Detergent-labile, supramolecular assemblies of KcsA: Relative abundance and interactions involved ☆

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A B S T R A C T

In this work, we illustrate the ability of the prokaryotic potassium channel KcsA to assemble into a variety of supramolecular clusters of defined sizes containing the tetrameric KcsA as the repeating unit. Such clusters, particularly the larger ones, are markedly detergent-labile and thus, disassemble readily upon exposure to the detergents commonly used in protein purification or conventional electrophoresis analysis. This is a reversible process, as cluster re-assembly occurs upon detergent removal and without the need of added membrane lipids. Interestingly, the dimeric ensemble between two tetrameric KcsA molecules are quite resistant to detergent disassembly to individual KcsA tetramers and along with the latter, are likely the basic building blocks through which the larger clusters are organized.

As to the proteins domains involved in clustering, we have observed disassembly of KcsA clusters by SDS-like alkyl sulfates. As these amphiphiles bind to inter-subunit, "non-annular" sites on the protein, these observations suggest that such sites also mediate channel–channel interactions leading to cluster assembly.

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1. Introduction

There is increasing evidence to support the notion that ion channels and neuroreceptors in their native cellular environments are likely to assemble into supramolecular complexes or clusters in which their function becomes modulated through homomeric or heteromeric molecular interactions [1,2]. According to observations on membrane protein receptors [3], clustered assemblies in which the activity of one receptor influences that of its neighbors, convert conformational changes from a single origin into intermolecular allosteric behavior. Indeed, some of the best characterized cases in this regard show that cooperative, coupled channel gating depends upon clustering. For instance, ryanodine receptors, the Ca^{2+} release channels in sarcoplasmic reticulum in skeletal and cardiac muscle, form arrays showing coupled gating as an important regulatory mechanism in excitation–contraction coupling [4,5]. Other instances where clustering and coupled gating affect channel function include inward rectifier Kir 4.1 potassium channels [6]; cystic fibrosis transmembrane conductance regulator chloride channels [7], nicotinic acetylcholine receptors [8], IP3 receptor Ca^{2+} channel [9,10] and several members of the ATP-gated channel and the G-protein-coupled receptor families [11,12], including rhodopsin [13]. Moreover, cooperative gating of clustered sodium channels has been used to explain non-Hodgkin and Huxley behavior in the dynamics of action potential initiation in mammalian neurons [14]. Nonetheless, the details on the nature and the mechanisms involved in regulating the assembly/disassembly processes of these biologically-relevant complexes remain largely unknown.

KcsA is a prokaryotic potassium channel structurally simpler than its eukaryotic counterparts and it has been used extensively to explain ion selectivity and permeation in ion channels at the molecular level [15]. The crystal structure of KcsA shows a homotetramer in which each 160 amino acids subunit defines two transmembrane segments connected by a pore region that contains an ion selectivity filter unmistakably homologous to the more complex eukaryotic K^+ channels [15]. Despite such apparent simplicity, it has been reported that the membrane-bound KcsA tetramer further assembles into still insufficiently defined clusters, both in vitro [16–18] and in vivo [19]. Such clusters form in a protein concentration-dependent manner [16] and seem responsible for the adoption of different modes of coupled gating, which causes large changes in channel function [16,17]. Indeed, KcsA clustering is likely behind the complex activity patterns detected in

many electrophysiological studies [20–26], which most often have not been analyzed in detail. The observed clustering of KcsA, along with the availability of its high resolution structure and the ease to express it at high yields, makes this protein a potentially good model to study these phenomena of protein clustering and coupled gating. However, evidencing KcsA clusters has so far required fairly sophisticated experimental techniques [16,19], which are not too amenable for use on a routine, quantitative monitoring of assembly/disassembly processes. Moreover, simpler techniques such as conventional SDS-PAGE of the expression products from the KcsA construct, renders KcsA as a major band corresponding to the well-known SDS-resistant tetramer of four identical subunits (Fig. 1A), but usually accompanied by just a minor band corresponding to dimers of such tetramers (labeled 2T in the figure) as the only evidence of supramolecular assembly [16,27–29]. These latter observations suggest that detergents such as those used in conventional electrophoresis analysis cause cluster disassembly and therefore prevent the observation of the native clusters.

Here we report on simple electrophoretic methods as alternative analytical procedures to evaluate rapidly the supramolecular assembly of KcsA tetramers into clusters. Indeed, instead of the well-known individual KcsA tetramers, clusters of different sizes are observed as major components of the KcsA batches. Such clusters appear to have KcsA tetramers and dimers of tetramers as the elemental building blocks through which the larger, cooperative clusters are assembled in a reversible process which does not need the presence of added lipids or a membrane environment.
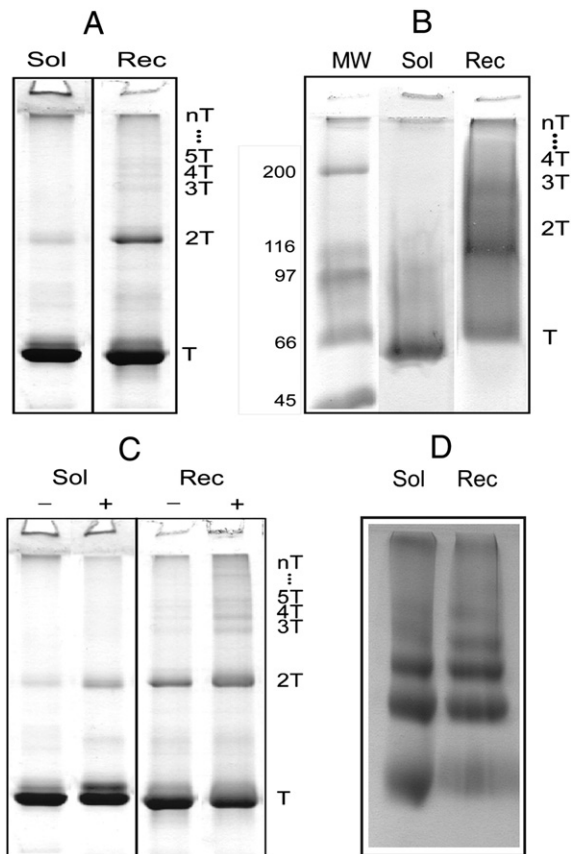
Fig. 1. Visualization of KcsA clusters by different electrophoretic methods. SDS-PAGE (A, C), PFO-PAGE (B), and BN-PAGE (D). "Sol" lanes correspond to purified KcsA solubilized in 5 mM DDM (and supplemented with 1 mM SDS in D), while "Rec" lanes correspond to KcsA reconstituted in asolectin vesicles. "Plus" sign lanes correspond to KcsA samples crosslinked with glutaraldehyde, while "minus" correspond to control samples without crosslinking. T stands for the KcsA tetramer, while 2T, 3T, 4T, 5T… nT refer to cluster species having 2, 3, 4, 5 … n-times the molecular weight of the KcsA tetramer. MW stands for molecular weight standards.

