

Mutational Analysis of Synaptobrevin Transmembrane Domain Oligomerization[†]Mark E. Bowen,[‡] Donald M. Engelman,[§] and Axel T. Brunger^{*:‡}

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Received October 2, 2002

ABSTRACT: Synaptobrevin 2 is thought to facilitate fusion of synaptic vesicles with the presynaptic membrane through formation of a soluble NSF attachment protein receptor complex (SNARE) with syntaxin 1a and a synaptosomal associated protein of 25 kDa (SNAP-25). Previous reports have described a homodimer of synaptobrevin that is dependent on the transmembrane domain. However, these reports disagree about the magnitude of dimerization, which makes it difficult to assess the biological relevance of this interaction. We used SDS–PAGE and the TOXCAT genetic assay to reexamine the homodimerization of the synaptobrevin transmembrane domain in detergents and the *Escherichia coli* inner membrane, respectively. To gauge the magnitude of synaptobrevin homodimerization, we used the well-characterized glycophorin A homodimer as a positive standard. In contrast to previous studies, we found synaptobrevin homodimerization in *E. coli* is very weak when compared to glycophorin A. Recombinant synaptobrevin forms a small amount of dimer and higher order oligomers in detergents that are highly dependent on solubilization conditions. We estimate a dissociation constant of 10 mM for synaptobrevin dimerization in detergent. Thus, the dimerization of synaptobrevin in membranes is very weak, questioning any possible functional role for this association in vivo.

Neurotransmitter release depends on the regulated fusion of synaptic vesicles with the presynaptic plasma membrane (1, 2). Many of the proteins involved in this process have been identified (3, 4). A set of three proteins, termed SNAREs (soluble NSF attachment protein receptors), has been shown to be essential for the final stages of synaptic vesicle fusion and sufficient to cause fusion of liposomes in vitro (5–7). These proteins are part of an evolutionarily conserved family thought to mediate all intracellular fusion events in eukaryotes (8, 9).

Neuronal membrane fusion involves a complex of three SNAREs, syntaxin, SNAP-25 (synaptosomal associated protein of 25 kDa) and synaptobrevin/VAMP (vesicle associated membrane protein). Synaptobrevin 2 was initially characterized as an integral membrane protein of 18 000 kDa found to reside on synaptic vesicles (8, 10, 11). These initial reports did not include any description of synaptobrevin oligomers. Syntaxin and SNAP-25 are primarily localized on the plasma membrane. Formation of the SNARE complex begins in trans (proteins on opposing membranes) and results in a highly stable cis complex where all proteins reside in the same membrane (12). The crystal structure of the cytoplasmic portions of the SNARE complex revealed a parallel four-helix bundle, which is thought to extend to the transmembrane domains of synaptobrevin and syntaxin (13).

The vast majority of information about SNAREs arises from studies of the soluble cytoplasmic domains, but it is now becoming clear that the transmembrane domains affect both the structure and the function of SNAREs. Synaptobrevin forms a complex with synaptophysin that is dependent on the presence of the transmembrane domain (14, 15). Several previous reports in the literature have described homodimerization of synaptobrevin that is dependent on the transmembrane domain. The first report described a putative synaptobrevin homodimer in detergent-treated brain extracts that was weak enough to require stabilization by chemical cross-linkers to be detected by immunoblotting (15). Later reports using recombinantly produced synaptobrevin described a homodimer that was stable in the absence of cross-linkers (14, 16–18). Using chimeras of synaptobrevin and *Staph* Nuclease A in a SDS–PAGE assay run in the presence of urea, Laage and Langosche identified a conserved amino acid motif necessary for homodimerization and calculated a dimer-to-monomer ratio of 0.62 (16). Similar results were obtained using a genetic assay lacking any positive standard (17). Dimerization was also reported for purified full-length synaptobrevin that was enhanced by detergent depletion in the presence of liposomes (18). In this report, overexposed immunoblots were used to visualize the dimer suggesting a much weaker interaction than in the previous study (16). The suggestion was made that this interaction motif could drive oligomerization of the SNARE complex in vivo or contribute to complex stability and lipid mixing (16–18).

