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The Role of Extramembranous Cytoplasmic Termini in Assembly and Stability of the Tetrameric K^+-Channel KcsA

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Abstract Membrane-active alcohol 2,2,2-trifluoroethanol has been proven to be an attractive tool in the investigation of the intrinsic stability of integral membrane protein complexes by taking K^+-channel KcsA as a suitable and representative ion channel. In the present study, the roles of both cytoplasmic N and C termini in channel assembly and stability of KcsA were determined. The N terminus (1–18 residues) slightly increased tetramer stability via electrostatic interactions in the presence of 30 mol.% acidic phosphatidylglycerol (PG) in phosphatidylcholine lipid bilayer. Furthermore, the N terminus was found to be potentially required for efficient channel (re)assembly. In contrast, truncation of the C terminus (125–160 residues) greatly facilitated channel reversibility from either a partially or a completely unfolded state, and this domain was substantially involved in stabilizing the tetramer in either the presence or absence of PG in lipid bilayer. These studies provide new insights into how extramembranous parts play their crucial roles in the assembly and stability of integral membrane protein complexes.

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Introduction

Potassium channels are widely distributed as oligomeric complexes in different cell types of all living organisms. The formation of these complexes is crucial for their function as these complexes contribute to the control of potassium flow, cell volume, release of hormones and neurotransmitters, resting potential, excitability and behavior (Sachs and Engelman 2006). How these complexes are formed and what determines their stability are still matters of debate. The potassium channel KcsA is an oligomeric membrane protein from *Streptomyces lividans*, which is a convenient model protein to study membrane protein assembly and stability (Valiyaveetil et al. 2002). The four subunits of KcsA are arranged symmetrically around the central pore. The transmembrane segment M2, nearest to the C terminus, contributes to the lining of the pore, while the one closest to the N terminus, M1, is exposed to the membrane bilayer (Doyle et al. 1998). The positively charged N-terminal helix which lies at the membrane interface and a large C terminus are included as cytoplasmic domains that are rich in charged or polar amino acid residues (Cortes et al. 2001). These domains could not be resolved in the crystal structure, which accounted only for the membrane-inserted 23–119 amino acids in the KcsA sequence (Doyle et al. 1998). Later, the structure of the full-length KcsA–Fab complex revealed a well-defined, four-helix C-terminal bundle that projected $\approx$70 Å toward the cytoplasm (Uysal et al. 2009).

The tetrameric structure of KcsA is highly stable in a wide range of detergents, even in SDS (Cortes and Perozo 1997). This high stability is caused not only by interactions between protein subunits but also by interactions between the protein and the surrounding lipid bilayer. For example, the efficiency of formation of the KcsA tetramer during assembly depends on the membrane lipid composition. It has been shown that the charged lipid phosphatidylglycerol (PG) is important for efficient membrane association and tetramerization of KcsA. Interestingly, these lipids also have been suggested to be required for KcsA channel activity and proper conformation of the protein (Valiyaveetil et al. 2002; Heginbotham et al. 1998). PG could be copurified with KcsA and was suggested to be present in the crystal structure of KcsA (Valiyaveetil et al. 2002). The interaction of PG may be either specific or more general, e.g., electrostatic interactions which play an important role in stabilizing the tetramer (Raja et al. 2007). The increased membrane association by introducing PG in the phosphatidylcholine (PC) bilayer can most likely be attributed to an increased targeting to the membrane by FtsY, a membrane-associated homologue of the eukaryotic signal recognition particle (SRP) receptor α-subunit, which is responsible for the cotranslational targeting of many integral membrane proteins (Millman et al. 2001). In addition, PG could contribute directly to targeting, e.g., by anchoring the positively charged N terminus to the *cis* side of the membrane, as observed in other protein insertion systems (Kusters et al. 1994; Ridder et al. 2001). The increased tetramerization by the presence of 20–30 mol.% PG could be due to a requirement of PG for correct folding of the KcsA monomer, in analogy to the results obtained for the *Escherichia coli* protease DegP (Skorko-Glonek et al. 1997).

In an in vitro transcription and translation *E. coli* system, KcsA monomers have been reported to be targeted cotranslationally via the SRP pathway to the internal bacterial membrane, where the assembly of the membrane-inserted monomers into highly stable tetramers proceeds efficiently and rapidly, through a process influenced by the membrane proton-motive force (Van Dalen et al. 2000). However, the molecular determinant(s) involved in promoting subunit tetramerization or channel assembly remain to be explored.

It has been shown that 2,2,2-trifluoroethanol (TFE) induces KcsA tetramer dissociation, presumably via increased lateral pressure in the head group region followed by a lower lateral pressure in the acyl chain region (Raja et al. 2007; Van den Brink-van der Laan et al. 2004a). In the present study, the effects of TFE were used as a tool to investigate KcsA tetramer assembly and its intrinsic stability, with special emphasis on the role of the N- and C-terminal domains in channel oligomerization in lipid bilayers.

Materials and Methods

Reagents

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids (Birmingham, AL). *n*-Dodecyl-*β*-D-maltoside (DDM) was from Anatrace (Maumee, OH). Ni^{2+}-NTA agarose was obtained from Qiagen (Chatsworth, CA) and Bio-Beads SM-2 Adsorbent was from Bio-Rad (Richmond, CA). TFE was obtained from Merck (Darmstadt, Germany).

Protein Expression, Purification and Reconstitution

Full-length (FL) and ΔN (Δ1-18) KcsA proteins were expressed with an N-terminal His-tag and purified as described previously (Raja et al. 2007; Raja 2010). The mutant ΔC (Δ125–160) was generated by chymotrypsin treatment (100 μg/ml, 60 min, room temperature) of DDM-solubilized FL-KcsA. The purity of all proteins was assessed by SDS-PAGE. Large unilamellar vesicles (LUVs) were prepared by extrusion (Hope et al. 1985). All proteins were reconstituted in different lipid mixtures with a protein concentration of 0.1 mg/ml, and proteoliposomes were obtained according to the method described previously (Raja et al. 2007).

Analysis of KcsA Tetrameric Stability by TFE

Small aliquots of proteoliposomes were incubated with variable concentrations of TFE for 1 h at room temperature. Samples were mixed with an electrophoresis sample buffer (50 mM Tris-HCl [pH 6.8], 50% glycerol, 0.01% bromophenol blue and 10% SDS) and run on 15% acrylamide gel in the presence of 0.1% SDS. After staining by silver nitrate, gels were scanned by a densitometer (Bio-Rad) and quantified with the program Quantity One (Bio-Rad). The tetramer intensity as an amount of total tetramer present in each sample was set to 100%. The amount of tetramer (%) was plotted against TFE (vol.%) for stability assay.