2. Materials and methods

2.1. Expression, purification and reconstitution of KcsA

Expression and purification of wild-type KcsA were as described previously [16]. To reconstitute KcsA, batches of large unilamellar vesicles of asolectin (soybean lipids, type II-S, Sigma) were prepared at 25 mg/ml in 10 mM Hepes, pH 7, 100 mM KCl, and stored in liquid nitrogen, as described previously [30]. Then, asolectin was solubilized at a 0.8 DDM (Calbiochem)/lipid molar ratio and mixed with purified DDM-solubilized KcsA in 1 mM DDM at 500:1 lipid to protein molar ratio. The detergent was removed using Bio-Beads SM-2 (Bio-Rad laboratories) at a mass ratio of 100:1 relative to the detergent in two rounds, one of 2 h at room temperature and a second one of 12 h at 4 °C, which resulted in the formation of proteoliposomes. Samples were stored in liquid nitrogen in 10 mM Hepes buffer, pH 7, 100 mM KCl.

Crude membrane extracts were obtained from *Escherichia coli* M15 (pRep4) cells harvested after 2 h of isopropyl β-D-thiogalactopyranoside induction, as described [27].

2.2. SDS-PAGE, PFO-PAGE and crosslinking

SDS-PAGE and PFO-PAGE analysis were carried out as reported [16,31]. For crosslinking "in vitro", 15 μg of purified KcsA, either as a solubilized protein or reconstituted into asolectin lipid vesicles at 1 mg protein/ml, was reacted with different concentrations of glutaraldehyde (ranging from 0.001 to 0.5%, w/v) for periods of time from 1 min to overnight and at room temperature [32]. The conditions chosen for routine experiments were 0.005% w/v glutaraldehyde and 1 h incubation at room temperature. For crude membrane extracts, cells were resuspended in 150 μl PBS, pH 7.4, divided into four aliquots and reacted with glutaraldehyde at concentrations of 0.005 and 0.0125% (w/v), for 1 h at 4 °C. These conditions were similar to those used to crosslink E-cadherin–catenin complexes in epithelial cells [33]. After crosslinking, the cells were lysed by adding lysozyme (final concentration 0.4 mg/ml) for 5 min and by submitting them to six temperature cycles (each including 1 min in liquid nitrogen and 1 min at 42 °C), followed by vortexing. Cell lysates were centrifuged for 15 min at 13,000 ×g, the supernatant discarded and the pellet solubilized in 40 μl of 10 mM Hepes buffer, pH 7, 100 mM KCl, containing 10 mM DDM, followed by several passages through a Hamilton syringe. The resulting homogenates were centrifuged for 15 min at 13,000 ×g and 5 μl aliquots of the supernatants were mixed with 5 μl of SDS sample buffer and used for SDS-PAGE. Western blot was done as reported [16], with the exception that the antibody solutions contained 3% (w/v) bovine serum albumin.

2.3. Blue native PAGE

Blue native PAGE (BN-PAGE) was performed in linear 4–16% (w/v) polyacrylamide-gradient gels as described [34,35]. Samples consisted of either reconstituted KcsA in asolectin lipid vesicles, or purified DDM-solubilized KcsA. When handling reconstituted samples, 30 μg of protein was centrifuged at 18,500 ×g for 10 min. Once the supernatant was discarded, the pellet was resuspended with either DDM or SDS, and incubated at 4 °C O/N for its solubilization. After centrifugation at 18,500 ×g for 10 min., the supernatant containing the solubilized protein was collected and supplemented with a 5% Coomassie Brilliant blue G stock solution in 750 mM aminocaproic acid just before to be applied onto polyacrylamide gradient gels. For purified DDM-solubilized KcsA, 20 μg of protein was directly supplemented with alkyl sulfates (when indicated) and Coomassie Brilliant blue G as above, and applied onto the gel. Electrophoresis was initiated at 85 V for 30 min, and continued at 200 V for 2.5 h at 4 °C. The protein bands in the gel were visualized by Coomassie blue staining. All gels were scanned and analyzed with ImageQuant TL v2005 software (Molecular Dynamics).

