

## Influence of C-Terminal Protein Domains and Protein–Lipid Interactions on Tetramerization and Stability of the Potassium Channel KcsA<sup>†</sup>

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**ABSTRACT:** KcsA is a prokaryotic potassium channel formed by the assembly of four identical subunits around a central aqueous pore. Although the high-resolution X-ray structure of the transmembrane portion of KcsA is known [Doyle, D. A., Morais, C. J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) *Science* 280, 69–77], the identification of the molecular determinant(s) involved in promoting subunit tetramerization remains to be determined. Here, C-terminal deletion channel mutants, KcsA  $\Delta$ 125–160 and  $\Delta$ 120–160, as well as 1–125 KcsA obtained from chymotrypsin cleavage of full-length 1–160 KcsA, have been used to evaluate the role of the C-terminal segment on the stability and tetrameric assembly of the channel protein. We found that the lack of the cytoplasmic C-terminal domain of KcsA, and most critically the 120–124 sequence stretch, impairs tetrameric assembly of channel subunits in a heterologous *E. coli* expression system. Molecular modeling of KcsA predicts that, indeed, such sequence stretch provides intersubunit interaction sites by hydrogen bonding to amino acid residues in N- and C-terminal segments of adjacent subunits. However, once the KcsA tetramer is assembled, its remarkable in vitro stability to detergent or to heat-induced dissociation into subunits is not greatly influenced by whether the entire C-terminal domain continues being part of the protein. Finally and most interestingly, it is observed that, even in the absence of the C-terminal domain involved in tetramerization, reconstitution into membrane lipids promotes in vitro KcsA tetramerization very efficiently, an event which is likely mediated by allowing proper hydrophobic interactions involving intramembrane protein domains.

Potassium channels are highly complex membrane proteins widely distributed in different cell types of practically all living organisms, where they contribute to the control of potassium flow, cell volume, release of hormones and neurotransmitters, resting potential, excitability, and behavior (1). Back in 1995, a rather simple member of the potassium channel family, the so-called KcsA,<sup>1</sup> was identified in *Streptomyces lividans* (2). The ease of heterologous expression of KcsA in an *Escherichia coli* system, its resistance to a variety of detergents, and its purification in large quantities as His tag derivatives have allowed remarkable achievements in this field such as its crystallization and structural determination at high resolution using X-ray diffraction methods (3). KcsA can also be reconstituted into lipid bilayers, allowing for its functional characterization as a voltage- and

pH-dependent potassium channel (2, 4–8). Thus, the possibility of securing both high-resolution structural and functional data on this molecule makes KcsA an obliged reference model in ion channel studies.

KcsA is a homotetramer in which each subunit is made up of 160 amino acids (Figure 1) defining two transmembrane segments connected by a pore region that contains an ion selectivity filter unmistakably homologous to the more complex eukaryotic potassium channels. The transmembrane segment M2, nearest to the C-terminal, contributes to the lining of the pore, while the one closest to the N-terminal, M1, is exposed to the membrane bilayer (3). Additionally, the N- and C-termini are included, respectively, in two relatively large cytoplasmic domains, rich in charged or polar amino acid residues. These latter domains were not resolved in the crystal structure, which accounted only for the membrane-inserted 23–119 amino acids in the KcsA sequence (3). Nevertheless, electron spin resonance studies provided evidence that these protein domains form a fenestrated “hanging basket”-like structure underneath the membrane (6), through the openings of which ions must pass prior to their arrival to the transmembrane channel pore.

In an in vitro transcription and translation *E. coli* system, KcsA monomers have been reported to be targeted cotranslationally via the signal recognition particle pathway to the internal bacterial membrane (9), where the assembly of the

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<sup>1</sup> Abbreviations: KcsA, potassium channel from *Streptomyces lividans*; DDM, dodecyl  $\beta$ -D-maltoside; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; T1, N-terminal or first tetramerization domain from the eukaryotic voltage-activated *Shaker* potassium channel.

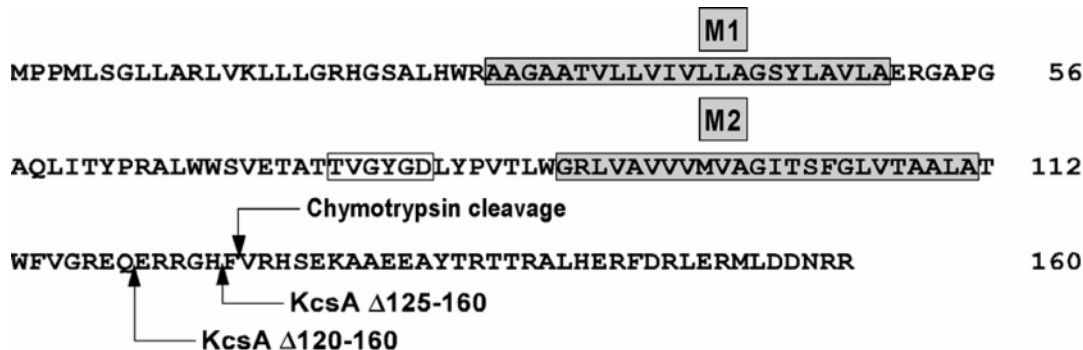


FIGURE 1: Amino acid sequence of the KcsA potassium channel from *S. lividans* (SWISS-PROT accession number Q54397). The boxes indicate the transmembrane segments M1 and M2 and the characteristic selectivity filter (TVGYGD). The arrows indicate the chymotrypsin cleavage site yielding 1–125 KcsA and the resulting C-termini in the KcsA Δ125–160 and Δ120–160 deletions. A hexahistidine tag (sequence MRGSHHHHHHGS) was bound to the methionine N-terminus in all cases.