The study of membrane proteins lags far behind studies of soluble proteins because of the difficulty in sample preparation and handling. Biochemical characterization of membrane proteins requires the presence of detergent and

[†] This work supported by a grant from the National Institutes of Health to A.T.B. (NIH RO1 MH63105-01).

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often lipids to maintain their native activities and binding propensities. As such, membrane proteins are known to form irreversible aggregates when detergent solubilization is suboptimal. With these difficulties in mind, we have attempted a careful reexamination of the interactions of the SNARE transmembrane domains in both detergent micelles and the bacterial inner membrane.

In our hands, the homodimerization of synaptobrevin is very weak when compared with the well-characterized Glycophorin A transmembrane dimer. Through a single-point mutation, we were able to change synaptobrevin into a strongly interacting transmembrane domain in the *E. coli* inner membrane but not in detergents. Additionally, we find that recombinant synaptobrevin is prone to the formation of dimers and other higher order oligomers that are highly dependent on the purification conditions. Our findings would suggest that the weak synaptobrevin homodimerization is not likely to be a major force for SNARE complex oligomerization in vivo.

EXPERIMENTAL PROCEDURES

TOXCAT. The synaptobrevin and syntaxin transmembrane constructs illustrated in Figure 1 were cloned into pccKAN using *Nhe* I and *Bam* HI restriction sites. Synaptobrevin transmembrane sequences for insertion into pccKAN were amplified from the full-length synaptobrevin sequence described below. All constructs were confirmed using bidirectional sequencing. Different length constructs were screened to identify residues necessary for dimerization (Figure 1A).

The TOXCAT assay was carried out as described previously (19). Expression cultures for TOXCAT analysis were prepared from dilution to an OD₆₀₀ of 0.05 of saturated overnight cultures grown in the presence of 200 μg/mL ampicillin. Cultures for analysis were grown in 200 μg/mL ampicillin to an OD₆₀₀ of 0.6 to allow for expression of the reporter gene. The chimeric constructs from pccKAN are expressed constitutively at low levels in the NT326 strain. Expression levels of all TOXCAT constructs were compared by western blotting using antiMBP (New England Biolabs). All constructs were expressed at similar levels (data not shown). A 200-μL cell culture normalized to an OD₆₀₀ of 0.6 was resuspended in 0.5 mL 100 mM Tris (pH 8.0). Cell lysis was induced by addition of a small drop of toluene followed by incubation at 30 °C for 30 min. CAT activity assays to assess dimerization were performed using Quant-CAT (Amersham) according to manufacturer's instructions. Error bars represent the standard deviation in CAT activity of three replicate cultures expressing the chimera.

To assay for proper orientation of the TOXCAT chimeras in the *E. coli* inner membrane, *malE* complementation was assessed. NT326 cells (*malE*⁻) containing the pccKAN plasmids were cultured on M9 agar plates containing 0.4% maltose, 1% ion sugar, and ampicillin. Proper orientation of the chimera would place the MBP portion of the construct in the periplasm allowing survival on maltose.

Expression and Purification. Synaptobrevin 2 was cloned using standard methodology from a rat cDNA library (Clontech) and inserted into pet28a (Novagen) for expression in the *E. coli* strain BL21 (DE3) (Novagen). Expression was done at 37 °C in TB media supplanted with kanamycin at

A	GpA	LIIFGVMAGVIGT
	GpA G83I	LIIFGVMAIVIGT
	Syb BC	IILGVICAILLIIII
	Syb mult	IIAGVIAAILLIIIIA
	Syb AD	WWKNLKMMIILGVICAILLIIIIIVYFST
	Syb AC	WWKNLKMMIILGVICAILLIIII
	Syb BD	IILGVICAILLIIIIIVYFST
	Syx	MIIICCVILGIIIAS
	Syb (ref. 17)	LGVICAILLIIIIIVYST