For crude membrane extracts, 200 μg of cell pellet was suspended in 2 ml of 20 mM Tris–HCl pH 8, 0.45 mM sucrose, 8 mM EDTA supplemented with PMSF (0.3 mg/ml). Cells were lysed and processed as shown above for the crosslinking experiments except that the solubilization buffer was 80 μl of 50 mM Bis–Tris pH 7, 750 mM aminocaproic acid, 10% glycerol, 40 mM DDM, and that the solubilized supernatant was supplemented with a 5% Coomassie Brilliant blue G stock solution in 750 mM aminocaproic acid and SDS at 1 mM final concentration. Electrophoresis was carried out as explained above. Proteins were then electroblotted from the gel onto a PVDF membrane at 30 V overnight. Blots were treated with 62.5 mM Tris–HCl pH 6.8, 2 % SDS for 1 h at 50 °C, dried and activated in methanol for 30 s to eliminate the Coomassie blue dye, which interferes in immunodetection. After washing in TBST (50 mM Tris–HCl pH 7.4, 25 mM NaCl, 0.05% Tween 20) blots were processed for Western blot by blocking in 3% (w/v) bovine serum albumin in TBST, then incubated with a mouse monoclonal anti-Tetra-His antibody (1:2000, Qiagen) and diluted in the blocking solution. After washing, the immunoblots were incubated with a secondary horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:10,000, Sigma). Immunoreactive proteins were visualized by chemiluminescent ECL select detection reagent (Amersham Biosciences) in a LIAS ChemLite 200F/400F (Avegene) imaging system.

2.4. 2D-PAGE

20 μg of purified DDM-solubilized KcsA was submitted to a BN-PAGE in the first dimension as detailed in Section 2.3. The corresponding gel strip containing the sample was excised and submitted to a SDS-PAGE in the second dimension. To do this, the strip was layered on top of a 13.5% polyacrylamide SDS-PAGE resolving gel and a 4% polyacrylamide stacking gel was polymerized all around the gel strip to fill the gap not covered by it. An identical gel strip from the BN-PAGE first dimension was stained and has been included on top of the Fig. 2 as a reference.

3. Results

3.1. Electrophoretic methods to detect KcsA clustering

Fairly sophisticated and time-consuming procedures, such as FRET between batches of fluorescently-labeled protein [16] or immunogold staining [19], have evidenced extensive KcsA clustering both "in vivo"

and "in vitro". Nonetheless, following purification from detergent-solubilized extracts, KcsA appears mostly as the well known, SDS-resistant tetrameric protein in conventional SDS-PAGE (Fig. 1A). Moreover, the only indication of supramolecular assembly is a minor band corresponding to a dimer of such tetramers (labeled 2T in the figure), which appears in the gels instead of the expected larger clusters. A possible explanation to reconcile the above observations is that SDS and other detergents used to solubilize the samples disrupt the channel–channel interactions holding together the larger supramolecular assemblies, thus, causing the release of the individual KcsA tetrameric molecules. Fig. 1A also shows that the 2T band is more prominent when these experiments are carried out with samples of KcsA reconstituted into lipid membranes.

In an attempt to find simple alternatives to SDS-PAGE to assay KcsA samples without causing a major disruption of clusters, we first tested PFO-PAGE, very similar to SDS-PAGE but substituting SDS by a milder detergent such as PFO, which is quite effective in solubilizing membrane proteins while partly preserving the interactions within quaternary structures or supramolecular complexes [31,36]. Just as in SDS-PAGE, the electrophoretic mobilities of proteins in PFO-PAGE are linearly dependant on the logarithm of their molecular masses [31] although the bands in PFO-PAGE are not as sharp as in SDS-PAGE and frequently trail off. The tetrameric KcsA in PFO-PAGE (T species; migrating at an apparent molecular weight of 66 kDa) still appears as the major component in the DDM-solubilized samples. Conversely, the profile of samples from KcsA reconstituted into membranes shows more prominent heavier bands than the SDS-PAGE does, and in particular the 2T band appears now as the most abundant KcsA species seen in the gel (Fig. 1B). A previous study combining PFO-PAGE and purified KcsA solubilized in decyl-maltoside (instead of the dodecyl-maltoside used here) already showed a clear predominance of supramolecular species compared to the individual KcsA tetramers [37]. Such a supramolecular population, however, appeared as a single band larger than 300 kDa which could not be resolved into clusters of different sizes under the experimental conditions used in that report.

In order to obtain sharper bands while preserving the KcsA clusters, we have used glutaraldehyde crosslinking techniques in otherwise conventional SDS-PAGE. The rational here is that disassembly by SDS of detergent-labile KcsA clusters into isolated tetramers could be prevented by crosslinking interacting tetramers within the
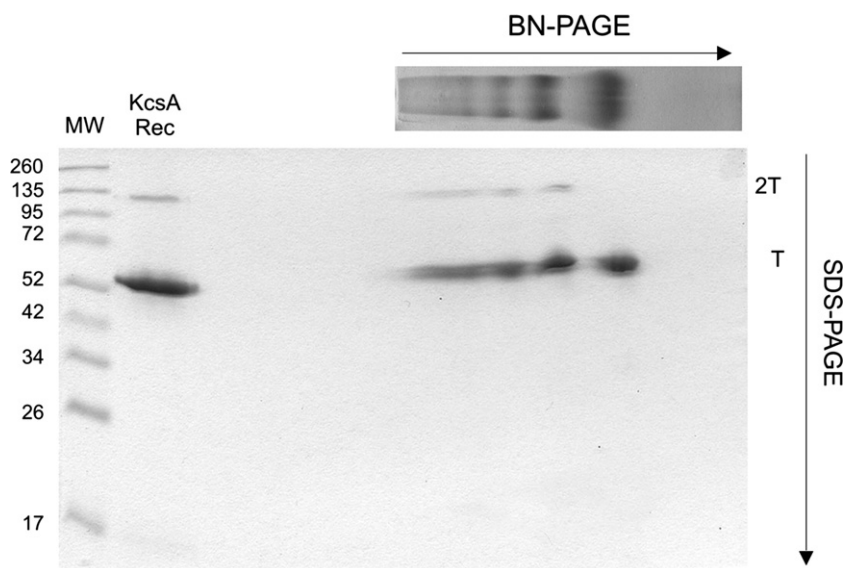
Fig. 2. Assignment of cluster bands in BN-PAGE gels by 2D-PAGE. Reconstituted KcsA in asolectin was solubilized with 40 mM DDM and subjected to a 2D-PAGE (see Methods) consisting first of a BN-PAGE, followed by SDS-PAGE in the second dimension. In the latter, commercial molecular weight standards (MW) and a sample of KcsA reconstituted in asolectin lipid vesicles (KcsA Rec) were run in separate wells as references (see text). Abbreviations and symbols are as in Fig. 1.

