The potassium channel T1 domain plays an important role in the regulated assembly of subunit proteins. We have examined the assembly properties of the Shaker channel T1 domain to determine if the domain can self-assemble, the number of subunits in a multimer, N, and the mechanism of assembly. High pressure liquid chromatography (HPLC) size exclusion chromatography (SEC) separates T1 domain proteins into two peaks. By co-assembly assays, these peaks are identified to be a high molecular weight assembled form and a low molecular weight monomeric form. To determine the N, of the assembled protein peak on HPLC SEC, we first cross-linked the T1 domain proteins and then separated them on HPLC. Four evenly spaced bands co-migrate with the assembled protein peak; thus, the T1 domain assembles to form a tetramer. The absence of separate dimeric and trimeric peaks of assembled T1 domain protein suggests that the tetramer is the stable assembled state, most probably a closed ring structure.

Voltage-gated K⁺ channel proteins are multisubunit ion channel proteins. The core channel consists of an apparent tetramer of a-subunit proteins that assembles to form the K⁺ ion-selective aqueous pore across the cell’s plasma membrane (1). In addition, β-subunit proteins, which are apparently not transmembrane proteins, can be attached to each α-subunit protein (2). Molecular cloning has revealed a large diversity of K⁺ channel subunit proteins. Sequence comparisons among α-subunits has revealed that the similarities among encoded proteins cluster into a variety of K⁺ channel subfamilies (3). Biophysical studies have indicated that these subfamilies are in fact functional subsets of channel proteins in that functional heteromultimeric channels have only been formed by co-expression of two α-subunit proteins from the same subfamily (4).

The mechanisms that govern the assembly and function of voltage-gated ion channels are poorly understood. Recently we and others have identified a conserved molecular domain, the T1 domain, encoded within the cytoplasmic N terminus of the α-subunit protein that plays an important role in the assembly of K⁺ channel subunit proteins (5–10). Our studies have suggested that the T1 domain, translated by itself, can self-assemble (6). Sucrose density gradients reveal the formation of a high molecular weight complex; co-immunoprecipitation studies show that a tagged T1 domain protein can co-precipitate another un-tagged T1 domain protein. In addition, the T1 domain contains the molecular recognition sequences required for the subfamily-specific assembly of voltage-gated K⁺ channel proteins (11). Chimeras made with swapped N-terminal sequences show the assembly specificity of the N-terminal donor; the soluble T1 domain translated by itself only co-assembles with T1 domain proteins made from the same subfamily. These results have prompted our hypothesis that the T1 domain is the primary site for organized tetramerization of K⁺ channel subunit proteins along subfamily-specific lines.

Other recent studies have questioned the role that the T1 domain plays in K⁺ channel subunit assembly. Subunit proteins synthesized with deleted N termini have been shown to function in Xenopus oocyte expression systems (9, 10, 12, 13). In addition, another group has suggested that S1, the first transmembrane domain, is necessary for assembly, based on their hydrodynamic and co-precipitation studies (7). In fact, no studies have directly examined the assembled T1 domain protein to show that it is forming a tetramer on its own. Tetramerization has only been inferred based on the migration rate of the protein in sucrose gradients or gel filtration columns. In addition, the molecular species responsible for the co-immunoprecipitation signal has not been identified. It is not clear to what extent dimers, trimers, tetramers, larger octomers, or complexes with other proteins contribute to the co-immunoprecipitation signal. Finally, what role the T1 domain plays in the normal assembly of other, non-Shaker type potassium channels has not been directly examined.

In this paper, we have developed methods to efficiently separate T1 domain proteins into different molecular weight species, in order to separate assembled and unassembled proteins. By combining molecular size separation with co-precipitation techniques we have determined the assembled species that are responsible for the co-precipitation of untagged T1 domains. Finally, using chemical cross-linking we have measured the number of subunit proteins assembled in the peak assembled fraction. These results have allowed us to formulate more specific models of the mechanisms by which the T1 domain functions in K⁺ channel assembly.

