio and analyzed using a two-electrode voltage clamp. No inactivating component of current was observed. *Middle*: Shaker-IR and K374E+E293K-wt subunits were expressed at a 9:1 ratio. A prominent inactivating component of current was observed. *Bottom*: The same amount of K374E+E293K-wt RNA as in the middle panel was injected in the absence of IR RNA. Currents were recorded during 100-ms steps from a holding potential of −80 mV to potentials between −60 and +80 mV in 20-mV increments. A representative experiment is shown. Peak current amplitudes at +80 mV were 24 µA (*top*), 27 µA (*middle*), and 2.7 µA (*bottom*). (B) Shaker-IR and K374E+E293K-wt subunits (■) or E293K-IR and K374E-wt subunits (○) were expressed at ratios ranging from 4:1 to

Taken together, the results with charge reversal and neutralization mutations suggest that the Shaker protein can tolerate interactions ranging from ionic to neutral at these positions.

We have previously shown that the charge neutralization mutation K374Q can be partially and inefficiently suppressed in combination with E283Q in S2 (Papazian et al., 1995). One interpretation of this result is that K374 and E283 experience a weak, presumably long-range, electrostatic interaction in the Shaker channel (Papazian et al., 1995). Using charge reversal mutations, we detected no maturation in the double mutant combination, K374E+E283R-IR (Fig. 2, A and B). Therefore, no long-range interaction was detected by using charge reversal mutations, perhaps because these are individually more detrimental to the protein than charge neutralization mutations.

Although extramembrane regions help to specify the structure of a membrane protein, evidence is accumulating that interactions between transmembrane segments are also important (Lemmon and Engelman, 1994). Such interactions include the tight packing of side chains, which is required for the dimerization of glycophorin (Lemmon et al., 1992a,b, 1994), and electrostatic interactions between ionizable side chains, which are formed during the assembly of the T-cell receptor (Cosson et al., 1991). In addition to these quaternary interactions, our data suggest that ionic interactions between transmembrane segments can help to specify the tertiary structure of a membrane protein.

Electrostatic interactions have been studied primarily in soluble proteins. Many soluble proteins contain buried or partially buried charged groups that interact electrostatically (Hendsch and Tidor, 1994; Nakamura, 1996). The contribution of such interactions to stability has been estimated by comparing the wild-type protein to one containing neutral or hydrophobic amino acid substitutions. Although a buried or partially buried ionic interaction may be somewhat more favorable energetically than a neutral hydrogen bond, such ionic interactions may actually be destabilizing compared to a protein containing hydrophobic substitutions that fill the same volume (Fersht, 1972; Anderson et al., 1990; Waldburger et al., 1995; Hendsch et al., 1996). Because electrostatic interactions may contribute less to protein stability than previously thought (Waldburger et al., 1995; Meeker et al., 1996; Nakamura, 1996), alternative roles in protein folding have been proposed (Hendsch and Tidor, 1994; Oliveberg and Fersht, 1996; Tissot et al., 1996). Thus electrostatic interactions may contribute to the specificity and kinetics of folding, either by limiting the number of possible native folds or by preventing the kinetic trapping of a
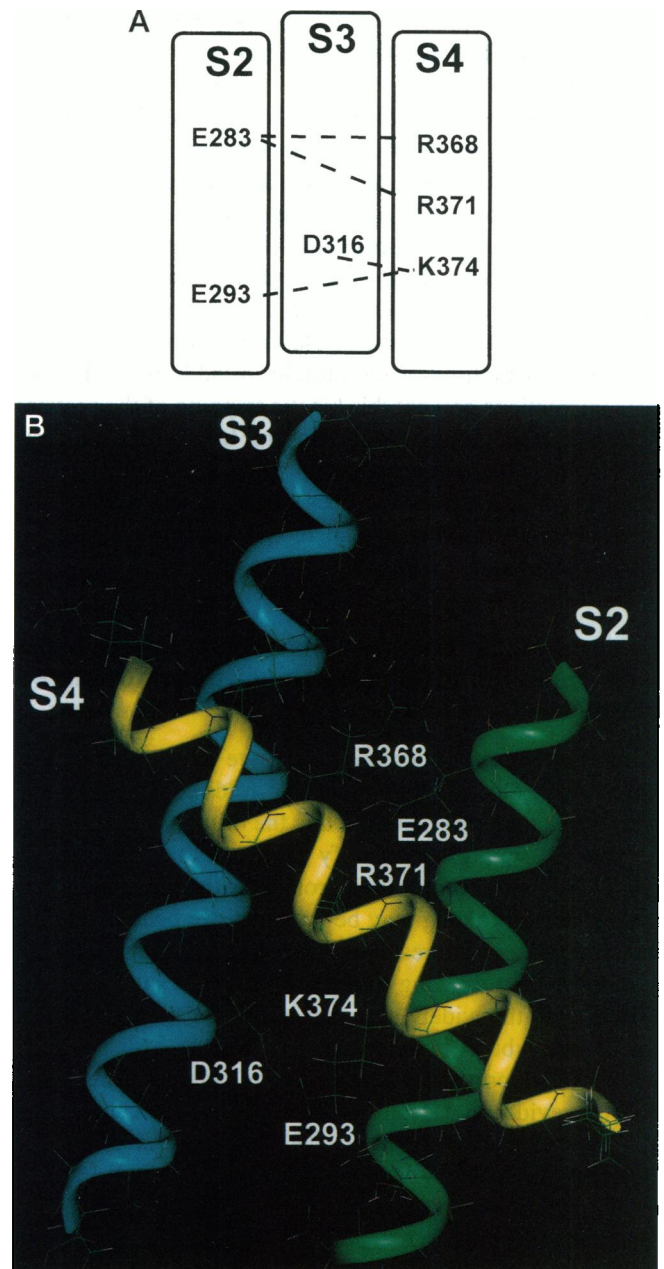