membrane-inserted monomers into highly stable tetramers proceeds efficiently and rapidly, through a process influenced by the membrane proton-motive force (10). Nonetheless, the molecular determinant(s) involved in promoting subunit tetramerization remains to be identified. Using a heterologous *E. coli* expression system, we now report that KcsA deletion mutants in which part of the cytoplasmic C-terminal is lacking assemble poorly into tetramers. However, we also found that in those C-terminal deletions unable to assemble as tetramers, the expressed monomeric subunits tetramerize very efficiently upon reconstitution into artificial lipid vesicles. This latter observation lends support to a role for the lipid environment in contributing to the assembly of oligomeric membrane proteins and reinforces the idea that membrane lipids and protein–lipid interactions could be important determinants in membrane protein folding (11).

## MATERIALS AND METHODS

**Constructs.** The wild-type KcsA construct was a gift from Drs. Marco Caprini and Antonio Ferrer-Montiel. It contained the *kcsA* gene of *S. lividans* cloned in frame into the pQE30 vector (Qiagen), which provided ampicillin resistance and an N-terminal hexahistidine tag. The KcsA Δ125–160 and Δ120–160 constructs were generated from the wild-type construct by reverse PCR using *Pfu* DNA polymerase (Fermentas), sense primer 5′-CGG TGA GGA TCC ATC CGC ATG CGA GCT CGG TAC CCC for both deletions, and antisense primers 5′-GCG GAT GGA TCC TCA GTG GCC CCG GCG CTC TTG TTC and 5′-GCG GAT GGA TCC TCA TGG TTC CCG GCC GAC GAA CCA, respectively, for the KcsA Δ125–160 and Δ120–160 deletions. These primers include the restriction sequence for *Bam*HI (5′-GGA TCC) to allow for the elimination of the desired C-termini. PCR products were precipitated by standard phenol/chloroform, digested with *Bam*HI (Fermentas), purified, and ligated overnight by T4 DNA ligase (Roche Diagnostics). *E. coli* XL1-blue strain cells were transformed and plated on LB agar containing ampicillin (50 μg/mL). Screening of 12 colonies was carried out for each ligation by extracting the plasmid and digesting it with *Bam*HI to check if the deletions had been introduced successfully. Also, the entire plasmid from positive colonies was digested with *Pvu*II (Fermentas), whose restriction sequence is present in the constructs at a single position, and run in a 10% PAGE in Tris–acetate buffer to check for the correct size of the

deletions. The constructs were also checked by dideoxynucleotide sequencing.

**Protein Expression and Purification.** A modification of published procedures (2, 12) was used here. Kanamycin-resistant *E. coli* M15 (pRep4) cells were transformed with the above constructs following standard heat-shock procedures and plated overnight on LB agar containing ampicillin (50 μg/mL) and kanamycin (25 μg/mL). A single colony was picked up and grown overnight in 100 mL of LB medium supplemented with antibiotics. This culture was diluted into 1 L of 2xYT medium supplemented with antibiotics and grown at 30 °C to exponential phase ( $OD_{600nm} = 0.6–0.8$ ). Protein expression was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside for 2 h. Cells were pelleted, washed with 1% NaCl, resuspended in 50 mL of lysis buffer (20 mM HEPES, pH 7.5, 0.45 M sucrose, 8 mM EDTA, 0.4 mg/mL lysozyme, and 0.75 mM PMSF), and kept on ice for 1 h. The mixture was sonicated on an ice bath using a Branson probe-type apparatus and centrifuged for 1 h at 100000g. Membrane proteins in this crude membrane pellet were solubilized in 20 mL of 20 mM HEPES, pH 7.5, 100 mM KCl, 10 mM imidazole, 0.75 mM PMSF, and 10 mM *n*-dodecyl β-D-maltoside (DDM; Calbiochem) for 1 h at room temperature. After centrifugation of insoluble remains (1 h at 100000g), the supernatant was incubated overnight with 3.5 mL of Ni<sup>2+</sup>-NTA agarose gel beads (Qiagen), placed into a column, and washed with 20 mM HEPES, pH 7.5, 100 mM KCl, 10 mM imidazole, and 1 mM DDM, until the absorbance at 280 nm was less than 0.01. The gel-bound protein was eluted using the previous buffer containing 1 M imidazole. Protein aliquots were mixed with an equal volume of electrophoresis sample buffer (20 mM Tris, pH 6.8, 20% glycerol, 0.1% bromophenol blue, and 4% SDS) and run in 13.5% PAGE in the presence of 0.1% SDS (13). Alternatively, the sample was dialyzed in buffer without imidazole and the protein concentration determined by the DC-Protein assay (Bio-Rad), relative to a bovine serum albumin standard. Yields were 2–4 mg of DDM-solubilized purified protein/L of culture for the wild-type KcsA construct and 0.9–1.0 and 0.5–0.6 mg/L, respectively, for the KcsA Δ125–160 and Δ120–160 deletions.