clusters prior to detergent treatment. This is similar to the approach used by Failer et al. [32], except that we use milder conditions (20-fold lower concentrations of glutaraldehyde) in order to prevent possible unspecific protein crosslinking. Fig. 1C illustrates that our mild glutaraldehyde treatment causes that molecular weight bands corresponding to integer values of the KcsA tetramer (labeled as 2T, 3T and so on, in the figure) appear more prominently upon crosslinking, as it would be expected from the occurrence of KcsA clusters prior to SDS treatment. Nonetheless, because of our mild cross-linking conditions, it is reasonable to assume that the crosslinked cluster bands seen in the gels result from detergent-induced disassembly of larger protein complexes insufficiently crosslinked under our experimental conditions and therefore, represents an underestimation of clustering. Again, cluster bands are more apparent when lipid-reconstituted samples, instead of detergent-solubilized ones, are used in the experiments. This suggests that KcsA clusters become partly disassembled in the DDM-solubilized purified samples, and therefore, cannot be crosslinked so efficiently.

As an additional tool to evaluate KcsA clustering we performed BN-PAGE, which has become a method of choice to define the oligomeric state of different proteins, and to study the organization of protein complexes in their native state [34,38–40]. Moreover, this technique has been used as an alternative to size exclusion chromatography (SEC) or dynamic light scattering (DLS) in evaluating protein aggregation states before crystallization [41]. In BN-PAGE, samples are first solubilized with a mild detergent, usually digitonin or DDM, but it is the negative charge from the Coomassie Brilliant Blue bound to the hydrophobic protein surfaces what determines the protein electrophoretic mobility. Fig. 1D shows that several bands can be clearly observed by BN-PAGE, which correspond to different KcsA supramolecular assemblies. Also, as observed with the other electrophoretic methods, when the protein is reconstituted into lipid vesicles ("Rec" line in the figure), the higher molecular weight bands in BN-PAGE are more intense than those from DDM-solubilized samples ("Sol" line). These results were highly reproducible when using different purified protein batches.

A known drawback of the BN-PAGE technique when dealing with membrane proteins is the difficulty to assign molecular weights to the gel bands [39], which are usually overestimated because membrane proteins bind lipids, detergents and Coomassie dye that contribute to an apparent weight larger than real [39,42]. Indeed, using a standard set of soluble proteins as molecular weight markers, the estimated molecular weight for the fastest migrating KcsA band was approximately 140 kD, instead of the theoretical 70 KDa expected for the tetrameric KcsA (data not shown). This is confusing because one could erroneously assign such band to the 2T species mentioned above. Therefore, in order to identify correctly the KcsA oligomers states we performed a two-dimensional electrophoresis (2D-PAGE), BN-PAGE in the first dimension and SDS-PAGE in the second one, since the latter allows unequivocal molecular weight assignments. Fig. 2 shows that in the BN-PAGE dimension there are several KcsA cluster bands that yield T and 2T species when submitted to the second SDS-PAGE dimension. The exception to this pattern is the fastest migrating band in BN-PAGE, which yields only T species in the SDS-PAGE dimension. In addition to confirming the efficiency of SDS on disassembling KcsA clusters, these observations bear on two important additional facts. First, they demonstrate that all the heavier bands in BN-PAGE result from the association of KcsA tetramers and dimers of tetramers (T and 2T species, respectively). Second, it is confirmed that the fastest migrating band in the BN-PAGE gels corresponds exclusively to T species (KcsA tetramers), as it is the only band which does not yield 2T species in the second dimension.

We also used samples from crude membrane extracts to test the presence of KcsA clusters in such native environment. In a first approach, intact E. coli cells expressing the KcsA construct were crosslinked with glutaraldehyde, lysed and a membrane fraction obtained by centrifugation. Such fraction was solubilized by DDM

and analyzed by SDS-PAGE and immunoblotting. Fig. 3A shows that, similarly to the observations on the purified protein (see Fig. 1C), electrophoretic bands heavier than the KcsA tetramer are observed in response to increasing the glutaraldehyde concentration, even under the mild cross-linking conditions used here. In a second approach, a crude membrane fraction was also obtained from E. coli cells and analyzed by BN-PAGE and immunoblotting. It can be seen that the band pattern of a control sample of purified KcsA is comparable to that of the crude membrane extract (Fig. 3B and C) except for the reduced mobility of bands in the latter, probably due to a larger amount of lipids bound to KcsA [39,42]. This result shows that the protein in a native environment is also assembled into similar clusters to those observed in the purified KcsA preparations. This is consistent with a recently published manuscript on clustering of membrane proteins in which very similar or identical transmembrane segments seem required for clustering to occur and therefore, "homoclusters" containing a single class of membrane proteins should be favored [43].