Tryptophan Fluorescence and Acrylamide Quenching

Proteoliposome samples were treated with TFE at desired concentrations. Fluorescence experiments were performed using a QuantaMaster QM-1/2005 spectrofluorometer (Photon Technology, Princeton, NJ), fitted with a xenon flash lamp at room temperature. A 0.3-cm excitation and emission path length quartz cell was used for all fluorescence measurements. The excitation wavelength was set at 280 nm, and emission spectra were collected between 300

and 400 nm, averaging three scans. The bandwidths for both excitation and emission monochromators were 5 nm. Acrylamide quenching of tryptophan fluorescence was performed to check the accessibility of tryptophans in lipid bilayers in the absence or presence of TFE, as described previously (Raja et al. 2007). The accessibility of tryptophans was monitored by analyzing the quenching data using a Stern–Volmer equation (Lackowicz 1999):

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 is the tryptophan fluorescence in the absence of quencher and F is the observed fluorescence at the concentration $[Q]$ of the quencher. K_{SV} is the collisional quenching constant, which was determined from the slope of Stern–Volmer plots. As a control, similar experiments were performed for an L-Trp solution (3 μM). All data were corrected for inner filter effects due to acrylamide absorbance according to the standard method (Lackowicz 1999).

Results

TFE-Induced Dissociation of ΔN-KcsA in Lipid Bilayers

To investigate which cytoplasmic domain affects the stability of KcsA, TFE-induced tetramer dissociation experiments were performed for ΔN and ΔC-KcsA mutants. Figure 1 illustrates a KcsA sequence in which the N- and C-terminal domains are highlighted. The silver-stained gels of ΔN-KcsA reconstituted in PC and PC:PG (7:3 mol.%) lipid bilayers and the effects of TFE on tetramer dissociation are shown in Fig. 2a. In both lipid bilayers, the amount of TFE required to dissociate the tetramer was ~30 vol.%. In addition, the monomer band intensity did not increase as a function of TFE concentration, suggesting that dissociated monomeric ΔN-KcsA is insoluble in the presence of TFE as also reported recently (Raja 2010). These results indicate that deletion of a positively charged N terminus abolishes the slight stabilizing effect of PG in FL KcsA (Raja et al. 2007), thus indicating the role of electrostatic interactions between the N terminus and charged PG in stabilizing the tetramer.

TFE-Induced Dissociation of ΔC-KcsA in Lipid Bilayers

Next, the role of the C terminus in determining KcsA tetrameric stability was investigated. The entire KcsA (1–160 residues) was enzymatically converted into ΔC-KcsA upon removal of 125–160 residues. Gel images of ΔC-KcsA reconstituted in PC and PC:PG (7:3 mol.%) bilayers are shown in Fig. 2b. In addition to a tetrameric band (T–C) in PC bilayers, some bands of slightly higher molecular weight appear, indicating that these bands result from partial chymotrypsin digestion of FL KcsA. It is also interesting that dissociation of ΔC-KcsA in both lipid bilayers is followed by the appearance of two monomeric bands. M1 band, ~15 kDa, corresponds to monomeric ΔC-KcsA (Δ125–160). M2 appears at a relatively lower molecular weight of ~12 kDa, which could be the result of a second cleavage product of chymotrypsin-treated monomer, as also observed in previous studies (Van den Brink-van der Laan et al. 2004b). In pure PC, the intensity of M2 is higher than that of M1 in the range of 0–20 vol.% TFE, which eventually disappears upon increasing TFE concentration. In PC:PG bilayers the band intensity of M1 is significantly higher than that of M2 at 0–20 vol.% TFE. However, the band intensity of M1 is significantly increased at 25–35 vol.% TFE, which was not observed in the pure PC system. Perhaps the interaction of PG promotes proper folding and better solubility of M1, which is similar to the effect of PG on FL monomeric KcsA, as reported previously (Raja et al. 2007). Since the dissociation of T–C KcsA in PC:PG bilayers is followed by an increase in M1 band intensity, the appearance of M2 is unrelated to tetrameric dissociation and it is possibly derived from chymotrypsin cleavage of unassembled monomers. In pure PC the tetramer is completely dissociated at 30 vol.% TFE, while in PC:PG (7:3 mol.%) the tetramer dissociates at 35 vol.% TFE. However, the

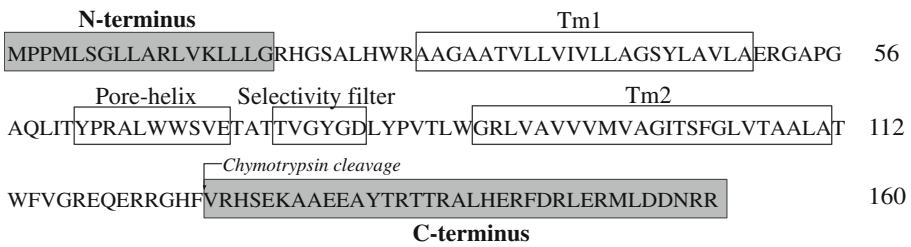

Fig. 1 Amino acid sequence of the KcsA potassium channel from *S. lividans* (SWISS-PROT accession number Q54397). *Open boxes* indicate the transmembrane segments Tm1 and Tm2 and the characteristic pore-helix and selectivity filter. *Gray boxes* represent the N (1–18 residues) and C (125–160 residues) termini