**EXPERIMENTAL PROCEDURES**

**T1 Domain Constructs**—The constructs CF2-Tag1, 1ABC(no tag), 31ABC, and IT1-T7tag were described previously (6, 11). In experiments not taking advantage of epitope tags, 1ABC(no tag) is abbreviated 1ABC. All clones contain stop codons immediately following the expressed sequence. For 1ABC, the stop codon was inserted into the EcoRI site of pCITE 2A (Novagen) by oligonucleotide-directed mutagenesis. 1ABC(poly-His) construct is identical to 1ABC, except it lacks a stop codon at the EcoRI site. This puts the potassium channel sequence in frame with the poly-His (6-His) tag in pCITE 2A (Novagen), with the stop codon provided by the vector immediately following the 6-His stretch.

In **Vitro Translation**—In vitro translation procedures were described previously (11). All proteins were synthesized with [³⁵S]Met. Microsomal membranes were not needed in these experiments, since all constructs lacked transmembrane domains.
T1 Domain Self-tetramerization

SDS-PAGE and Autoradiography—Electrophoresis and autoradiography procedures were performed similarly to those described previously (11). Gradient gels were poured in Bio-Rad minigel multisters, from the bottom, as recommended by the manufacturer. Acetone precipitations were performed to concentrate large volume samples using bovine serum albumin (BSA) as a carrier, as described previously (11). In vitro translation experiments, lactalbumin was used as a substitution for BSA to avoid interference with the higher molecular weight bands.

Immunoprecipitations—Immunoprecipitation experiments were performed using procedures described previously (11).

Ni2+ Affinity Chromatography—Affinity columns were prepared by placing Ni2+ affinity resin (Novagen), sufficient to form a 50-μl bed volume, into a 0.65-μm Ultrafree-MC microcentrifuge spin filter (Millipore Corp.). The resin was washed in distilled H2O to remove storage buffer. All Ni2+ columns were prepared using 1 ml of wash buffer, and elution buffer was removed by brief centrifugations (<2 min) at 4,000 rpm in a microcentrifuge at 4°C. Washed resin was then charged with Ni2+ by equilibration in 250 μl of charge buffer (50 mM NiSO4) for 10 min at 4°C. Charge buffer was removed and exchanged with 250 μl of bind buffer (500 mM NaCl, 20 mM Tris, pH 7.9, 1% CHAPS, 1.5 mM imidazole) to prepare the column for use. Following removal of the bind buffer, the column was ready for use. Each column was used for only one purification.

In vitro translation samples were prepared by mixing 1-2 μl of in vitro translation reaction with 200 μl of bind buffer. The sample was then spun down by centrifugation at 60,000 rpm for 20 min in a mini-ultracentrifuge (Beckmann). For HPLC samples, individual fractions were adjusted to bind buffer conditions, by the addition of salts, CHAPS, and imidazole, before applying to the resin. Sample supernatant was mixed with the resin for 10 min at 4°C to ensure complete binding of poly-His-tagged proteins to the resin. Unbound material was then spun through the column, and the resin was washed in a series of steps: first 1 × wash with 350 μl of bind buffer, followed by 3 × washes with 350 μl of wash buffer (bind buffer with 1 mM imidazole). Samples were eluted in 350 μl of elution buffer (bind buffer with 1 M imidazole). All fractions were collected and analyzed by SDS-PAGE gel and autoradiography. Samples were concentrated for gel analysis either by acetone precipitation or immunoprecipitation with the common anti-1B antiserum (11).

Chemical Cross-linking—Samples were prepared for cross-linking by adding 8 μl of in vitro translation reaction to 800 μl of cross-link buffer (150 mM NaCl, 20 mM HEPES, pH 7.9, 1% CHAPS, 5 mM imidazole). Hemoglobin, the dominant protein in the in vitro translation mix, was removed by passing the material through charged Ni2+ affinity resin equilibrated in cross-link buffer. Hemoglobin binds to the resin under these conditions, and non-poly-His tagged T1 domain proteins pass through the resin. Disuccinimidyl suberate (DSS, Pierce) was added to all samples of cleared sample (total volume 100 μl), from a 25 mM stock in dimethyl sulfoxide, to vary DSS concentration from 0 to 2.5 mM. The cross-linking reaction was incubated on ice for 30 min. The reaction was quenched by adding 5 μl of 1× Tris and incubating for 60 min in vivo at 37°C to quench cross-linkages. Samples were analyzed by SDS-PAGE gradient gels (5-20%) and autoradiography. Samples were concentrated for gel analysis either by acetone precipitation or immunoprecipitation with the common anti-1B antiserum (11).