**Chymotrypsin Hydrolysis.** The entire 1–160 KcsA was converted into 1–125 KcsA by treatment with chymotrypsin type II (Sigma) for 3 h at 37 °C, using a protein subunit:

enzyme ratio of 50:1 (by weight). The reaction was stopped by addition of 5 mM PMSF. Specific removal of the 126–160 protein segment was demonstrated by MALDI tryptic-peptide mass fingerprinting in excised electrophoresis bands from entire 1–160 and hydrolyzed 1–125 KcsA preparations (14, 15).

**Reconstitution of Proteins into Asolectin Lipid Vesicles.** Large unilamellar vesicles of asolectin (soybean lipids, type II-S, Sigma) were prepared (16) at 25 mg/mL in 10 mM HEPES, pH 7.5, 100 mM KCl (reconstitution buffer) and stored in liquid N<sub>2</sub>.

Purified DDM-solubilized protein was mixed with the above asolectin vesicles previously resolubilized in 5 mM DDM, at a lipid:protein subunit molar ratio of 2000:1, for 2 h. Reconstituted liposomes were formed by removing the detergent by gel filtration on Sephadex G-50 (fine, 15–20 mL bed volume), previously swollen overnight in buffer without detergent (7). The detergent-solubilized lipid/protein mixture (2 mL) was loaded on top of the column, and the reconstituted liposomes were eluted in the void volume at 2 mL/min. The protein-containing reconstituted fractions were pooled and centrifuged for 30 min at 300000g. The pellet was resuspended into 1 mL of reconstitution buffer, divided into 100  $\mu$ L aliquots, and stored in liquid N<sub>2</sub>.

**Thermal Stability of KcsA.** Routinely, aliquots of DDM-solubilized or asolectin-reconstituted KcsA samples were mixed with an equal volume of SDS-containing electrophoresis sample buffer (see above), incubated for 20 min at selected temperatures, cooled on ice for approximately 10 min, and examined by SDS-PAGE. After Coomassie Brilliant Blue staining, the gels were scanned and the intensities of the bands measured by densitometry.

**Molecular Modeling.** A full-length KcsA structural model was obtained with the commercial program Insight II (Biosym/MSI) using the  $\alpha$ -carbon trace (PDB entry 1F6G) from Cortes et al. (6) as the template. The crystallographic structure determined for 23–119 KcsA (PDB entry 1BL8) was then superimposed and transplanted to the model to get the correct dihedral angles and side chain rotamers within the transmembrane portion of the protein (residues L24 to V115). The orientation and optimization of the side chains corresponding to the cytoplasmic N- and C-termini was carried out in three steps: First, those residues making van der Waals clashes were selected and fitted with “quick and dirty” algorithms. Second, the model was energy minimized (100 steps of steepest descent and 100 of conjugate gradient, cutoff of 10 Å for nonbonded interactions) with the GROMOS 43A1 force field, implemented in Swiss PDB viewer v3.7 (17), to relax the backbone and side chains. Third, final side chain orientations were found with the computer-designed algorithm PERLA (described in detail at <http://proteindesign.embl.de>) as described previously (18–20). The model was tested by PROCHECK (21), showing a Ramachandran plot with 82.9% of the residues in the most favored regions and 17.1% in additional allowed regions.

## RESULTS

Following purification from DDM-solubilized bacterial lysates, the N-terminal His tag derivative of full-length 1–160 KcsA is obtained primarily as an SDS-resistant tetramer, as seen by conventional SDS-PAGE of unboiled

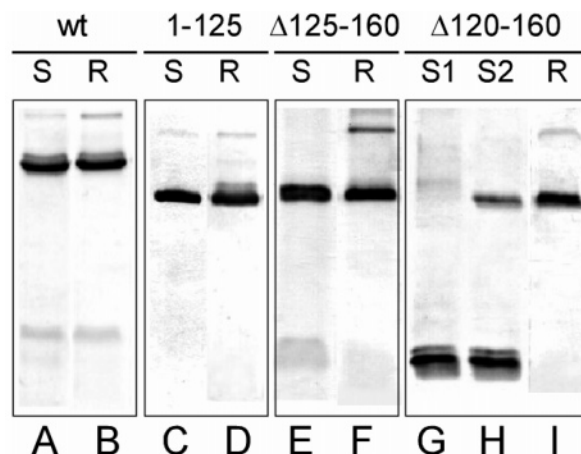


FIGURE 2: Representative SDS-PAGE profile of nonheated aliquots of wild-type 1–160 KcsA (lanes A and B) and 1–125 KcsA (lanes C and D) and the deletion products KcsA  $\Delta$ 125–160 (lanes E and F) and KcsA  $\Delta$ 120–160 (lanes G–I). “S” lanes correspond to the DDM-solubilized and purified proteins (lanes A, C, E, G, and H), while “R” lanes correspond to samples reconstituted into asolectin lipid vesicles (lanes B, D, F, and I). Lanes G and H (also labeled as S1 and S2) correspond to separate pools of different chromatographic fractions from the purified KcsA  $\Delta$ 120–160 deletion (the one in lane G being almost exclusively monomeric) obtained by eluting the Ni<sup>2+</sup> affinity gel with an imidazole gradient. Lane I, which is almost exclusively tetrameric, corresponds to reconstitution into asolectin lipid vesicles of all chromatographic pools of the solubilized protein (as in G and H) mixed together.

samples (Figure 2, lanes A and B) (4, 22). Western blots show strong reactivity of this protein band against commercial anti-His-tag monoclonal antibodies, thus confirming it as the expression product of the KcsA construction (not shown). Routinely, this major tetrameric species is accompanied by monomeric KcsA as a minor component, and in sufficiently loaded gels, higher molecular weight, SDS-resistant KcsA multimers can also be observed.