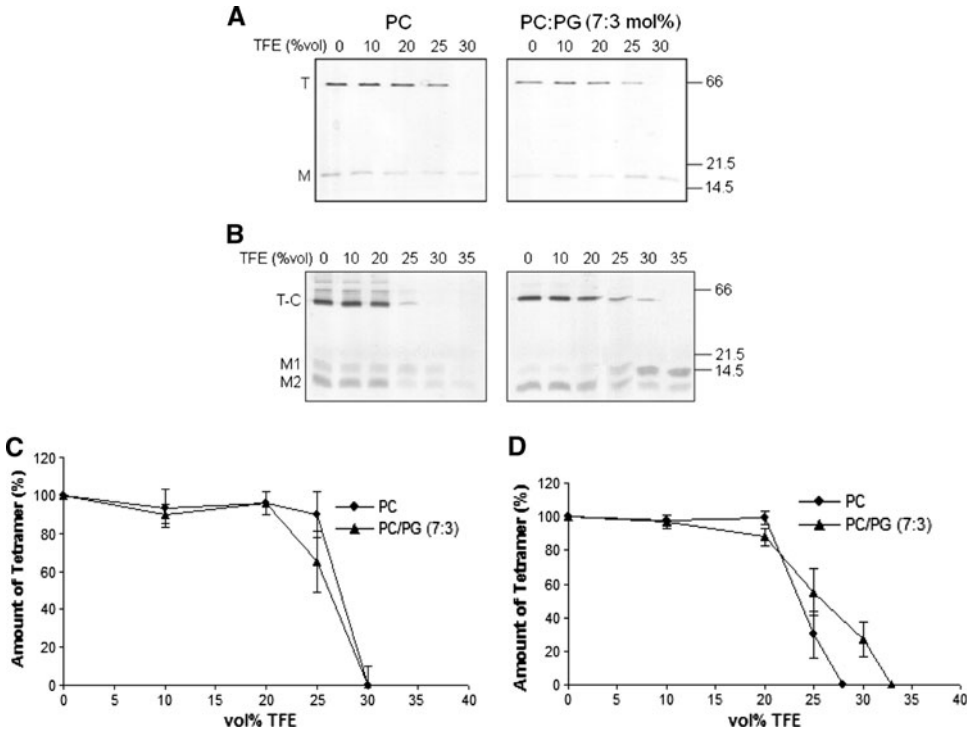

Fig. 2 TFE-induced tetramer dissociation of ΔN-KcsA (**a**) and ΔC-KcsA (**b**) in PC or PC:PG (7:3 mol.%) lipid bilayers. TFE-treated samples were analyzed by SDS-PAGE and stained with silver nitrate. Tetrameric (T), chymotrypsin cleaved tetrameric (T–C) and monomeric (M, M1 or M2) KcsA are indicated; and a protein marker (in kDa) is shown on the *right*. Silver-stained gels were quantified for ΔN- (**c**) and ΔC-KcsA (**d**) reconstituted in pure PC or PC:PG (7:3 mol.%) bilayers. The intensities of tetramer bands were assigned as a relative value of 100% observed for a TFE-untreated (0 vol.% TFE) sample. Data points correspond to the average ± SD of three experiments

tetramer intensity is significantly decreased at 25 vol.% TFE in both lipid systems compared to FL and ΔN-KcsA. These results indicate that the stability of ΔC-KcsA is significantly decreased in both lipid bilayers.

For comparison, Fig. 2c and d summarize the data of tetramer dissocation of ΔN- and ΔC-KcsA in pure PC and PC:PG lipid bilayers, respectively. Since the N terminus is associated with the membrane, the slight stabilizing effect in FL (Raja et al. 2007) and ΔC-KcsA could be due to electrostatic interactions between the N terminus and charged PG. Therefore, the role of the N terminus was determined as a function of PG concentration in the membrane.

Influence of Charge Density on the Stability of the ΔN-KcsA Tetramer

The potential role of the N terminus in stabilizing the tetramer was further analyzed by titrating an increasing amount of PG (mol.%) in PC bilayers, as shown in Fig. 3. Silver-stained gels were quantified, and the amount of TFE (vol.%) at which 50% tetramer was stable as a function of PG concentration in the membrane was determined. Interestingly, the stability of ΔN-KcsA remained unchanged throughout the range of PG, which is in contrast to the slightly increased tetrameric stability of FL-KcsA in the range 30–50 mol.% PG (Raja et al. 2007). The data indicate that electrostatic interactions between the N terminus and PG play an important role in stabilizing the tetrameric

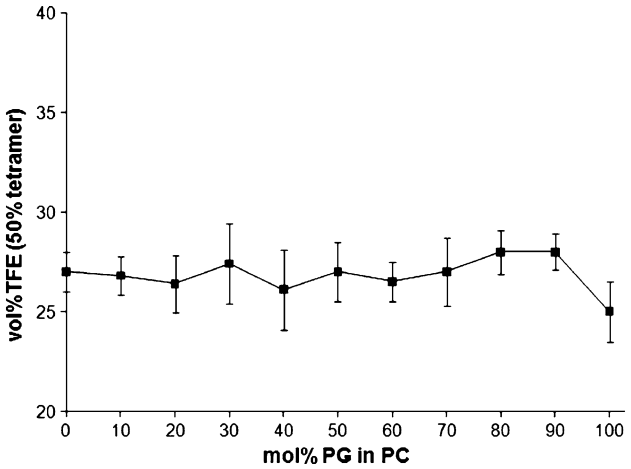

Fig. 3 Influence of anionic PG on tetramer stability of ΔN-KcsA. ΔN-KcsA was reconstituted in LUVs containing varying amounts of PG in PC (mol.%). Samples were analyzed as described in the legend of Figure 2, and the TFE concentration (vol.%) required to dissociate the 50% tetramer was calculated. Data points correspond to the average ± SD of three experiments

structure of KcsA, thus abolishing the PG-dependent stabilizing effect in the presence of the N terminus.

Analysis of Channel Reassembly by SDS-PAGE

To investigate which cytoplasmic terminus is involved in channel reassembly after TFE-induced tetramer dissociation,

SDS-PAGE experiments were performed to monitor reassembled tetramer upon TFE dilution. Figure 4 illustrates silver-stained SDS gels of FL, ΔN and ΔC-KcsA in either the presence or the absence of a TFE concentration which was required to completely dissociate tetramer into monomers as determined from TFE-induced tetramer dissociation experiments of FL-KcsA (Raja et al. 2007) and ΔN and ΔC-KcsA as shown in Fig. 2. Tetrameric reassembly was monitored upon diluting TFE-induced tetramer-dissociated samples to reduce denaturant concentration to 5 vol.% TFE. The (%) values of reassembled tetramer compared to untreated tetramer were calculated and are compiled in Table 2. The amount of reassembled tetramer of ΔC-KcsA was found to be significantly higher than FL and ΔN-KcsA, indicating that the presence of the C terminus might interfere with proper refolding. Furthermore, channel reassembly of ΔN-KcsA was slightly higher than that of FL-KcsA, suggesting that at high TFE concentration the N terminus might be misfolded, thus inhibiting channel refolding.