3.2. Use of detergents to study the assembly and disassembly of KcsA clusters

In order to explore how the individual channels are clustered, we have also used detergents to disassemble KcsA clusters in a progressive, concentration-dependant manner. This is a similar approach to that used for decades in unfolding proteins by denaturants or chaotropic agents from which so much folding information has been derived. In these experiments we determine the ability of either DDM or SDS, a milder and a harsher detergent, respectively, i) to solubilize KcsA samples for electrophoretic purposes and ii) to disrupt channel–channel interactions within the clusters. For these purposes, reconstituted KcsA samples were exposed to increasing detergent concentrations and the resulting band patterns analyzed by BN-PAGE. Fig. 4A shows that a minimum of 20 to 40 mM DDM is needed to solubilize properly the sample. The term *properly* used here means that if less detergent is used, the protein is mostly lost when the samples are pelleted before being applied to the gel, or remain at the interface of the separating gel upon completion of the electrophoresis run (Fig. 4A, first lane). On the other hand, a concentration of DDM above 150 mM is needed to start disassembling significantly the KcsA clusters. Then, bands corresponding to 3, 4 and 5T KcsA species begin to disappear from the gel, although
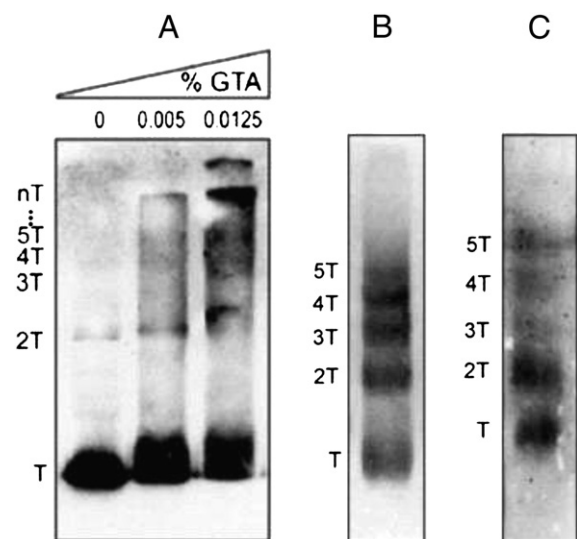
Fig. 3. KcsA clusters in crude membrane extracts from *Escherichia coli* cells. Immunoblot of a 7.5% SDS-PAGE with crude membrane extracts from crosslinked intact *E. coli* cells expressing KcsA (A). Immunoblot from a BN-PAGE with 5 μg of purified DDM-solubilized KcsA (B), and a crude membrane extract of *E. coli* cells expressing KcsA (C). Abbreviations and symbols are as in Fig. 1. GTA stands for glutaraldehyde.
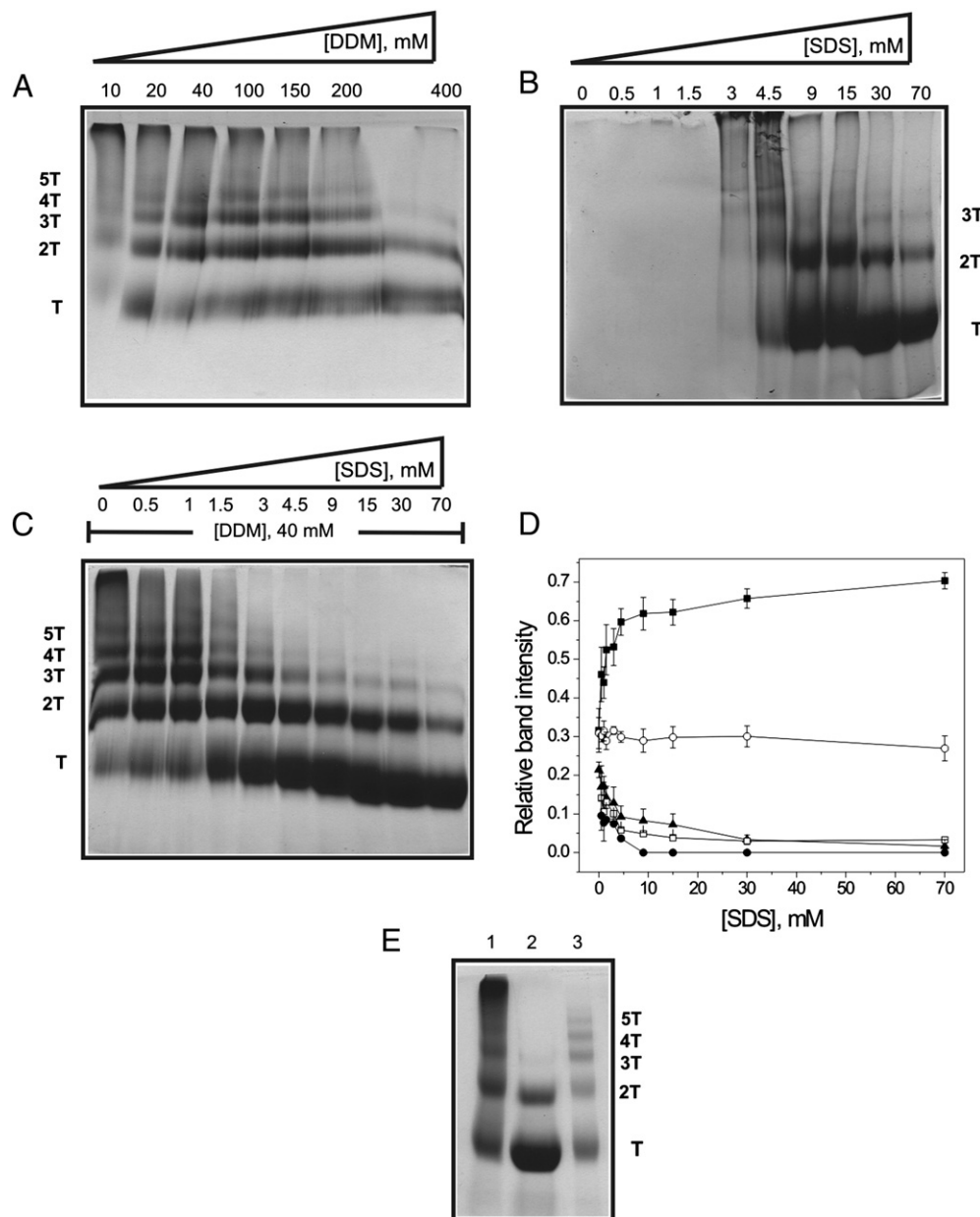
Fig. 4. Detergent-induced disassembly of KcsA clusters. Reconstituted KcsA in asolectin was incubated with different concentrations of DDM (A) or SDS (B and C) before being applied to a BN-PAGE. Samples were solubilized with 40 mM DDM before adding SDS (C). Panel D shows the analysis of BN-PAGE gel bands such as that from C. The results are expressed as the intensity of each band relative to the sum of all the bands present within each lane. Values represent the average ± SEM of 5 different gels. Symbols indicate T (■), 2T (○), 3T (▲), 4T (□) and 5T (●) KcsA species. BN-PAGE was also used to test the reversibility of the assembly/disassembly process (panel E). For that purpose, purified KcsA solubilized in 20 mM Hepes, pH 7.0, 100 mM KCl, and 5 mM DDM (lane 1) was incubated with 70 mM SDS for 5 min (lane 2) and then dialyzed overnight against the same buffer but without SDS (lane 3).