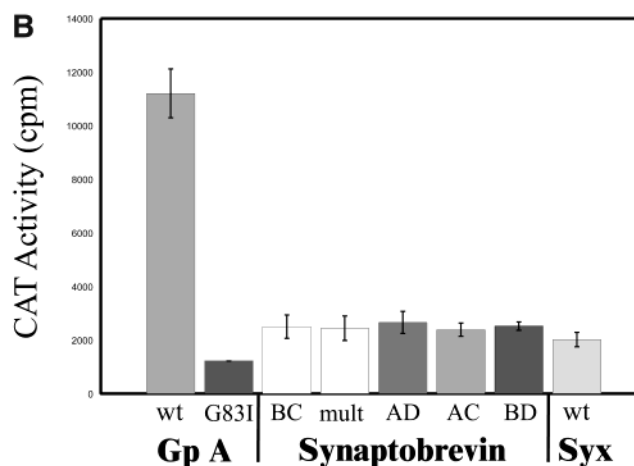


FIGURE 1: (A) Transmembrane sequences used in the TOXCAT analysis. Chimeric proteins containing the transmembrane domain sequences indicated were created to analyze relative strengths of interaction in the *E. coli* inner membrane. The GpA chimera contains residues 75–87 of the transmembrane domain from glycophorin A. G83I refers to a point mutant of the glycophorin A sequence. Synaptobrevin constructs of different lengths were examined to identify residues contributing to dimerization. Numbering of residues is from full-length rat synaptobrevin 2. Syb BC contains residues 97–111. mult refers to a triple point mutant (L99A/C103A/I111A) of the synaptobrevin BC construct. Syb AD contains residues 89–114. Syb AC contains residues 89–111. Syb BD contains residues 97–114. Syx contains residues 267–280 from the syntaxin 1a transmembrane domain. Syb (17) is the sequence used in ref 17. (B) Quantitative assay of transmembrane dimerization. CAT activity (units) measured for cultures expressing TOXCAT chimeras containing the transmembrane sequence indicated. GpA refers to Glycophorin A transmembrane domain. The sequences tested are as indicated in Figure 1A.

50 μg/mL and IPTG to 0.5 mM. Protein was extracted from *E. coli* under native conditions using detergents as indicated. Protein was purified using Ni-NTA agarose (Qiagen) with high stringency washing according to the manufacturer's instructions. Protein identity was confirmed using the anti-synaptobrevin monoclonal antibody CL69.1 kindly provided by Reinhard Jahn. Protein concentrations were determined from the absorbance value at 280 nm in 6 M guanidine hydrochloride and 20 mM sodium phosphate at pH 6.5 using a molar extinction coefficient of 13 940 M⁻¹ cm⁻¹. Mutagenesis of cysteine 103 was accomplished using the Quick Change kit (Stratagene) as directed by the manufacturer.

Electrophoresis. SDS-PAGE was carried out on either minigels (Bio-Rad) or Phast gels (Pharmacia) as directed by the manufacturer. Samples were incubated at 37 °C in 2% SDS with 0.2 M DTT. Running buffer included 0.1% SDS. No SDS was cast in the gels. Western blots were done using

standard wet transfer protocols and developed with the ECL kit (Amersham).

RESULTS

Previous studies of synaptobrevin dimerization relied on recombinant protein purified after overexpression in *E. coli*. As expression and purification of membrane proteins can often result in aggregated or improperly folded material, we sought to investigate dimerization *in vivo*. To accomplish this we used the TOXCAT system, which was designed to discriminate between strongly and weakly associating transmembrane sequences incorporated in the *E. coli* inner membrane (19). TOXCAT relies on low-level constitutive expression of a chimeric DNA binding protein containing the transmembrane sequence of interest. Dimerization of transmembrane sequences within the TOXCAT chimera results in transcription of the CAT reporter gene allowing relative strengths of association to be measured. Previous studies using the well-characterized transmembrane domain from glycophorin A (GpA) found that results from the TOXCAT system were in good agreement with studies carried out in detergent micelles (19–21).