TFE-Induced Changes in Tryptophan Fluorescence Emission

The properties of Trp fluorescence have been extensively exploited in investigations of channel unfolding or refolding of KcsA in detergent micelles or lipid bilayers (Raja et al. 2007; Barrera et al. 2005). To gain insight into tetramer folding upon N- or C-terminal deletion, Trp fluorescence experiments were performed in either the presence or the absence of varying concentrations of TFE in PC:PG (7:3 mol.%) bilayers. Each monomer of KcsA contains five Trp (W) residues located at the membrane–water interfacial regions (see Fig. 1).

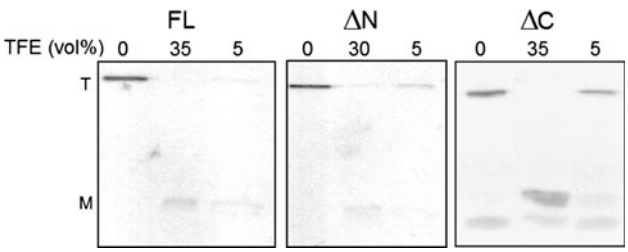

Fig. 4 Representative silver-stained SDS-PAGE analysis of KcsA reassembly in PC:PG (7:3 mol.%) bilayers. As control, TFE-untreated samples (0) are also shown. Channel unfolding experiments were performed at 35 vol.% TFE for FL, 30 vol.% for ΔN and 35 vol.% for ΔC-KcsA. The amount of TFE used for tetramer dissociation was based on TFE-induced tetramer dissociation data shown in Fig. 2. Channel refolding was monitored upon dilution of TFE-containing samples to reduce denaturant concentration to 5 vol.%. The amounts of reassembled tetramer (%) are compiled in Table 2. Tetrameric (*T*) and monomeric (*M*) KcsA are indicated

FL-KcsA: Trp Fluorescence and Effect of TFE

For comparison, the effects of TFE on Trp fluorescence of FL-KcsA from a previous study (Raja et al. 2007) are compiled in Table 1 and Trp fluorescence spectra in the presence and absence of TFE (v/v) are shown in Fig. 5a. Fluctuations in fluorescence intensities and strong shifts in emission maxima clearly indicated distinct conformational changes in the protein via positioning of Trp residues into a more hydrophilic environment, as shown previously (Raja et al. 2007).

ΔN-KcsA: Trp Fluorescence and Effect of TFE

Qualitatively, similar changes were observed in Trp fluorescence of ΔN-KcsA (Fig. 5b, also see Table 1) as a function of TFE concentration. These results suggested that FL and ΔN-KcsA exhibit similar folding properties, thus indicating that in the absence of the N terminus the protein behaves similarly to TFE-induced tetramer unfolding.

ΔC-KcsA: Trp Fluorescence and Effect of TFE

For ΔC-KcsA, Trp fluorescence spectra in the presence and absence of TFE are shown in Fig. 5c and the values of fluorescence quenching and shifts in emission maxima are compiled in Table 1. As shown, fluorescence intensity decreased upon increasing TFE concentration; hence, no increase or fluctuation in fluorescence was observed, as was the case for FL and ΔN-KcsA. Furthermore, strong red shifts observed for FL and ΔN-KcsA at 10–30 vol.% TFE could not be monitored for ΔC-KcsA, suggesting dissimilar or minor conformational changes in this protein compared to FL and ΔN-KcsA.

TFE-Induced Tetramer Unfolding and Acrylamide Quenching

Within the limits of experimental resolution, changes in fluorescence intensities and red shifts in emission maxima suggested that KcsA might undergo several distinct conformational changes by exposing Trp residues to a more hydrophilic environment during tetramer unfolding. This was assessed in a more direct manner by means of acrylamide, a hydrophilic quencher of Trp fluorescence. This quencher has the advantage that it has a very low permeability to lipid membranes. In addition, no charge interaction takes place between the charged lipid head groups (Lackowicz 1999). The Stern–Volmer quenching plots of FL, ΔN and ΔC-KcsA in the absence and presence of TFE were linear; and representative plots of the effect of 30 vol.% TFE are illustrated in Fig. 6a–c, respectively.

Table 1 Effect of TFE on Trp fluorescence and acrylamide accessibility of FL, ΔN- and ΔC-KcsA in a lipid bilayer

TFE (v/v)	Fluorescence quenching (%)[a]			Red shift (nm)[b]			K_{SV} (M^{-1})[c]		
	FL	ΔN	ΔC	FL	ΔN	ΔC	FL	ΔN	ΔC
0	–	–	–	–	–	–	6 ± 0.8	4.5 ± 1	5.5 ± 1
10	58 ± 10	55 ± 8	4 ± 2	2 ± 0.2	3 ± 0.4	1 ± 0.5	9 ± 2	6.75 ± 2	9 ± 0.8
20	30 ± 8	37 ± 5	33 ± 8	4 ± 0.9	3 ± 0.3	1 ± 0.2	11 ± 2	12 ± 1.4	10 ± 1.4
30	40 ± 8	47 ± 8	36 ± 6	4 ± 0.7	4 ± 0.3	2 ± 0.6	16 ± 2.5	15 ± 2	17 ± 2.5
40	33 ± 4	25 ± 9	56 ± 11	5 ± 0.6	4 ± 0.4	6 ± 1.2	11 ± 2.5	8 ± 1.5	20 ± 2.5

[a,b] Parameters of Trp fluorescence were derived from data shown in Fig. 5

[c] Stern–Volmer constants were determined from the slopes of the linear regression lines from plots of $F_0/F = 1 + K_{SV}[Q]$. Data points correspond to the average ± SD of at least three experiments

The Stern–Volmer constants as a function of TFE concentration are compiled in Table 1.

The quenching constants and fluorescence emission spectra of all three proteins did not differ significantly, indicating that deletion of the N or the C terminus does not substantially alter Trp positioning at the membrane–water interfacial regions and, therefore, protein folding. In FL and ΔN-KcsA, quenching constants increased at 10–30 vol.% TFE. A decrease in acrylamide quenching was observed at 40 vol.% TFE, which is an indication of protein aggregation at high TFE concentration (Raja et al. 2007). Taking into account the effects of TFE on Trp fluorescence emission intensities and red shifts in maxima monitored for FL and ΔN-KcsA, acrylamide quenching data indicate that TFE apparently promotes exposure of Trp residues via several distinct conformational changes. It is interesting to observe that ΔC-KcsA exhibited an increase in quenching constant at 40 vol.% TFE, thus indicating maximal exposure of Trp residues upon tetramer unfolding. This again suggests that the C terminus accounts for TFE-induced major conformational changes during tetramer unfolding, as observed by maximum fluorescence quenching at high TFE concentrations.