For HPLC analysis, samples were cross-linked at 1.5 mM DSS, as described above, and then injected into the HPLC following quenching.

Size Exclusion HPLC—Silica-based size exclusion HPLC chromatography columns and guard columns were obtained from Bio-Rad. HPLC was performed on a Waters HPLC at 0.5 ml/min with one fraction collected per min. Columns were equilibrated in standard buffer (150 mM NaCl, 20 mM Tris, pH 7.6, 1 mM EDTA) at 4°C. Samples were prepared for injection by mixing 5 μl of in vitro translation reaction with 10 μl of standard buffer and cleared by centrifugation (60,000 rpm at 4°C for 30 min). The supernatant was filtered through a 0.45-μm microcentrifuge spin filter (Millipore) and injected into the HPLC. The column was checked before each series of runs by injection of molecular weight size standards. Three sets of standards were used: TGM standard (thymoglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B-12 to 100,000), FCAC standard (α-lactalbumin, catalase, ovalbumin, and chymotrypsinogen); and TAOR standard (thymoglobulin, aldolase, ovalbumin, and ribonuclease A). The position of the hemoglobin peak was detected at 280 nm to verify the consistent separation of our samples. In vitro translated proteins could only be detected by gel analysis of fractions collected from the column. All fractions from the excluded volume to the void volume were collected and analyzed by SDS-PAGE gel and autoradiography. Samples were concentrated for gel analysis either by acetone precipitation or immunoprecipitation with the common anti-1B antiserum (11).

- Data Quantitation and Analysis—[35S]Labeled samples were analyzed by either film densitometry of autoradiographs or direct PhosphorImager analysis of gels. Phosphor image capture was performed on a Molecular Dynamics PhosphorImager. Images were captured using an Envision ENV25Pro scanner. All images were analyzed using the Molecular Dynamics ImageQuant version 3.3 software package. Bands were quantitated using image integration with background subtraction from a neighboring lane of protein by location. Both methods were extensively compared and found to give comparable results. The data were plotted with Deltagraph and fitted with cubic spline curves for presentation.

HPLC runs were standardized by detection of protein standards using an absorbance detector set at 280 nm, before a series of experimental runs were performed. Because of the small amount of synthesized protein relative to the total protein in the in vitro translation mix, T1 domain proteins were not directly detected by UV absorbance but rather by [35S]autoradiography of SDS-PAGE gels run on the fractionated column eluate. To calculate the distribution coefficient, Kd, which describes the average access of a protein to the total column volume, we needed to measure the void and total volumes for the column. Void volume was detected as the first appearance of protein aggregates; the total volume was detected by the appearance of vitamin B-12. Kd is (peak volume − void volume)/(total volume − void volume) (14). For in vitro translated proteins, peak protein fraction volumes were set at the midpoint volume for the fraction and normalized back to the absorbance detector by subtracting the volume in the line after the detector up to the fraction collector. In internal control for this correction was the peak hemoglobin fraction. Protein peaks that were evenly divided between two fractions were considered to peak at the point where the fraction changed. Because these Kd measures for T1 domain proteins were only accurate to half a fraction volume, these numbers are considered approximate.

RESULTS

Soluble N-terminal peptides from the Shaker type K+ channel subunit protein AKv1.1, containing the T1 domain (see Fig. 1) were synthesized in vitro using a rabbit reticulocyte lysate system, as described previously (6, 11). All proteins translated efficiently without the addition of microsomal membranes and were soluble without added detergent. Full N-terminal peptides that were studied include CF22Tag, an epitope tagged N-terminal peptide (6); 1ABC(no tag), the full N-terminal protein; and 1ABC(pro-His), the 1ABC protein cloned in frame with the C-terminal 6-His sequence of the pCTIE-2A vector (Novagen). In addition, two constructs encoding the Shaker T1 domain with minimal additional sequences were examined: the epitope-tagged T1 domain protein, 1T1-T7Tag (11), which uses the pCTIE-2A initiator Met, and 31A1BC (11), which uses the AKv3.1a initiator Met and 10 amino acids, before the Shaker T1 domain. There were no detectable differences in the behavior of any of these constructs except for epitope tag sensitivity and longer retention of T1 only proteins on SEC HPLC due to their smaller size. The results presented are based on over 110 individual HPLC runs, with a minimum of three experiments for any individual result.