FIGURE 7 Model of electrostatic interactions between charged residues in segments S2, S3, and S4. (A) A schematic diagram summarizing the results of the suppression experiments is shown. The data suggest that K374 in S4 interacts closely with E293 in S2 and D316 in S3, and that E283 in S2 interacts closely with R368 and R371 in S4. (B) One possible packing arrangement of S2, S3, and S4 that accommodates the interactions inferred from suppression analysis. In this model we assumed that S2, S3, and S4 are α-helical, side chains are fully extended, and standard ψ and φ angles apply. Only the side chains of ionizable residues are shown. The model was generated using the program Insight II (Biosym/Molecular Simulations) on a Silicon Graphics computer.

9:1, and the percentage of inactivating current relative to the total current, measured at +40 mV, was determined. Data points represent the mean ± SD, $n = 3$–20. If no error bars are shown, the SD was smaller than the size of the symbol. The solid line, which indicates the amount of inactivation expected if subunits in the IR and wt backgrounds associate randomly, was calculated assuming that subunits distribute binomially among channels at all expression levels, that only one wt subunit is needed to inactivate the channel (MacKinnon et al., 1993), and that the steady-state current is 20% of the peak current amplitude in wt Shaker channels at +40 mV (Papazian et al., 1991). (C) The extent of maturation was determined after expression of the mutants D316K-wt, K374E-wt, D316K + K374E-wt, and after coexpression of separate RNAs for D316K-wt and K374E-wt in a 1:1 ratio. Results were quantified by densitometry as described under Fig. 2. Data points represent the mean ± SD, $n = 2$.

protein in an unproductive conformation during folding (Hendsch and Tidor, 1994; Oliveberg and Fersht, 1996; Tissot et al., 1996).

Our results argue that electrostatic interactions contribute significantly to the kinetics and specificity of folding of a membrane protein. We have shown that Shaker mutants that mature do so over the same time course as the control, whereas nonsuppressed constructs do not mature over a long period of time. This suggests that nonmaturing mutations trap the protein in misfolded, unproductive conformations that do not unfold and refold to the native state within the expected lifetime of the protein. In addition, only specific interactions prevent this kinetic trapping of the protein: K374E is rescued by D316K, but not by D310K, six residues away in the sequence. In this view, a mutation such as E283R, which reduces but does not eliminate maturation, would divert some but not all molecules from productive folding pathways.

Electrostatic interactions between charged residues in S2, S3, and S4 stabilize the structure of Shaker K+ channels

In interpreting the suppression data, we have assumed that interactions that are important for folding persist in the native structure, and therefore that the primary mutation and its specific suppressor identify positions that are in close proximity in the fully folded protein. Although some interactions during folding may be transient, we believe that the interactions between residues in S2, S3, and S4 are likely to persist for the following reasons. First, our results are compatible with the widely accepted model for the topology of the Shaker subunit in the membrane (Fig. 1). This topology model is supported by a significant body of direct and indirect evidence (Hoshi et al., 1990; MacKinnon and Yellen, 1990; Zagotta et al., 1990; Isacoff et al., 1991; Yellen et al., 1991; Santacruz-Toloza et al., 1994; Larsson et al., 1996; Manuzzu et al., 1996). We have identified interacting residues in two clusters. Residues in one cluster, E283, R368, and R371, are closer to the extracellular side of the membrane according to the topology model, whereas residues in the other cluster, E293, D316, and K374, are closer to the cytoplasmic side. Second, rescued double mutants have effects on the properties of the ionic current that are consistent with the persistence of these interactions in the native structure (Papazian et al., 1995). Third, evidence has been presented that pairs of residues identified by intragenic suppression are in close proximity (Sahin-Toth and Kaback, 1993; Liu et al., 1995). For instance, intragenic suppression identified likely charge pairs between transmembrane segments in the lactose permease, a membrane transport protein from *Escherichia coli* (King et al., 1991; Lee et al., 1992). The proximity of the interacting residues in the native state has since been confirmed (Sahin-Toth and Kaback, 1993). Finally, Peled-Zehavi et al. (1996) have shown that peptides corresponding to S2, S3, and S4 specifically and stably associate with one another in bilayers.