The KcsA tetramer is also remarkably resistant to heat (10, 12, 22–24). Figure 3A shows that indeed dissociation by heat of DDM-solubilized tetramers into monomers, when dissolved in SDS-containing electrophoresis buffer prior to undergoing the heating routine (see the Materials and Methods), shows a midpoint temperature of approximately 76 °C under our experimental conditions (Figure 4A). Such a midpoint temperature for dissociation under those conditions is increased by approximately 10 °C if the purified KcsA is reconstituted into asolectin lipids prior to dissolving it into SDS-containing electrophoresis sample buffer and submitting it to the above heating routine (Figures 3B and 4A). Moreover, heating intact reconstituted samples in the absence of SDS, and then dissolving them into SDS-containing electrophoresis sample buffer for the SDS-PAGE analysis, shows that the midpoint temperature for the disappearance of KcsA tetramers (which under these conditions results primarily in SDS-insoluble, high molecular weight aggregates) occurs at temperatures exceeding 100 °C (not shown). This suggests that the presence of lipids either when codissolved in the SDS solution during heating or, even more noticeably, when organized as intact KcsA reconstituted vesicles confers a higher thermal stability to the tetrameric protein. Such an increased thermal stability, however, does not affect the tetramer-to-monomer ratio in the wild-type KcsA population, which remains high and fairly constant (roughly 80% tetrameric KcsA) both in the DDM-solubilized

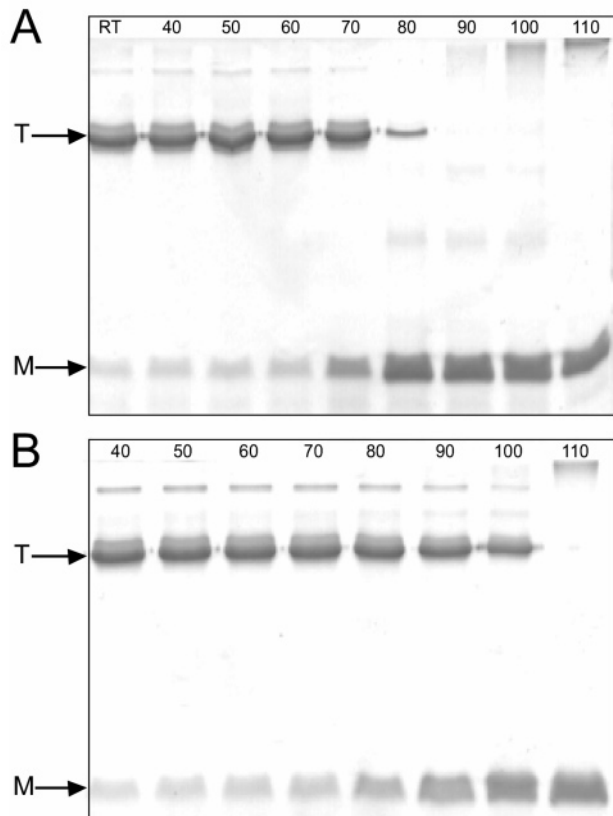


FIGURE 3: Representative electrophoretic gels used to study the stability of the different tetrameric KcsA proteins to detergent- and heat-induced dissociation. In this case, the behavior of tetrameric, full-length 1–160 KcsA is shown to illustrate the experimental approach. Panels A and B are representative SDS–PAGE profiles of the dissociation into monomers of tetrameric KcsA both in a DDM-solubilized form (A) or reconstituted into asolectin lipid vesicles (B). In either case, the samples were processed at the indicated temperatures ( $^{\circ}\text{C}$ , shown above the different lanes), as described in the Materials and Methods. RT stands for room temperature, and M and T stand for KcsA monomer and tetramer, respectively.

and in the asolectin-reconstituted samples (Figure 5A). Similar effects of lipids on KcsA stability have been previously attributed to the presence of anionic lipids, mainly phosphatidylglycerol, which is reported (i) to interact preferentially with the protein channel (24), (ii) to be required for channel function as measured in terms of increased rubidium fluxes (7, 25), and (iii) to copurify with KcsA when isolated from the *E. coli* expression system (25).

The entire 1–160 KcsA can be enzymatically converted into 1–125 KcsA by proteolytic treatment with chymotrypsin (Figure 2, lanes C and D) (3). Interestingly, it is observed that the minor monomer population present in the full-length KcsA preparations disappears almost completely during the chymotrypsin hydrolysis, suggesting that the unassembled monomer has additional accessible sites for chymotrypsin cleavage, which are not accessible in the assembled KcsA tetramer (26). Because of this, the estimation of tetramer-to-monomer ratios in these preparations is meaningless and has not been included in Figure 5.