The sequences used in this study are indicated in Figure 1A. Also included is the sequence from a previously published report, which appears to have omitted the phenylalanine at position 114 (17). The GpA transmembrane domain served as a positive control for a stable dimer, while the well-characterized, nondimerizing mutant G83I of GpA serves as a negative control for a nondimerizing transmembrane sequence. To ensure that sequences contributing to dimerization were not overlooked, a series of synaptobrevin sequences of different lengths were examined. The boundaries of the transmembrane domain inserted into the TOXCAT chimera are indicated in Figure 1A. Thus, construct AD begins at W90 and ends at S115 using the numbering from full-length synaptobrevin. The longest construct was 25 residues, and the shortest was 15 residues. The triple mutant L99A/I110/I111A (mult), previously reported to block homodimerization (16), was also tested in the context of the 15 residue transmembrane domain chimera. In addition, we tested the central 15 residues from the syntaxin 1a transmembrane domain.

All cultures for the TOXCAT analysis were grown concurrently and processed in parallel. All the chimeras were expressed and incorporated into the membrane at similar levels as assayed by western blotting. The longest constructs showed slightly higher levels of expression. Proper orientation of the chimeras in the inner membrane was tested using a *malE* complementation assay on M9-maltose agar plates. All constructs allowed for survival on maltose. The CAT activity of cells expressing the synaptobrevin and syntaxin chimeras is shown in Figure 1B. The GpA wild-type transmembrane sequence gave $11\,200 \pm 900$ units of CAT activity, while the GpA G83I mutant gave only 1200 ± 6 units. These two values can serve as controls for strongly dimerizing and nondimerizing transmembrane sequences, respectively. All the synaptobrevin constructs gave between 2400 and 2700 units of CAT activity. The length of transmembrane sequence placed in the chimera did not significantly affect the CAT activity. In addition, the CAT activity of the triple mutant (mult) was similar to that of the

wild-type sequence (BC), indicating that there was little difference in the degree of dimerization of these constructs in this assay. The syntaxin transmembrane domain gave 2000 units of CAT activity, which is slightly lower than synaptobrevin. All of the synaptobrevin and syntaxin constructs gave higher CAT activity than the GpA G83I mutant. This suggests that there is some association. However, none of these constructs gave more than 25% of the CAT activity seen for the GpA wild-type sequence.

As our results from the TOXCAT analysis are in conflict with previously published results (16, 17), dimerization was further examined using full-length synaptobrevin 2 produced recombinantly in *E. coli*. The two reports on dimerization of recombinant synaptobrevin used different detergents for the extraction from the *E. coli* cell pellet (16, 18). To see if this difference could explain the different estimates of the magnitude of dimerization, we directly compared the effect that detergent had on the oligomerization state of the purified protein. Cells from a single synaptobrevin-expressing culture were split in two and processed in parallel. One sample was extracted and purified in the presence 2% Thesit (16), while the other sample was extracted and purified with 1.5% sodium cholate (18). These concentrations of detergent were identical to those used previously in studies of synaptobrevin dimerization (16, 18). Buffers for both purifications were identical in all other respects and included 10 mM β -mercaptoethanol. The eluted proteins were adjusted to the same concentration and then 10-fold serially diluted four times to examine the concentration dependence of the dimer-to-monomer ratio. Samples were analyzed by SDS-PAGE and immunoblotted with a monoclonal antibody to synaptobrevin (Figure 2A).