Effect of N- or C-terminal Deletion on Channel Reassembly

A complicating factor in analyzing KcsA (re)assembly from gel electrophoresis experiments is the presence of SDS detergent in the media. To gain insight into channel reassembly/reversibility phenomena, acrylamide quenching experiments were performed. Proteoliposomes were first treated with 25, 30, 35 or 40 vol.% TFE and then diluted with a vesicle buffer to a final TFE concentration of 5% (v/v) to allow reassembly of KcsA subunits back into tetramers. In principle, decrease in fluorescence quenching or less accessibility of Trp residues to acrylamide (Trp protection) would be expected if unfolded (monomeric) KcsA refolds back into tetramer upon TFE dilution. The percent reversibility values, calculated from acrylamide quenching constants, are complied in Table 2; and plots of channel reversibility in FL, ΔN and ΔC-KcsA upon dilution of 30 vol.% TFE to 5 vol.% TFE are shown in Fig. 6a–c, respectively. For FL, the channel reversibility was found to be efficient upon diluting 25–30 vol.% TFE-containing samples. The channel reversibility was drastically reduced and finally even abolished at 35–40 vol.% TFE, and no reversibility could be observed. In ΔN-KcsA, channel reversibility was effectively decreased in the range of 30–35 vol.% TFE and, similar to FL-KcsA, no reversibility could be monitored at 40 vol.% TFE, indicating that the N terminus might be required for channel refolding. Completely different behavior was observed for ΔC-KcsA. Deletion of the C terminus supported channel reversibility such that unfolded tetramer in the presence of 40 vol.% TFE could be exclusively recovered upon TFE dilution.

Since TFE-induced unfolding elicited strong red shifts in emission maxima, the channel reversibility from the unfolded to the refolded state was further analyzed by following changes in the protein fluorescence spectral shifts upon TFE dilution. It should be expected that efficient reversibility/reassembly would result in a recovery of red-shifted emission spectra and an inefficient channel reassembly would still exhibit red-shifted emission spectra similar to channel unfolding behavior. The calculated emission maxima upon diluting 25, 30, 35 and 40 vol.% TFE-containing samples to 5 vol.% are shown in Fig. 6d. In FL-KcsA, red shifts caused by 20, 25 and 30 vol.% TFE were completely reversed upon TFE dilution. However, dilution of 35 and 40 vol.% TFE-containing samples still exhibited strong red shifts similar to protein unfolding behavior, thus indicating that high TFE concentrations result in irreversible protein denaturation or aggregation. ΔN-KcsA exhibited efficient reversibility in red shifts when 20 and 25 vol.% TFE-containing samples were diluted to 5 vol.% TFE. However, increasing TFE to 30–40 vol.% TFE significantly inhibited reversibility in spectral shifts, and the protein still exhibited strong red shifts

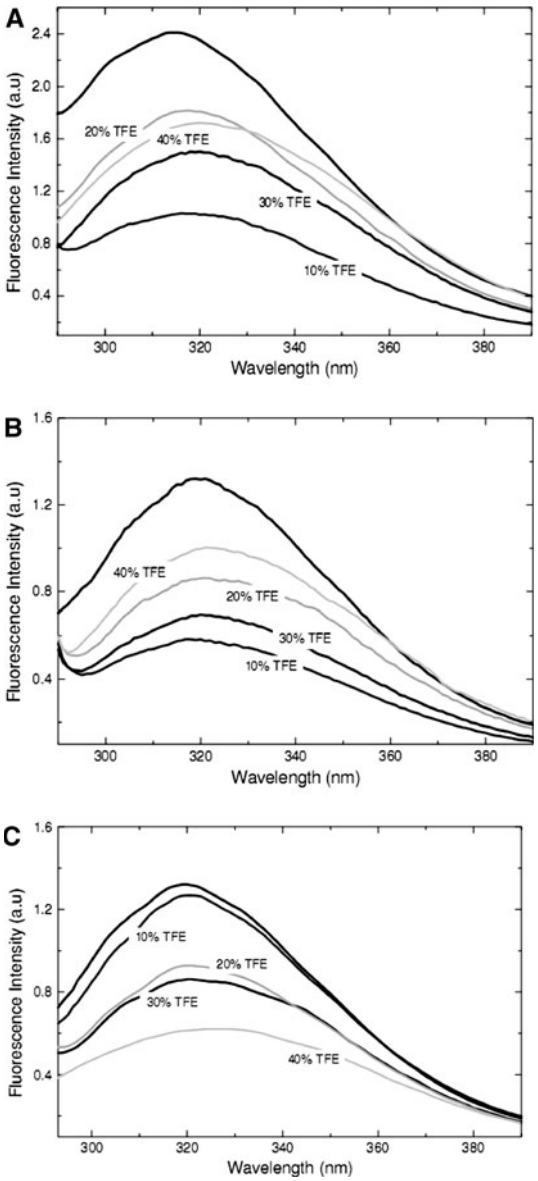

Fig. 5 Effect of TFE on Trp fluorescence of FL-KcsA (**a**), ΔN-KcsA (**b**) and ΔC-KcsA (**c**) in PC:PG (7:3 mol.%) bilayers. Representative corrected emission spectra are shown. Samples were investigated in the absence or presence of varying concentrations of TFE (vol.%)

similar to TFE-induced unfolding. In contrast, ΔC-KcsA exhibited different behavior since efficient reversibility in emission maxima was observed even at higher TFE concentrations. These results clearly indicate that decrease in channel reversibility of FL and ΔN-KcsA is most likely due to the presence of a large C-terminal domain, which might aggregate during tetramer unfolding at high TFE concentrations. It should be noted that the percentage of reversibility calculated from acrylamide quenching experiments is significantly higher than the percentage of tetramer recovered from refolding experiments shown in Fig. 4, indicating an interference of denaturant SDS in tetramer stability. Thus, fluorescence experiments provide a reliable measure of channel assembly compared to denaturing experiments.