the 3T band for instance, is still somewhat visible even at 400 mM DDM (Fig. 4A). These observations indicate a relative resistance to DDM of the channel–channel interactions in the KcsA clusters.

When SDS was used in these experiments instead of DDM, it was observed that concentrations of SDS lower than 3 mM do not suffice to properly solubilize the reconstituted samples. From 3 to 4.5 mM SDS, large KcsA clusters are detected, although the bands are not sharp enough and still some protein does not enter the gel. Finally, when SDS is at concentration above 9 mM the samples are satisfactorily solubilized, but most of the protein appears already disassembled in the form of T and 2T species, not too different from conventional SDS-PAGE, where SDS is used at around 70 mM (Fig. 4B).

In designing a more precise monitoring of detergent-induced disassembly of KcsA clusters we have combined the best features of the two

detergents from above. Thus, moderate concentrations of DDM (40 mM) was first used to properly solubilize the reconstituted membranes while leaving the clusters fairly unaffected, followed by addition of increasing concentrations of SDS to have a precise monitoring of cluster disassembly in the BN-PAGE. Fig. 4C shows that at the lowest SDS concentrations, a somewhat continuous trail of protein material is seen at the top of the gel, suggesting the existence of very large KcsA clusters under those conditions. Clusters corresponding to 2, 3, 4 and 5T species are also seen at those low SDS concentrations as defined bands in the gel. Monitoring the intensity of the individual bands as the SDS concentration is increased (Fig. 4D), shows an increase in the T band concomitant with a decrease in most other bands, following an apparent precursor-to-product relationship. It seems that the larger the clusters, the more detergent-labile they are; for instance, at around

4.5 mM of SDS, the trail of larger KcsA clusters and the 5T species have practically disappeared from the gel, while the 4T and 3T species, although diminished, remain quite noticeable. In contrast, the 2T species exhibits a different behavior which is worth mentioning. First, it is more abundant than the other clusters bands, in fact, quite similar to that of the T band when no SDS is present. Second, 2T species are much more resistant to SDS than any other cluster, as it remains fairly noticeable even at the highest SDS concentrations, when all other clusters have long disappeared. Third, the 2T species show a moderate but reproducible increase at low SDS concentrations, while all other clusters decrease. This unique feature suggests that when disassembled by the detergent, larger cluster species such as 3T, 4T or 5T give rise to 2T, in addition to T species. This interpretation is also supported by the two-dimensional gel in Fig. 3, where both T and 2T species are observed from the disassembly of the larger clusters when subjected to the second dimension, SDS-PAGE.

Extending the parallelism with the studies on unfolding/refolding of proteins by denaturants, we decided to evaluate the reversibility of the clustering process by exploring the possible re-assembly of detergent-disassembled Kcsa clusters upon detergent removal. To do so, solubilized KcsA treated with 70 mM SDS, where the larger clusters have disassembled into T and 2T species, was dialyzed against a DDM buffer to eliminate SDS. This results in the re-assemble of KcsA into larger clusters of 3T, 4T, and 5T, indicating that the assembly/disassembly of clusters is indeed a reversible process (Fig. 4E).

3.3. Effect of alkyl sulfates of different chain length on KcsA clustering

As shown above, SDS is quite efficient in disassembling KcsA clusters. However, we previously reported on the ability of SDS to stabilize the tetrameric structure of the individual KcsA channel by binding to the so called "non-annular" sites [44], which correspond to crystallographically-characterized inter-subunit crevices within the protein quaternary structure [45]. In an attempt to evaluate in more detail the correlation between these two phenomena, we have studied the effects of SDS-like alkyl sulfates, ranging 8 to 14 carbon atoms chain length, on the cluster pattern of DDM-solubilized KcsA by BN-PAGE. Fig. 5 shows that the disassembly of clusters by the alkyl sulfates is strongly dependent on their chain lengths. Thus, increasing concentrations of octyl sulfate have little or no effects on KcsA clusters. On the contrary, clusters disassemble to a certain extent in presence of decyl sulfate at high concentrations, or much more effectively, in presence of the dodecyl or tetradecyl analogues, even at fairly low concentrations. These effects of the alkyl sulfates in *destabilizing* KcsA clusters, closely parallel those reported for these molecules in *stabilizing* the KcsA tetramers [44], suggesting that the occupancy of the inter-subunit "non-annular" sites by the alkyl sulfates interferes with channel–channel interactions involved in clustering.