SDS–PAGE analysis of the dissociation into monomers of 1–125 KcsA tetramers cleaved with chymotrypsin in all of its four subunits shows that the SDS and heat resistance characteristic of full-length KcsA are essentially retained in the DDM-solubilized 1–125 KcsA proteolytic derivative

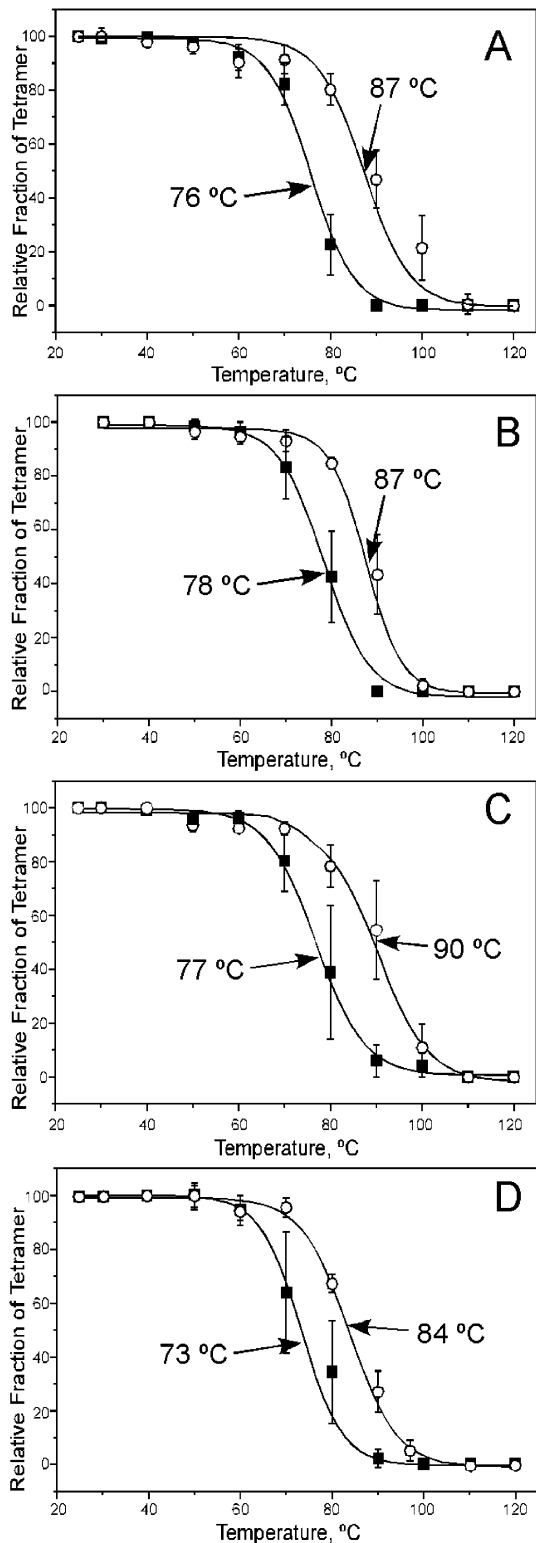


FIGURE 4: Stability of the different KcsA preparations to detergent- and heat-induced dissociation. The results show the fraction of tetramer remaining at the different temperatures in DDM-solubilized (■) or in asolectin-reconstituted (○) KcsA samples, as determined from densitometry scans of SDS gels such as that shown in Figure 3. Arbitrarily, we assigned a relative value of 100 to the intensity of the tetramer band seen at room temperature in each sample. The results for wild-type 1–160 KcsA (A) and 1–125 KcsA (B) and the KcsA  $\Delta 125$ –160 (C) and  $\Delta 120$ –160 (D) deletions are shown. Numbers within the panels indicate the estimated midpoint temperatures for tetramer dissociation ( $^{\circ}\text{C}$ ). The number of observations (number of different gels processed) ranged between 2 and 6 for the different samples.

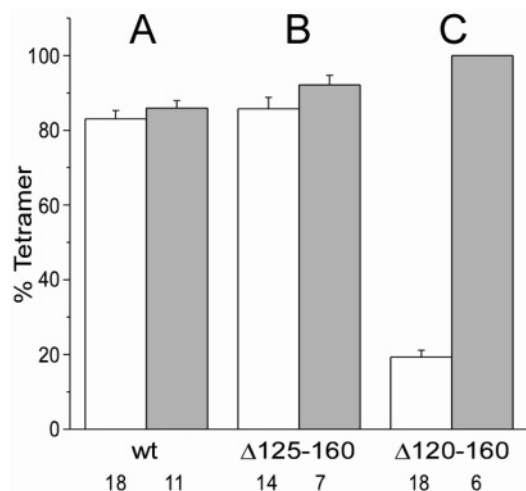


FIGURE 5: Percent of tetramer over the tetramer-plus-monomer population observed by SDS-PAGE analysis in nonheated samples of full-length 1–160 KcsA (A) or in the KcsA  $\Delta$ 125–160 (B) and  $\Delta$ 120–160 (C) deletions. Open bars correspond to DDM-solubilized and purified proteins, while gray bars correspond to the same samples but reconstituted into asolectin lipid vesicles. Results are given as mean  $\pm$  standard error, and the numbers below each bar are the number of experimental observations.

(Figure 4B) (9), which also shows the increased stability mentioned above upon reconstitution into asolectin lipid vesicles (Figure 4B). This suggests that, once the tetramer is assembled, proteolytic removal of the 126–160 protein segment does not destabilize the proteolyzed KcsA tetramer to undergo SDS- or heat-induced dissociation into subunits.

KcsA deletions lacking large portions of the protein's C-terminal can also be obtained by genetic engineering of the cDNA heterologously expressed in the *E. coli* system. It should be noted here that, just as the full-length or the chymotrypsin-cleaved KcsA (2, 4, 7, 8, 27), the C-terminal deletions used in this work, i.e., KcsA  $\Delta$ 125–160 and  $\Delta$ 120–160, give rise to functionally responsive potassium channels upon reconstitution into an artificial membrane (23).