HPLC Size Exclusion Chromatography of N-Terminal Proteins—Synthesized proteins were size-fractionated by size exclusion chromatography on silica-based HPLC columns (Bio-Rad). Columns with three different pore sizes were tested, Bio-Silect 125–5 (fractionation range 5–100 kDa), Bio-Silect 250–5 (fractionation range 10–300 kDa), and Bio-Silect 400–5 (fractionation range 20–1000 kDa). Fig. 2 shows an autoradiogram and a densitometry plot of a typical separation of 1ABC protein on a Bio-Silect 250–5 column. Two peaks of 1ABC protein are readily apparent, suggesting that the protein is
primarily found as two different sized species. Based on the fraction that the protein peaks were located in, approximate values were obtained for the fraction of the total volume that the protein has access to, $K_d$ (14). For full-length N-terminal proteins, such as 1ABC or CF2-Tag1, on a Bio-Silect 250–5 column, the low molecular weight protein peak has an approximate $K_d$ of 0.53, whereas the high molecular weight protein peak has an approximate $K_d$ of 0.24. For full length N-terminal proteins on Bio-Silect 400–5 columns, the $K_d$ for the low molecular weight peak is approximately 0.66, and the high molecular weight peak is approximately 0.46. Bio-Silect 125–5 columns do not adequately fractionate the higher molecular mass peak for full length N-terminal proteins ($K_d < 0.1$), suggesting that it is >100 kDa, as expected for a tetramer. For the T1 domain only proteins, such as 31ABC or 1T1-T7tag, similar results are obtained except that the two peaks are retained for slightly longer times, as expected for the smaller size of these proteins. Fractionations of soluble T1 domain proteins were unchanged by the addition of 1% CHAPS detergent to the sample or running buffer. We next sought to determine directly whether the two protein peaks correspond to differently assembled states of the T1 domain proteins.

Co-immunoprecipitation of Proteins Separated on HPLC—The first experiment was to employ a co-immunoprecipitation assay on fractions corresponding to the high and low molecular weight T1 domain protein peaks following separation on SEC HPLC. In this co-immunoprecipitation assay, CF2-Tag1 was co-translated with the T1 domain only protein 1T1-T7 tag. Co-assembly of these proteins was measured by the ability of the anti-Tag1 antiserum to co-precipitate the 1T1-T7tag protein, which it does not directly recognize, based on the stable assembly of this protein with CF2-Tag1, which is recognized by the
antiserum. A typical result for this assay is seen in the immunoprecipitation of unfractionated proteins in Fig. 3.

To determine which peak(s) contain assembled proteins, we co-translated 1T1-T7tag and CF2-Tag1 and separated the proteins on a Bio-Silect 400–5 SEC HPLC column. High molecular weight and low molecular weight fractions were separately pooled, and assembly-tested in the two protein populations by anti-Tag1 antiserum immunoprecipitation. Both 1T1-T7tag and CF2-Tag1 proteins are present in both peaks, as verified by immunoprecipitation with the common anti-1B antiserum. However, 1T1-T7tag proteins are only co-immunoprecipitated from the high molecular weight protein fractions and not from fractions corresponding to the low molecular weight peak. Therefore, the proteins in the low molecular weight peak are not co-assembled and are probably monomeric. Identical results were obtained from proteins separated on Bio-Silect 250–5 and 125–5 SEC columns.