Effect of Truncating Both N and C Termini on KcsA Tetrameric Stability

Gel electrophoresis, Trp fluorescence and acrylamide quenching experiments suggested that during tetramer unfolding the C terminus might aggregate, especially at high TFE concentration. It is also likely that the N terminus, which might flip out of the membrane upon TFE insertion between the lipid headgroups, then interacts with the C terminus, thus promoting protein aggregation at high TFE concentrations. However, what happens to the tetrameric stability when both termini are deleted? First, FL-KcsA reconstituted in PC:PG (7:3 mol.%) bilayers was treated with chymotrypsin as control (Fig. 7). As shown, the C terminus which was located extravesicularly was cleaved by chymotrypsin, thus generating (Δ125–160) KcsA tetramer as indicated by T–C (lane 2). Next, ΔN-KcsA solubilized in DDM was subjected to chymotrypsin digestion. The tetramer was found to be stable upon deletion of both termini, thus generating ΔNC-KcsA. However, the stability of this "minimum tetramer" was drastically decreased. Upon incubating DDM-solubilized ΔNC-KcsA with 5 vol.% TFE, the tetramer completely dissociated into its monomers (~13 kDa). Surprisingly, when ΔN-KcsA reconstituted in PC:PG (7:3 mol.%) bilayers was subjected to chymotrypsin hydrolysis, this minimum tetramer was immediately dissociated into monomers. Similar results were obtained when DDM-solubilized ΔNC-KcsA was reconstituted in PC:PG bilayers (not shown). Dissociation of ΔNC-KcsA was followed by two additional chymotrypsin cleaved products. M1 corresponds to the uncut monomeric ΔN-KcsA (~15 kDa); M2 relates to monomeric (Δ1–18) KcsA, which is possibly cleaved at 137 position (~14.5 kDa) since the C terminus contains two cleavage sites for chymotrypsin, while M3 corresponds to monomeric (Δ1–18) KcsA cleaved at 125 position (~13 kDa). Taken together, these results suggest that the C terminus plays a crucial role in determining the channel folding properties and it apparently stabilizes the tetramer in the absence of the N terminus in lipid bilayers. Likewise, the N terminus seems to stabilize the tetramer in the absence of the C terminus; therefore, deletion of both termini leads to an unstable tetramer.

Discussion

Increased membrane binding of a protein due to electrostatic interactions between the anionic lipids and positively

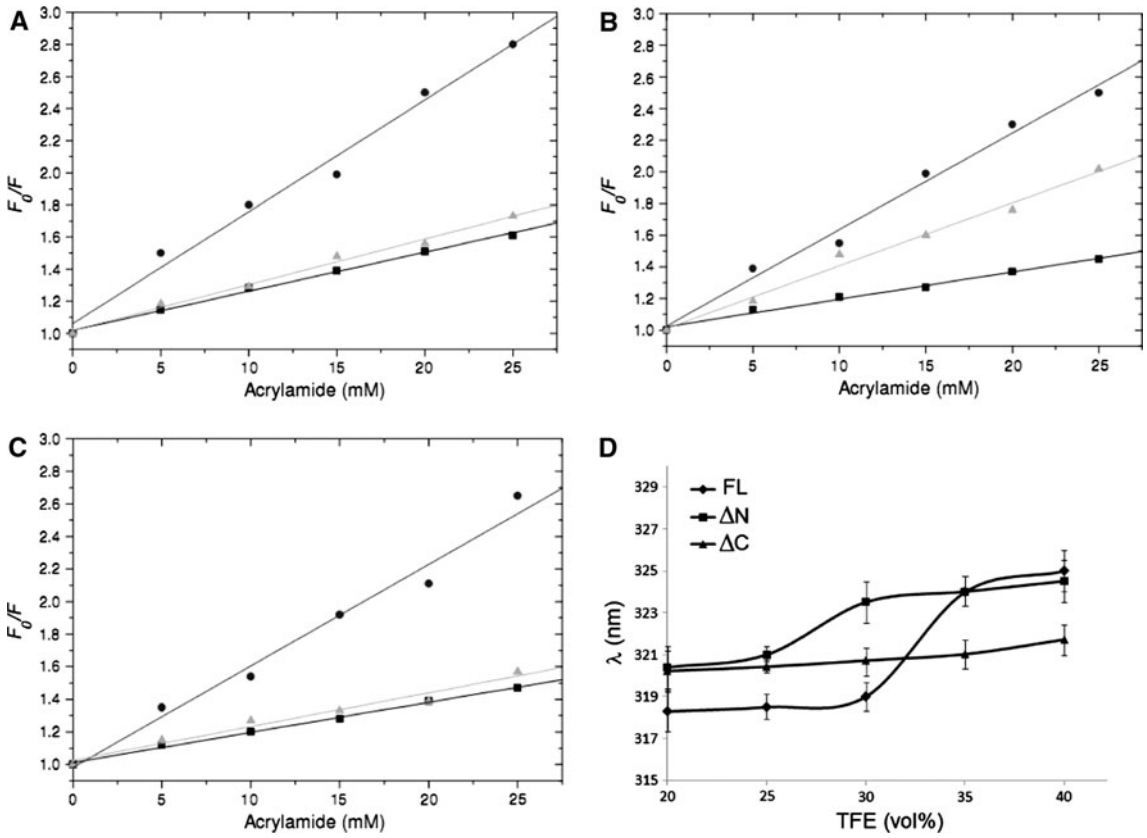

Fig. 6 Representative Stern–Volmer plots of Trp fluorescence quenching by acrylamide in FL-KcsA (**a**), ΔN-KcsA (**b**) and ΔC-KcsA (**c**) in PC:PG (7:3 mol.%) bilayers. Samples were investigated with (*filled circle*) or without (*filled square*) 30 vol.% TFE or upon diluting 30 vol.% TFE-containing samples to achieve a final concentration of 5 vol.% TFE (*filled triangle*). The slopes of the best fit linear regression lines for each data set (K_{SV} values) are shown in Table 1. **d** Average fluorescence emission wavelength (λ) as a function of TFE-induced unfolding at indicated TFE concentration followed by refolding upon dilution to reduce TFE concentration to 5 vol.%. Channel refolding upon dilution of 20 vol.% TFE to 5 vol.% resulted in similar emission wavelength (nm) as determined for 0 vol.% TFE for all proteins. Data points correspond to the average ± SD of three or four experiments