Maturation provides a consistent and reliable indication that the protein is in a native conformation (Fig. 5; Papazian et al., 1995) and therefore can be used as an assay for proper folding and oligomerization. Channels containing rescued subunits differ in their functional properties from Shaker-IR, however (unpublished results). This implies that their detailed structures may also differ. Voltage-dependent ion channels are not expected to have a single native structure, because biophysical analysis indicates the existence of a number of functionally important conformations (Bezanilla et al., 1994; Sigworth, 1994; Zagotta et al., 1994). The distribution of channel molecules among these states is controlled by voltage. In general, the altered functional properties of mutant channels can be accounted for by changes in the relative stabilities of conformational states or in the energetic barriers separating them (Schoppa et al., 1992). Thus Shaker-IR and rescued subunits may exist in different native conformations in cells at rest.

Structure and function of the voltage-sensing domain in Shaker K+ channels

The charged residues that participate in electrostatic interactions in the Shaker protein are important not only for folding but also for function. Voltage-dependent activation of one channel is accompanied by the transfer of a large amount of gating charge, equivalent to about 13 e_0, across the transmembrane electric field (Schoppa et al., 1992; Aggarwal and MacKinnon, 1996; Seoh et al., 1996). A significant portion of this charge is carried by E293 in S2, and R365, R368, and R371 in S4, possibly with a smaller contribution from D316 in S3 (Aggarwal and MacKinnon, 1996; Seoh et al., 1996). Therefore, structural interactions between S2, S3, and S4 are of particular interest because these segments contribute to the channel's voltage-sensing domain. Because we have shown that some of the interactions between these segments occur within the tertiary structure, our results imply that each subunit has its own voltage sensor, which is often assumed in kinetic models of voltage-dependent activation (Bezanilla et al., 1994; Sigworth, 1994; Zagotta et al., 1994).

A number of models for the physical mechanism of activation have been proposed to account for the large gating charge movement that accompanies activation. Early models invoked large-scale movements of the S4 backbone (Catterall, 1986; Durell and Guy, 1992), which in some proposals were accompanied by changes in the secondary structure of the segment (Guy and Conti, 1990). Recent experimental evidence indicates that charged residues involved in gating move across a significant fraction of the transmembrane electric field (Perozo et al., 1994; Aggarwal and MacKinnon, 1996; Larsson et al., 1996; Seoh et al., 1996; Yang et al., 1996). In fact, some positions are exposed on opposite sides of the membrane, depending on the voltage (Yang et al., 1996). These observations suggest alternative models of activation in which the voltage sensor is

exposed to solvent-filled spaces that intrude into the transmembrane region of the protein and serve to focus the electric field across a distance that is less than the thickness of the bilayer (Goldstein, 1996; Seoh et al., 1996; Yang et al., 1996). One estimate suggests that this distance may be less than 11 Å in voltage-dependent Na^+ channels (Yang et al., 1996). By this means, the gating charge could move a significant fraction or all of the way across the field with much smaller conformational changes, which might involve rearrangements of long side chains without requiring large-scale movements of the backbones of transmembrane segments (Goldstein, 1996; Seoh et al., 1996; Yang et al., 1996).

We find that positions that make the most significant contributions to the gating charge (E293, R365, R368, and R371) are also the most tolerant of mutations, including charge reversal mutations. This correlation is consistent with the proposal that these residues are exposed to solvent, at least in some conformations. Evidence that position 362 is exposed to solvent has been presented previously (Manuzzu et al., 1996), and we find that the mutation R362E does not eliminate either maturation (Fig. 3) or function (data not shown). In contrast, charge reversal mutations at E283, D316, and K374, residues that contribute less (or not at all) to the gating charge, are significantly more detrimental to protein maturation. These residues may play a role that is more important structurally than functionally, and may be less exposed to solvent or have less conformational flexibility than those that carry gating charge.

Our suppression data lead to preliminary models for the packing of segments S2, S3, and S4 that provide a basis for the intrusion of solvent into the transmembrane region. Fig. 7 *B* shows one of several possible packing arrangements for S2, S3, and S4 that are consistent with the interactions inferred from the suppression data. This model assumes that S2, S3, and S4 are α-helical, that the side chains are fully extended, and that all of the interactions can be accommodated concurrently without large-scale backbone movements. The significant feature common to packing arrangements that fit the data and these assumptions is that S4 is tilted relative to S2 and S3. Tilting the S4 segment could provide space for solvent intrusion near the voltage sensor. It should be noted that our data do not constrain the positions of S2 and S3 relative to one another. For instance, packing models in which S3 is to the right of S2 in a view analogous to that of Fig. 7 *B* can also accommodate the interactions (not shown). In addition, the angle at which S4 is tilted can be reduced if it is not assumed that the side chains are fully extended. It is encouraging, however, that a very divergent approach has also led to the proposal that the S4 segment is tilted. Spectroscopic studies of peptides corresponding to S2, S3, and S4 have demonstrated that not only do they interact specifically with each other in lipid bilayers, they also assume helical conformations with S4 tilted at a significant angle compared to S2 and S3 (Peled-Zehavi et al., 1996). Further experimentation will be needed

to determine the significance of this coincidence. With this sort of structural arrangement, both changes in the degree of tilt between the helices and movements of the ionized side chains could contribute to the physical mechanism of voltage-dependent activation.

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