Ni²⁺ Affinity Column Analysis of Subunit Assembly—In order to determine a more efficient and rapid co-purification assay based on the ability of poly-His-tagged T1 domain proteins to selectively bind to a Ni²⁺ affinity resin. Without poly-His-tagged T1 domain proteins to assemble, T1 domain proteins do not bind to the Ni²⁺ affinity resin (Fig. 4A). By co-translating untagged, and poly-His-tagged T1 domain proteins, the co-assembly of these proteins can rapidly be determined by the purification of untagged proteins on the Ni²⁺ affinity column. Fig. 4B shows the co-purification of 1ABC(no tag), with 1ABC(poly-His) on a Ni²⁺ affinity column. Therefore, the poly-His tag does not disrupt the ability of the proteins to assemble.

Ni²⁺ Column Analysis of Proteins Separated on HPLC—The Ni²⁺ affinity column co-purification analysis was next applied to fractions from the HPLC separation of co-translated 1ABC-(no tag) and 1ABC(poly-His). The raw fractions, before application to the Ni²⁺ affinity resin, show that both proteins are separated in similar manners, suggesting that the proportion of assembled and unassembled proteins was similar for each. The fractions were applied to Ni²⁺ affinity columns, washed, and eluted. Elution of the Ni²⁺ column shows that 1ABC(poly-His) proteins are purified identically to their original profile; however, 1ABC(no tag) proteins are only co-purified in the higher molecular weight fractions. Quantitation of the counts for the various proteins, following Ni²⁺ affinity column co-purification analysis on the different fractions, shows that 1ABC(no tag) T1 domain protein co-purifies as a monophasic peak that is identical to the high molecular weight peak identified above (see Fig. 5). This suggests that the high molecular weight peak is the sole form of stably assembled T1 domain proteins and the low molecular weight peak is completely composed of monomeric proteins.

Chemical Cross-linking of T1 Domain Proteins—In order to directly measure the Nₐ of the assembled T1 domain protein complex, we chemically cross-linked in vitro translated T1 domain proteins. Fig. 6 shows the cross-linking of 1ABC with varying concentrations of DSS. SDS-PAGE gradient gel analysis shows that cross-linked 1ABC protein runs as a regularly spaced ladder of clear bands, with additional cross-linked material visible at higher molecular weights. The lowest band is the 1ABC monomer, visible in the absence of cross-linker. The three higher bands are even sized multiples of the 1ABC monomer and most likely represent multimerically cross-linked 1ABC proteins. Consistent with this interpretation, the dimer band is the first to appear, with progressive movement of protein to trimer and tetramer-sized molecular weights with additional cross-linking. The tetramer band is always more diffuse than the monomer, dimer, or trimer bands and sometimes appears to be a doublet, suggesting that at least two different cross-linkings of the tetramer are possible. Higher molecular weight complexes represent either further multimerizations of the basic monomer or coupling of 1ABC to other proteins, possibly artifacts of the coupling procedure. Identical

![Image](image_url)

**Fig. 3.** Co-immunoprecipitation analysis of HPLC protein peaks identifies assembled fractions. CF2-Tag1 and 1T1-T7tag were co-translated then run on a Bio-Silect 400–5 SEC column to separate according to size. Fractions corresponding to the positions of the high molecular weight peak (fractions 19, 20, and 21) and the low molecular weight peak (fractions 23, 24, and 25) were pooled separately. Both peaks and the original unfraccionated translation were probed to determine if the proteins were co-assembled. Anti-1B is a common antiserum to the B subdomain of the T1 domain in both proteins, and it is a positive control for the presence of both proteins (11). Anti-Tag1 only recognizes CF2-Tag1; thus co-precipitation of 1T1-T7tag by this antiserum is indicative of assembly. The minus antibody lane (—) is the negative control. Both proteins are found in the two peaks separated on SEC HPLC, as verified by precipitation with anti-1B; however, 1T1-T7tag is not co-precipitated in the low molecular weight peak. Thus only in the high molecular weight peak are the two proteins co-assembled.