Table 2 Channel reversibility monitored by acrylamide quenching and SDS-PAGE

Protein	Reversibility (%) by acrylamide quenching[a]				Reassembly by SDS-PAGE (% tetramer)[b]
	25 vol.% TFE	30 vol.% TFE	35 vol.% TFE	40 vol.% TFE	
FL-KcsA	90 ± 6	86 ± 4	15 ± 5	0	8 ± 6
ΔN-KcsA	80 ± 2	45 ± 10	35 ± 8	0	18 ± 8
ΔC-KcsA	90 ± 8	82 ± 6	80 ± 5	74 ± 8	60 ± 14

[a] Experiments were performed upon incubating samples with 25, 30, 35 or 40 vol.% TFE and then diluting to achieve a final concentration of 5 vol.% TFE. Plots of acrylamide quenching of 30 vol.% TFE followed by buffer dilution to achieve 5% TFE (v/v) are shown in Fig. 6

[b] The amount of tetramer (%) was calculated from relative amounts of tetramer present in the absence of TFE as shown in Fig. 4. For FL and ΔC-KcsA, tetramer dissociation experiments were performed at 35 vol.% TFE, whereas 30 vol.% TFE was used to destabilize ΔN-KcsA. These samples were diluted to reduce TFE concentration to allow reassembly at 5 vol.% TFE (v/v). All data points correspond to the average ± SD of three or four experiments

charged residues seems to play an important role in protein translocation (Ridder et al. 2001; van Dalen et al. 2002). Interaction of a protein with the bilayer surface generally promotes the formation of a secondary structure (White and Wimley 1999). Therefore, it is very likely that the protein will have a substantial amount of helical content when it is bound to the membrane, resulting in a helix dipole moment, which is equivalent to ~0.5 positive charge on the N terminus and 0.5 negative charge on the C terminus. Overall, the N-terminal part of the protein will thus be positively charged while the C-terminal part is negative. Thus, in the presence of negatively charged

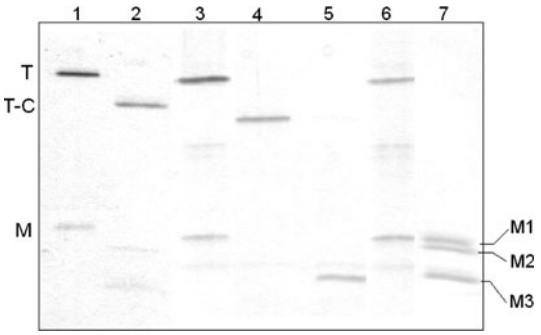

Fig. 7 SDS-PAGE analysis of proteolytic cleavage of the extravesicular C terminus of KcsA in PC:PG (7:3 mol.%) bilayers. *Lane 1*, FL-KcsA reconstituted in PC:PG (7:3 mol.%) bilayers; *lane 2*, chymotrypsin cleavage of sample from lane 1; *lane 3*, DDM-solubilized ΔN-KcsA; *lane 4*, chymotrypsin cleavage of sample from lane 3; *lane 5*, sample from lane 4 was incubated with 5 vol.% TFE for 1 h at room temperature; *lane 6*, ΔN-KcsA reconstituted in PC:PG (7:3 mol.%) bilayers; *lane 7*, chymotrypsin cleavage of sample from lane 6. Samples were directly analyzed by SDS-PAGE. Monomeric (*M*), tetrameric (*T*) and chymotrypsin cleaved tetrameric (*T-C*) KcsA are indicated and a protein marker (in kDa) is shown on the *right*

lipids, this charge distribution in the protein possibly results in interaction of the N terminus and repulsion of the C terminus by the bilayer, thus facilitating translocation of the slightly positively charged N terminus (Ridder et al. 2001).

The N terminus of KcsA forms an alpha-helix anchored at the membrane–water interface (Cortes et al. 2001). In the present study, the expression level of ΔN-KcsA was drastically decreased compared to FL-KcsA, suggesting that deletion of the N terminus interferes with efficient channel assembly or targeting of the channel in the inner membrane (Perozo et al. 1999). Previously, a slight stabilizing effect in the presence of acidic PG was observed for FL-KcsA (Raja et al. 2007). However, this effect was abolished upon removal of the N terminus, as shown in the present study, thus indicating that electrostatic interaction between the helix dipole and the acidic PG slightly increases tetramer stability. This effect can be explained by a recent description of the roles of ionic interactions between the N-terminal helix and other acidic lipids in stabilizing the tetrameric structure (Raja 2010). In addition, it has been shown that PG headgroups are able to form electrostatic interactions with Arg64 and Arg89 at the interfaces of KcsA monomers (Deol et al. 2006), explaining the specific interaction and copurification of PG with KcsA (Valiyaveetil et al. 2002). The presence of PG on the *cis* side of the membrane, according to the positive-inside rule, establishes electrostatic interaction with the membrane-associated, positively charged N terminus (Von Heijne 1986). Because TFE can strengthen electrostatic interactions (Akitake et al. 2007), its presence may make this interaction even stronger, especially at moderate TFE concentration, resulting in stabilizing these electrostatic interactions at the membrane surface.

Changes in Trp fluorescence and acrylamide quenching are quite remarkable, implying that long-range conformational changes (from the site of the perturbation/truncation to the periphery–membrane interface) can be detected upon TFE-induced unfolding or refolding. Additionally, these changes in fluorescence characteristics are supported by denaturation/SDS-gel electrophoresis experiments, which indicate that such deletions do affect the folding of the protein and, therefore, its oligomeric assembly and stability.

Furthermore, the TFE-induced changes in Trp fluorescence and acrylamide quenching during channel unfolding and refolding are in total agreement with previously reported KcsA behavior in lipid membranes, again indicating that TFE concentration up to moderate level (~30 vol.%) unfolds or destabilizes the tetramer and that this process is almost completely reversible, which can be determined by reversibility in native protein fluorescence emission wavelength (Barrera et al. 2008). However, high TFE concentrations (~35–40 vol.%) cause unfolded monomers to aggregate irreversibly into a highly helical state such that TFE-induced red shifts in protein fluorescence cannot be recovered upon TFE dilution (Barrera et al. 2008). Interestingly, such an irreversible aggregation seems to be caused by misfolding of the large C terminus, as investigated in this study. Hence, removal of this domain apparently protects protein from misfolding, thus allowing efficient reassembly of the tetramer.