4. Discussion

Simple electrophoretic assays that minimize the effects of detergents on protein cluster assembly including PFO-PAGE, crosslinking/SDS-PAGE and BN-PAGE have been used here to address clustering of KcsA channels, including the relative abundance of the various species detected, their stability against different detergents, or the reversibility of the process. Despite specific advantages or disadvantages for a given application, all these assays illustrate the ability of KcsA to assemble into a variety of defined, detergent-labile supramolecular complexes or clusters. Such clusters are readily detected either in solubilized preparations of purified KcsA, in reconstituted samples, and in intact cells, suggesting that they represent the native, membrane-bound KcsA species existing "in vivo". The detection of clustering by these procedures is also in agreement with more complex and time-consuming studies, such as those based on FRET [16], AFM [18], or in situ immunogold staining

[19], as well as with functional measurements from patch-clamping reconstituted KcsA in giant liposomes [16], where single channel events represent just a fraction of the observed KcsA gating events, which are dominated by different modes of coupled gating resulting likely from channel clustering.

An interesting feature of KcsA clustering is the variety of the resulting supramolecular assemblies. Such assemblies appear always more abundant when analyzing KcsA reconstituted into membranes instead of detergent-solubilized samples, suggesting that a membrane milieu could favor a shift in the equilibrium towards the assembled KcsA species.

In addition to the T species, (i. e., the individual KcsA tetrameric channel), at least 2T, 3T, 4T and 5T species have been clearly detected. All these protein bands show reactivity in Western blots against anti-His antibodies, thus confirming them as expression products of the KcsA construction (Fig. 3B). Such diversity is reminiscent of that seen in the voltage-dependent anion channel from the mitochondria outer membrane (VDAC), which assembles into clusters containing anywhere from 1 to 20 individual molecules, seemingly related to a diversity of functional capabilities of this protein [46–48].