![Image](image_url)

**Fig. 4.** Ni²⁺ affinity column co-purification analysis demonstrates T1 domain protein assembly. T1 domain proteins were in vitro translated and then applied to Ni²⁺ affinity columns. The columns were washed at increasing stringency, and then bound proteins were eluted at a high imidazole concentration (1 M imidazole). A, affinity chromatography of 1ABC(no tag) on Ni²⁺ affinity resin. L, sample loaded; F, column flow-through; B, bind buffer wash; W, washes with wash buffer; E, elution with elution buffer. 1ABC(no tag) does not bind to the Ni²⁺ affinity resin; therefore, no protein is present in the elution step. B, affinity chromatography of 1ABC(no tag) co-translated with 1ABC(poly-His) on Ni²⁺ affinity resin. Although 1ABC(no tag) does not directly bind to the Ni²⁺ affinity resin, it is present in the elution step because of its stable assembly with 1ABC(poly-His), which does bind to the column.
results were obtained with other T1 domain proteins.

HPLC Analysis of Chemically Cross-linked T1 Domain Proteins—We next combined chemical cross-linking with separation of proteins on SEC HPLC to directly characterize the specific molecular composition of the two peaks of T1 domain proteins observed above and to determine if higher molecular weight complexes, created during cross-linking, co-migrate with the T1 domain protein peaks or migrate at novel positions, suggestive of artifactual couplings. In Fig. 7, A1ABC domain proteins were cross-linked in 1.5 mM DSS and then run on a Bio-Silect 250–5 SEC column. Protein in the monomer band shows the characteristic biphasic peaks at identical positions as un-cross-linked samples. The dimer, trimer, and tetramer peaks are monophasic and co-migrate with the higher molecular weight monomer peak. Higher molecular weight cross-linked species do not co-migrate with either peak; rather they migrate at novel higher molecular weight positions and thus probably represent species that are artifically linked during the cross-linking.

The SEC HPLC separation of the proteins shows that T1 domain proteins can be cross-linked up to the tetramer size and still migrate in the high molecular weight peak, thus identifying the higher molecular weight peak as the tetrameric form of the protein. Furthermore, the fact that the dimer and trimer proteins also show a single peak at the tetramer position indicates that they are partially cross-linked tetramer proteins that are separated during the SDS-PAGE analysis.

**DISCUSSION**

The T1 domain was originally identified through its role in K+ channel subunit assembly (5, 6). Later, it was shown to be a critical regulator of subunit heteromultimerization, by controlling the subfamily specific heteromultimerization of K+ channel subunit proteins (11). This work has sought to examine the T1 domain, separate from the K+ channel transmembrane domains, to measure directly the self-assembly properties of the T1 domain.

HPLC analysis shows that the T1 domain exists predominantly in two forms, a high molecular weight form and a low molecular weight form. Both co-immunoprecipitation and cross-linking analysis clearly identify the high molecular weight form as an assembled state of the protein and the low molecular weight form as the unassembled, monomeric form of the T1 domain. Chemical cross-linking shows that all the assembled T1 proteins cross-link in an evenly spaced ladder; the assembled state are completely contained within the high molecular weight peak fractions. 

![Figure 5](image5.png)

**FIG. 5.** Ni2+ affinity column co-purification analysis of HPLC fractions shows assembled protein separation profile. A1ABC(no tag) and A1ABC(poly-His) were co-translated and separated on a Bio-Silect 250–5 SEC HPLC column. Fractions from the column were split in half; half the fraction was analyzed for the protein migration, and the other half was applied to a Ni2+ affinity column. A, autoradiography of proteins separated on a Bio-Silect 250–5 SEC column. B, band volume quantitation of experiment in panel A. The co-translation autoradiograph shows the chromatography of the T1 domain proteins before application to the Ni2+ affinity columns. OT lane shows the unfraccionated translation. The following lanes are successive fractions from the column, starting at the void volume. Upper band is A1ABC(poly-His), and the lower band is A1ABC(no tag). Both proteins are present in the translation and show essentially identical SEC separation patterns with high and low molecular weight protein peaks. The Ni2+ column flow-through for the HPLC fractions shows only a single protein at the A1ABC(no tag) molecular weight. In the Ni2+ column elution, both A1ABC(no tag) and A1ABC(poly-His) are present in fractions corresponding to the high molecular weight peak; however, only A1ABC(poly-His) is present in the fractions corresponding to the low molecular weight peak. Therefore, T1 domain proteins in the assembled state are completely contained within the high molecular weight peak fractions. 1ABC(poly-His):Ni2+ column eluted; 1ABC(no tag):Ni2+ column eluted; 1ABC(poly-His)Ni2+ column eluted; 1ABC(no tag):Ni2+ column flow-through.