The N terminus was found to be crucial for efficient channel (re)assembly, although the activity of the KcsA channel was not altered when the N-terminal helix was truncated (Perozo et al. 1999). The functional importance of the N terminus in promoting channel assembly can be exemplified by T1 domains, which correspond to a large N-terminal cytoplasmic protein stretch, in eukaryotic voltage-gated *Shaker* potassium channel (Kreusch et al. 1998). First, rapid T1 tetramerization occurs first and then transmembrane domains of channel subunits are brought into close proximity to allow full tetramerization of the channel. Furthermore, *Shaker* channels lacking their T1 domains poorly assembled into tetrameric units (Zerangue et al. 2000).

In many potassium channels, including KcsA, the C termini are also involved in tetramerization. For example, inwardly rectifying potassium (Kir) channels, which like KcsA belong to the 2TM1P-type channels, also have tetramerization domains at their C termini (Tinker et al. 1996). Deletion mutation and molecular modeling of FL-KcsA indicated putative involvement of the 120–124 sequence stretch in tetramerization of KcsA subunits (Molina et al. 2004), which was not truncated in the present

study. According to molecular modeling (Molina et al. 2004), the interactions between residues 120–124 and the N- and C-terminal segments of the adjacent monomer are mainly electrostatic, which involve interactions between T112, E118, Q119, E120, R121, R122 or H124 on the C terminus and A23, S22 or H25 on the N terminus. Thus, every 120–124 stretch in each monomer could form up to four to five hydrogen bonds with the adjacent monomer, which might lead to a priming event in channel tetramerization.

In the crystal structure of FL-KcsA (Uysal et al. 2009), the helices below the inner bundle gate at position 115 bend significantly and adopt a conformation parallel to the axis of symmetry. This helical segment might play an important role during the opening of the channel by providing the necessary flexibility for the conformational changes that occur during channel activation. However, truncating the channel at position 124 has a direct impact on the conformation of the closed gate via weakening of the intersubunit van der Waals contacts between side chains below the inner bundle gate, which changes the conformation of the gate and destabilizes the closed conformation. This information can be combined with the intrinsic stability of the KcsA channel, demonstrating that a decrease in oligomeric stability is most likely related to a destabilizing effect on the closed channel conformation upon C-terminal truncation.

To simplify the system, channel unfolding and refolding events are proposed in schematic models (Fig. 8). During partial unfolding at moderate levels of TFE, the N terminus is not inserted into the lipid bilayer anymore due to the partitioning of TFE in the lipid headgroups (Fig. 8b); and at higher levels of TFE, the pore region is flipped out of the membrane, as depicted by strong red shifts in emission maxima and quenching by acrylamide, whereas the C terminus undergoes distinct conformational changes due to direct interaction of TFE with the protein, as suggested previously (Van den Brink-van der Laan et al. 2004a). Such changes will possibly be accompanied by a transition from partial unfolding to complete unfolding, thus leading to a more cylindrically shaped structure of the KcsA subunit (Fig. 8c). Since the C terminus is the site for major conformational changes (Uysal et al. 2009), removal of this domain (Fig. 8d) may eliminate such changes in the protein, which might affect the stability of channel conformation and promote efficient reassembly of channel subunits from either partially (Fig. 8e) or completely (Fig. 8f) unfolded tetramer.

The channel's intrinsic tetrameric stability upon removal of the C terminus by enzymatic cleavage is in good agreement with previous observations obtained by thermal denaturation experiments on C-terminally truncated KcsA

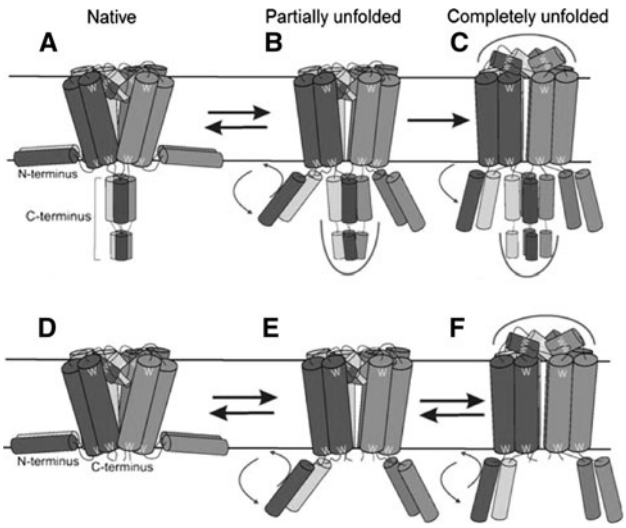

Fig. 8 Schematic modeling of KcsA unfolding and refolding in membranes. **a** KcsA tetramer is shown in the form of four cylindrical subunits. **b** During partial unfolding, the N terminus sticks out of the membrane interface and the C terminus undergoes distinct conformational changes. Such a state of tetramer is reversible. **c** Denaturation or complete unfolding at higher TFE concentration leads to irreversibility in channel tetrameization, presumably via aggregation or misfolding of the C terminus. However, in the absence of the C terminus (**d**) tetramer can refold from either a partially (**e**) or a completely (**f**) unfolded state. In the denatured/completely unfolded state (**c**, **f**) the flipping out of the pore region has also been depicted and the positions of Trp residues (W) are indicated at membrane–water interfacial regions

(Perozo et al. 1999), suggesting once again that the stability of the channel does not depend to a great extent upon the presence of an intact C terminus. Furthermore, removal of 125–160 residues not only facilitates efficient tetramerization of KcsA subunits, as observed previously (Molina et al. 2004), but also enhances the efficiency of tetrameric reassembly, thus allowing proper interactions among intramembrane protein sites in the membrane.

An intriguing observation of this study is that truncation of both termini drastically decreases the channel stability in detergent micelles; however, this "minimum tetramer" is immediately dissociated into monomeric subunits upon insertion into the membrane. Since N- and C-termini exert their considerable stabilizing effects, it is reasonable to assume that unavailability of these extramembranous parts somehow alters the protein conformation in the membrane, thus resulting in tetramer dissociation. This effect seems completely opposite to "lipid-facilitated" tetrameric formation, which occurs in the absence of any cytoplasmic domain (Shai 2001). An important consideration in future mechanistic analyses is required for a comprehensive understanding of how alteration in protein–lipid interaction affects the assembly and stability of these oligomeric complexes.

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