KcsA clusters are detergent-labile, as they disassemble mostly to the well-known KcsA tetramer depending upon detergent type and treatment. On the contrary, such tetramers are extremely resistant to detergents and other protein denaturants [45] and therefore, KcsA tetramers could be considered an "artifactual" final product from all detergent-based treatments, including those used in the solubilization and purification of the protein bacterial lysates. That is the reason why purified KcsA in conventional SDS-PAGE (where SDS is present at approximately 70 mM or higher concentration) shows the SDS-resistant, T species as the major component ([16,27–29], and Fig. 1A). In fact, the detergent-lability of the KcsA clusters constitutes also a drawback in the electrophoretic techniques used here, as they all use a given detergent at a given concentration. In this respect, SDS has been found to be particularly disruptive of the channel–channel interactions supporting clustering, but DDM or even PFO are disruptive too, although to a lesser extent. Therefore, the estimated KcsA cluster sizes and the relative abundances reported here are likely to represent an underestimation of clustering because of partial detergent-induced disassembly. Detergent-lability, on the other hand, can also be used as a tool to explore how individual KcsA molecules are packed into clusters. The progressive disassembly by SDS under controlled conditions (Fig. 4C) shows that the larger clusters are more detergent-labile than the 2T species, which indeed could be considered detergent-resistant. This suggests that KcsA tetramers are packed differently into clusters depending on the number of KcsA units clustered and involves different channel–channel interactions with variable resistance to disruption by SDS. In addition to a detergent resistance higher than any other cluster, the 2T species is unique in that along with the T species, it seems a final product from the disassembly of larger clusters. Interestingly, the mixture of T and 2T species resulting from detergent disassembly can re-assemble back into the larger clusters upon detergent removal, thus, indicating the reversibility of the assembly/disassembly process even in the absence of lipids. The peculiarities of the 2T KcsA species are also reminiscent of the dimeric species of VDAC [49]. The high resolution structure of VDAC in a native-like bilayer environment shows a tightly packed dimer, which acts as a building block to form larger protein clusters. However, inter-dimer interactions in the VDAC clusters are weaker than intra-dimer ones and therefore, facilitate the dynamic assembly/disassembly of dimer units to and from those protein clusters. We believe that 2T species of KcsA, along with the individual T species, could play a similar role to that of VDAC dimers as building blocks of the larger KcsA clusters. Indeed, this would be in agreement with the "ladder-like" activity pattern observed by patch-clamp of reconstituted KcsA giant liposomes (see Fig. 7 in reference [16]). "Ladder-like" recordings are occasional observations (only 6 recordings from of a total of 93 patches exhibiting ion channel activity) in which the number of open channels within the patch increases during
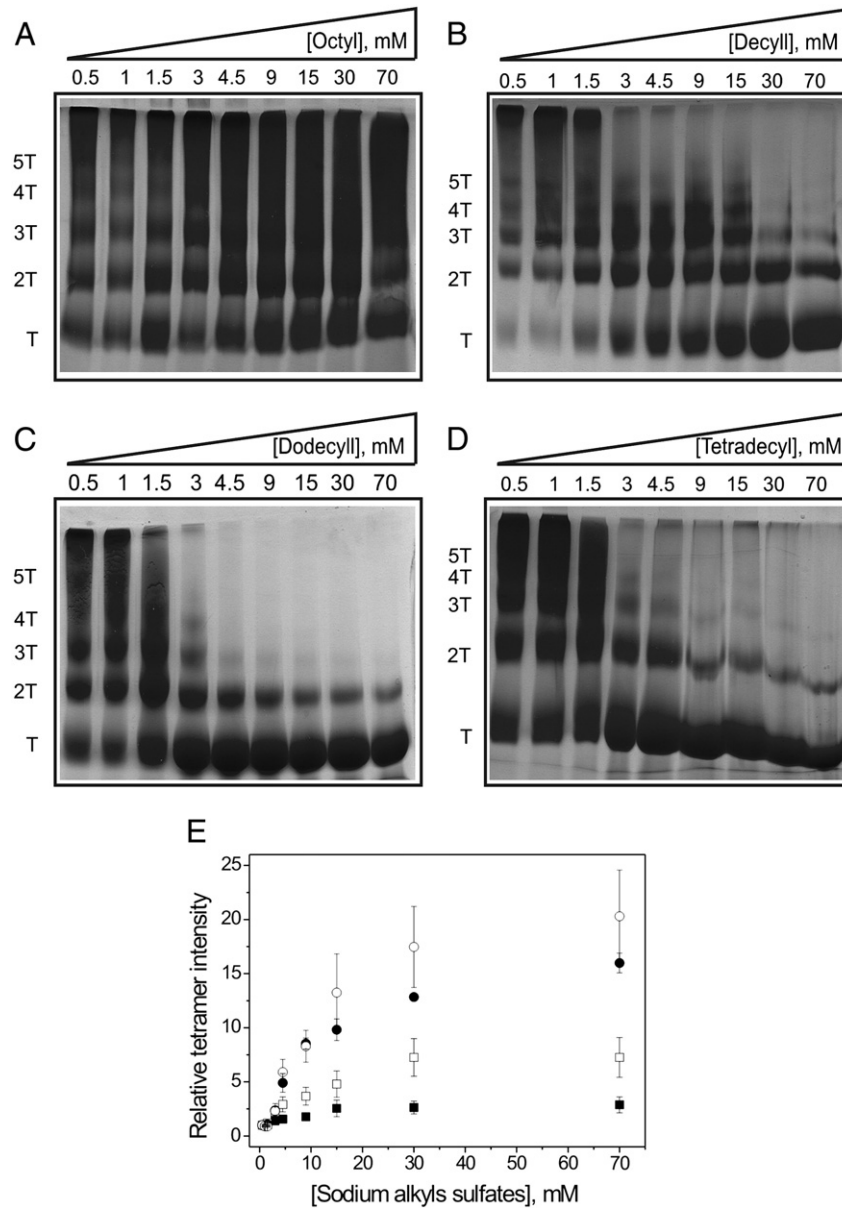
Fig. 5. Effect of different sodium alkyl sulfates on KcsA clusters. Purified DDM-solubilized KcsA was incubated with increasing concentrations of n-alkyl sulfates of different chain lengths before being applied to BN-PAGE (A, B, C and D). Panel E shows the analysis of the gel bands, expressed as the intensity of the tetramer relative to the sum of all the bands present within each lane. Values represent the average ± SEM of 5 different gels. Symbols indicate sodium alkyl sulfates with a different chain length: octyl (■), decyl (□), dodecyl (●), and tetradecyl (○).

the time course of the recording in response to repetitive electrical stimulation, illustrating how a complex coupled-gated array of KcsA channels becomes assembled at the tip of the patch-pipette. Interestingly, the size of the current steps entering or leaving the "ladder-like" ensemble of channel activity reveals currents corresponding either to the unitary KcsA current level (T species) or to twice as much such unitary currents (2T species), each in approximately 50% of the cases. This apparent correlation between cluster analysis and functional data from patch-clamp experiments, suggests that clustering is a reversible and dynamic process in which the establishment of channel–channel interactions causes profound changes in channel activity.

As to putative KcsA domains involved in clustering, we have studied the effects of SDS-like alkyl sulfates to gain information on this issue. We previously showed that these molecules bind to inter-subunit "non-annular" sites on the protein, stabilizing the tetrameric structure of individual KcsA molecules [44]. Hydrophobic interactions seem to

be critical, since acyl chain carbons from C9 to C14 are necessary for this process to occur [44]. Through BN-PAGE, we also find that acyl chain longer than eight carbons are necessary to destabilize KcsA clustering, showing a clear antagonism between i) the ability of SDS-like alkyl sulfates to bind these non-annular sites and stabilize the KcsA tetrameric structure and ii) their ability to destabilize protein clusters. We conclude from these studies that such sites mediate channel–channel interactions leading to cluster assembly and that binding of the amphiphylic ligands to such sites shifts the equilibrium to promote cluster disassembly.

5. Conclusions

The highlights of this study are i) the observation that cluster formation is reversible and can be induced in DDM without lipids being present, ii) the documentation that dimers of tetramers are more

stable than other clustered forms and iii) that destabilization of the tetramer dimers by alkylsulfates is correlated to stabilization of the tetramer itself, in line with effects resulting from non-annular binding of these alkylsulfates.

The finding that KcsA, one of the structurally simplest ion channels, already shows such a complex clustering behavior and its relation to functionally-relevant coupled gating reinforces the idea that supramolecular assemblies of ion channels and the interactions that hold them together may be more important than originally thought for the understanding of the biological processes they mediate. In such endeavor, KcsA might again be an excellent model to identify structural motifs responsible for clustering-mediating, channel–channel interactions or to establish the mechanisms by which such interactions lead to coupled gating of ion channels.

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