![Figure 6](image6.png)

**Fig. 6.** Chemical cross-linking of T1 domain proteins. A1ABC was in vitro translated and then cross-linked with increasing concentrations of DSS, as described under "Experimental Procedures." Cross-linked proteins were run on a 5–20% linear gradient SDS-PAGE gel, and the positions of the proteins were determined by autoradiography. The first lane shows the migration of molecular mass standards. The monomer band is the only band present in the non-cross-linked sample. At the lowest level of cross-linking, a band at the expected position for a dimer is seen. At higher cross-linker concentrations, bands are seen at the expected positions for the trimer and tetramer, with additional material present as a diffuse smear at high molecular weights. The protein band at the tetramer position is broader than the other bands and appears to be a doublet.
translated, then cross-linked with 1.5 mM DSS as described under "Experimental Procedures." This cross-linked material was then separated by size on a Bio-Silect 250–5 SEC HPLC column. Fractions were acetone-precipitated and then run on a 5–20% linear gradient SDS-PAGE gel and subjected to autoradiography to determine the migration pattern of the different protein species. A, autoradiograph of the HPLC fractions following separation of cross-linked 1ABC. B, densitometry analysis of bands from the gel in panel A. Band volume for proteins at the different molecular weight sizes was measured and plotted without normalization versus the fraction number. The monomer band shows two peaks at the expected high and low molecular weight positions seen in uncross-linked samples. Dimer, trimer, and tetramer bands all show a single peak in the fraction corresponding to the high molecular weight peak fraction for uncross-linked samples. The higher molecular weight smear of cross-linked protein peaks in earlier fractions that do not correspond to the position of protein peaks in uncross-linked samples. Therefore, the high molecular weight T1 domain peak is identified as being composed of T1 domain protein homotetramers.

A closed circular structure of the assembled T1 domain

domain HPLC profile suggests that there is no particular stability associated with this structure. This observation argues against subunits assembling as dimers and the dimers assembling as tetramers in an isologous association. Rather, the results suggest that T1 domain proteins assemble by the nomenclature of single subunit proteins in a repeated A-B interface interaction pattern until the fourth subunit protein completes the closed ring. Such heterologous association has been observed in the multimerization of other membrane proteins, such as neuraminidase (15).

Closer analysis of the cross-linked tetrameric band, following autoradiography of SDS-PAGE gels reveals that it often appears to be a doublet or a broader band than any of the other T1 domain bands. This suggests that the tetramer can be cross-linked into two different structures. One structure is presumably the linear form, common to the monomer, dimer, and trimer cross-linked proteins. The second structure probably represents the fully cross-linked, native form of the assembled T1 domain. Based on the presumed form of the functional K⁺ channel, with an axis of symmetry down a central aqueous pore, and the unique stability of the fully assembled state of the T1 domain, the native form of the assembled T1 domain is probably a closed circular, ring, structure.

A closed circular structure of the assembled T1 domain would provide additional stability to the tetramer by providing stabilizing subunit-subunit interactions on both sides of the T1 domain, thus requiring two separate interactions to be broken in order to disrupt the structure. Indeed, co-purification analysis on HPLC fractions, using a poly-His-tagged subunit protein co-translated with an untagged subunit protein, shows that co-purification is limited to the tetramer peak. Thus interactions between T1 domain proteins that are not completed to the tetrameric state must dissociate within the time frame for these experiments. Cross-subfamily interactions between T1 domains or point mutations that disrupt the tetramer may be filtered out by this stringent assembly requirement. Selectivity against assembly of K⁺ channel subunit proteins across subfamilies or disruption of assembly by mutagenesis can therefore occur either through incompatible interface amino acids or through subtle structural differences between proteins with compatible interfaces that prevent the closing of the ring, either through torsion or interface angle incompatibility.

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