A first difference we noticed regarding the expression of the above KcsA deletions relates to the yield obtained in terms of DDM-soluble purified protein, which decreases significantly compared to that of full-length KcsA preparations (see the Materials and Methods for yield estimates). Different from full-length KcsA, a significant portion of the proteins expressed in both deletions is recovered as DDM-insoluble material (likely to be part of the inclusion bodies) that goes into the pellet discarded from centrifugation of the cell lysates prior to the affinity chromatography step. Such pellets of DDM-insoluble material, however, are soluble in SDS and can still be analyzed by SDS-PAGE, which shows that they are composed almost exclusively of KcsA monomers (not shown).

In addition to the lower yield in terms of DDM-soluble protein, which occurs in both deletions, SDS-PAGE analysis reveals that the tetrameric protein is still predominant in the purified KcsA  $\Delta$ 125–160 deletion (Figure 2, lanes E and F), but not in the shorter KcsA  $\Delta$ 120–160 deletion (Figure 2, lanes G–I), in which tetramers are only a minor species and, consequently, the tetramer-to-monomer ratio decreases dramatically (Figure 5B,C). Interestingly, such a decreased tetramer population in the KcsA  $\Delta$ 120–160 deletion is not due to a major loss in tetramer stability. Indeed, SDS-PAGE

analysis of the heat resistance to dissociation of the deletion tetramers (Figure 4C,D) indicates that, regardless of the differences in the relative abundance of tetrameric protein in the KcsA  $\Delta$ 125–160 and  $\Delta$ 120–160 deletions, the midpoint temperatures for their dissociation into subunits are still fairly high and comparable to or slightly lower than those exhibited by full-length KcsA. On the basis of the above observations, it seems reasonable to conclude that the 120–124 ERRGH sequence stretch, which is lacking in the KcsA  $\Delta$ 120–160 deletion, is involved in determining whether monomeric KcsA subunits would associate as tetramers in the *E. coli* expression system used in these studies. However, once the tetramer is assembled, the presence of a longer or a shorter C-terminal has only a limited influence on the overall stability of the protein.

Molecular modeling of full-length KcsA further supports our proposal for the putative involvement of the 120–124 sequence stretch on tetramerization of KcsA subunits. Figure 6 shows that the model predicts the existence of *intersubunit* interaction sites at the region of interest, in the vicinity of the cytosolic channel pore. It is observed that the interactions between residues 120–124 and the N- and C-terminal segments of the adjacent monomer are mainly electrostatic: R121 forms two hydrogen bonds with the N-terminal polypeptide backbone (carbonyl groups of A23 and S22 in the adjacent subunit), R122 hydrogen bonds to Q119 also in the adjacent subunit, and finally H124 hydrogen bonds to both E120 in the same subunit and S22 in the adjacent one. The nearby E118 is predicted to interact with H25, which in turn forms a hydrogen bond with T112. Thus, every 120–124 stretch in each monomer could form up to 4–5 hydrogen bonds with the adjacent monomer, reinforcing the notion that these residues might play a role as a molecular determinant in the oligomerization process.

On the other hand, studies of reconstitution of the deletion proteins into lipid vesicles indicate that the need for the presence of the 120–124 sequence stretch to allow KcsA subunits to assemble as tetramers is not such a critical requirement in the environment provided to the protein in the reconstituted vesicles, into which such an assembly process is clearly favored. Indeed, reconstitution of the deletion proteins into asolectin lipid vesicles shows that, opposite the observations on the DDM-solubilized, purified proteins, tetramers become again the predominant molecular species in all the reconstituted deletion samples (Figure 5B,C). This is again most noticeable in the KcsA  $\Delta$ 120–160 deletion (Figure 5C), which goes from being predominantly monomeric in the DDM-solubilized, purified preparation to almost exclusively tetrameric in the reconstituted samples (Figure 2, lanes G–I). This suggests that, besides protein–protein interactions, protein–lipid interactions could play an important role in promoting oligomerization and assembly of preformed membrane protein subunits. This phenomenon seems reminiscent of that mediated by the so-called intramembrane association domains in tetramerization of eukaryotic channels, which can also be achieved in deletion mutants lacking their “priming” T1 tetramerization domain (28). Whatever the case might be, once the KcsA deletion tetramers are assembled, either as a minor species in the DDM-solubilized expression products or as the major species in the reconstituted vesicles, their detergent and heat resistances to dissociation into subunits (Figure 4) are similar

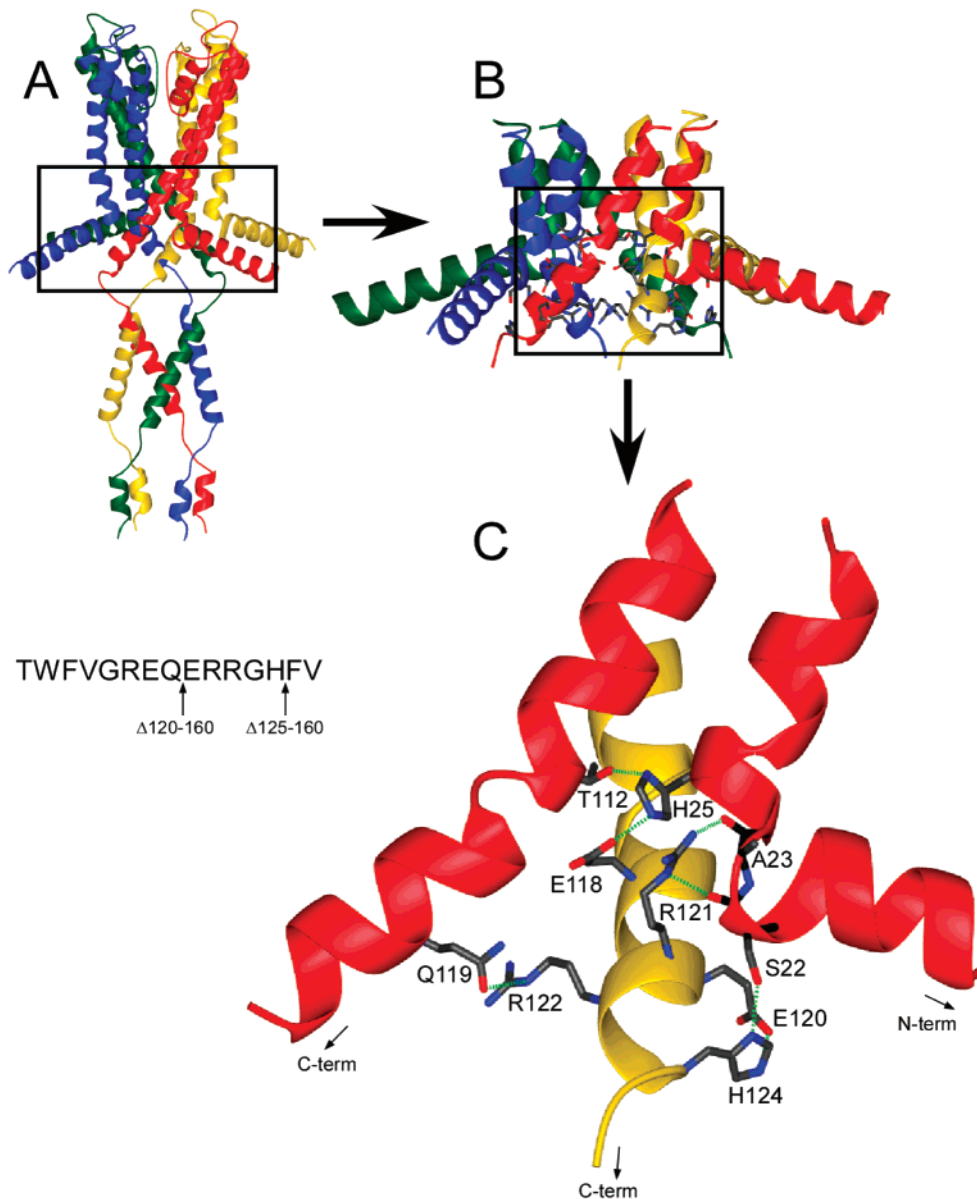


FIGURE 6: Full-length KcsA model (A) used to illustrate the putative interactions anchoring adjacent monomers in the closed state of the protein channel (see the Materials and Methods). Panel B focus on the cytosolic mouth of the channel, while panel C zooms in on an even closer view of the 120–124 sequence stretch in one of the subunits (yellow ribbon) interacting with the contiguous monomer (red ribbon). Only the main residues involved and the predicted hydrogen bonds (green, discontinuous line) are shown.

and only slightly lower than those of the full-length tetrameric channel (23), suggesting once again that the stability of the assembled tetramer does not depend to a great extent upon the presence of an intact C-terminal as a protein constituent.

## DISCUSSION

Oligomerization of compatible subunits to form functional ion channels is a complex event of molecular recognition resulting from the successful concurrence of many elemental steps including membrane targeting, folding, posttranslational modification, and assembly. The occurrence and the order in which these processes take place may differ in each case, but it is assumed that the nascent polypeptide chains contain specific sequences to determine their overall processing and cell surface expression. The physiological relevance of this is obvious, and in voltage-dependent potassium channels in particular, it has been shown that deletions, as well as

mutations of sequences involved in trafficking to the plasma membrane, lead to impaired channel function and relate to genetic diseases (see ref 29 and references therein).

In this paper we report on two main observations related to KcsA tetramerization. First, we noticed that going from the C-terminal deletion KcsA  $\Delta 125-160$  to the KcsA  $\Delta 120-160$  greatly impairs the ability of the protein to assemble as a tetramer in the *E. coli* heterologous expression system. This suggests a possible role for the ERRGH 120–124 sequence as a putative “tetramerization” domain. Second, it is observed that, even in the absence of such an apparently critical sequence stretch, reconstitution into lipid vesicles of the predominantly monomeric KcsA  $\Delta 120-160$  deletion results in the highly efficient formation of functionally responsive tetramers. Compared to the wild-type protein, such deletion tetramers exhibit a similar or slightly diminished stability to heat- and detergent-induced dissociation. Taking the two observations together, it seems that hydro-

phobic interaction between the transmembrane protein segments is the most important factor in holding the tetramer together, but under conditions less favorable than those provided by reconstitution into lipid vesicles to facilitate subunit recognition and assembly, the ERRGH 120–124 sequence becomes a key element for efficient tetramerization.

In the eukaryotic voltage-activated *Shaker* potassium channel, tetramerization is mediated by the so-called T1 or first tetramerization domains (30). T1 domains supervise proper tetrameric assembly of compatible channel subunits and correspond to a large N-terminal cytoplasmic protein stretch (31, 32), which by itself readily forms tetrameric structures. Zerangue and co-workers (33) propose that tetramerization takes place in a multistep process in which rapid T1 tetramerization occurs first, and then it brings transmembrane domains of T1-compatible channel subunits into close proximity to allow full tetramerization of the channel. According to this proposal, T1 domains act *on the kinetics of the assembly process* by priming the slower concentration-dependent process of tetramerization between transmembrane segments of T1-compatible channel subunits (33). This kinetic mechanism is supported by the observation that tetrameric assembly of *Shaker* channels lacking their T1 domains occurs poorly at “normal” (low) protein concentration, but becomes more efficient in expression systems yielding a higher concentration of channel subunits.

In principle, the above notions seem consistent with our findings on the tetrameric assembly of KcsA, except that it is the C-terminal segment of KcsA that appears to play the role of the N-terminal, *Shaker* T1 domain. Such a possibility is not unprecedented since it is known that other potassium channels different from *Shaker* have their C-terminals involved in tetramerization. For instance, in the EAG (“ether-a-gogo”) family, which like *Shaker* belongs to the so-called 6TMIP (six transmembrane domains, one pore region) potassium channels, the deletion of a short domain near the C-terminus impairs tetramerization (34). Kir (inwardly rectifying) potassium channels, which like KcsA belong to the 2TMIP-type channels, also have tetramerization domains at their C-terminals (35).

Regardless of their location at the N- or C-terminal, a key feature of putative tetramerization domains should be to mediate intersubunit interactions so that compatible subunits may recognize each other and be properly oriented for the subsequent occurrence of hydrophobic interactions between transmembrane regions of the protein, which are major determinants in holding the oligomer together. Such priming intersubunit interactions seem to be attained differently in different channels. For instance, in the N-terminal, T1 domains of *Shaker* (33), as well as in the C-terminal tetramerization domains of EAG channels, intersubunit contacts are based on coiled-coil structures (34), while no coiled coils have been detected in the large cytoplasmic C-terminal of a prokaryotic KirBac1.1 channel (36), which has been structurally determined at high resolution and forms intersubunit  $\beta$ -structures. In KcsA and according to our molecular modeling, it seems likely that multiple hydrogen bonding involving the 120–124 residues and N- and C-terminal regions of adjacent subunits could be a priming event leading to tetramerization. Site-directed mutagenesis experiments are currently in progress in an attempt to provide further evidence for this hypothesis.

On the other hand, it has already been mentioned that, during KcsA tetramer formation, the full-length KcsA monomer first inserts into the target membrane and then into the membrane where tetramerization occurs (10). Therefore, since membrane targeting, monomer insertion, and tetramerization are linked, sequential processes, it is also conceivable that rather than being strictly involved in tetramerization, putative tetramerization domains might facilitate membrane targeting and/or folding of nascent monomeric components. In this regard, it has been reported that in a eukaryotic *in vitro* translation and translocation system, the C-terminal of KcsA affects the efficiency at which the M2 transmembrane segment spans the endoplasmic reticulum membrane and achieves its final structure (37). Thus, an alternative possibility would be that the 120–124 ERRGH sequence stretch is influencing monomer folding rather than monomer tetramerization in strict terms. This should lead the improperly folded monomers to form insoluble inclusion bodies, which is the usual way prokaryotes discard misfolded expression products, and would explain our lower yields in terms of DDM-soluble protein, as well as the increased amount of DDM-insoluble, monomeric KcsA seen in our two deletions.

Our second major observation is that, in the absence of the putative tetramerization domain, reconstitution into lipid vesicles of the purified KcsA  $\Delta$ 120–160 deletion protein in a predominantly monomeric form secures tetramerization very efficiently. The high efficiency of this phenomenon is unprecedented and reinforces the emerging concept that, in addition to contributing to the stability or the conformation of oligomeric membrane proteins, protein–lipid interactions may be important in promoting oligomerization of such proteins and in allowing intramembrane domain interactions to determine a correct oligomeric assembly (38). In particular for KcsA, it has been shown that, as long as the monomers are previously membrane-inserted, tetramers can be assembled in pure lipid bilayers as target membranes in an *in vitro* transcription and translation system (9), this process being more efficient in the presence of negatively charged phosphatidylglycerol (24). Also, the *in vitro* refolding into tetramers of previously trifluoroethanol-unfolded KcsA monomers has been found to occur only when in the presence of lipid vesicles, but in this case with a low (20–30%) efficiency and without any specific lipid requirement (25). The driving forces in this “lipid-facilitated” tetramerization of KcsA should be equivalent to those promoting the well-known oligomerization of compatible, synthetic transmembrane segments of oligomeric membrane proteins (39), which obviously occurs in the absence of any cytoplasmic tetramerization domain.

In conclusion, we found that the lack of the cytoplasmic C-terminal domain of KcsA and, most critically, the 120–124 sequence stretch prevents tetramerization of monomeric channel subunits in an *E. coli* expression system. We also observed that, once the KcsA tetramer is assembled, its *in vitro* stability does not depend to a large extent on whether the putative tetramerization domain continues being part of the protein, but is further increased by the presence of lipids. Finally and most interestingly, we also found that, in the absence of such a critical C-terminal domain, reconstitution into membrane lipids facilitates very efficiently tetramerization of KcsA subunits, which is likely mediated by allowing proper interactions among intramembrane protein sites in the hydrophobic reconstitution medium.

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