Oligomeric material is clearly visible for both detergents. A small amount of trimer is also visible in the 2% Thesit extraction. Additional exposure revealed higher order oligomers in both samples (data not shown). The concentration of protein loaded is quite high for western blotting resulting in saturation of the monomer band and making quantitation problematic. Using the public domain NIH IMAGE program to analyze the blot, we found that synaptobrevin purified in 2% Thesit had 2-fold more dimer than the sample purified in 1.5% cholate. This difference is small but reproducible ($n = 3$). In addition, in 2% Thesit the intensity of the dimer band decreased 10-fold upon 10-fold dilution, suggesting that this may not truly be an equilibrium. With this caveat, we attempted to place limits on the dissociation constant by using the intensity of the monomer band to estimate the concentration of dimer. Using NIH image, we found that in 2% Thesit the dimer band intensity is nearly identical to the intensity of the 100-fold diluted sample, giving a dimer-to-monomer ratio of 0.01. This is significantly different from the previously published estimate of 0.62 (16). A sample containing 1% dimer at 90 μ M protein would have a dissociation constant on the order of 10 mM.

The synaptobrevin transmembrane domain contains a cysteine residue at position 103, which is at the center of the identified dimerization motif (16). To determine whether intermolecular disulfide formation stabilizes the weak dimerization of recombinant synaptobrevin, we examined the effect of a reducing agent on the purification of synaptobrevin. Cells from one synaptobrevin growth were split in two and processed in parallel with or without 10 mM β -mercapto-

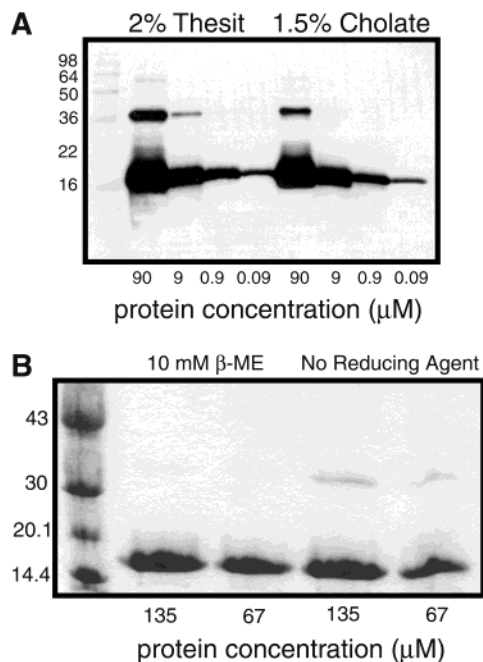


FIGURE 2: (A) Effect of detergent on extraction and purification of recombinant synaptobrevin. Cells from one growth were split and processed in parallel with either 2% Thesit (16) or 1.5% sodium cholate (18). Samples at 90 μM were serially diluted by 10-fold in buffer with the appropriate detergent before addition of SDS sample buffer. Protein concentration (μM) for each lane is indicated beneath the gel. Samples were run on 15% acrylamide gels and transferred to nitrocellulose for blotting with a synaptobrevin monoclonal antibody. (B) Effect of reducing agents on the purification of recombinant synaptobrevin examined by SDS-PAGE. Cells from one growth were split and processed in parallel either with or without β -mercaptoethanol as a reducing agent during purification. Samples were run on 20% acrylamide Phast gels. SDS-PAGE sample buffer contained 0.2 M DTT. The left two lanes show the results for synaptobrevin purified with reducing agents run at 135 and 67 μM . The right two lanes show the results for synaptobrevin purified without reducing agents also run at 135 and 67 μM .

ethanol as a reducing agent. Both samples were purified on Ni-NTA agarose using 2% Thesit in all buffers. Dimerization was assessed by SDS-PAGE using 1% SDS and 200 mM DTT in the loading buffer. The samples were not boiled before PAGE as boiling produced a variety of higher order oligomers in both samples (data not shown). Instead, samples were left at 37 $^{\circ}\text{C}$ for 10 min prior to electrophoresis. The SDS-PAGE results using Coomassie staining are shown in Figure 2B. For the sample processed in β -mercaptoethanol, the protein runs as a single species with the predicted molecular weight of monomeric synaptobrevin. Despite a high concentration of DTT in the sample buffer, the protein purified without reducing agents gave an additional faint band with the molecular weight of a synaptobrevin dimer visible by Coomassie staining. While oxidation apparently increased the amount of dimer, it still remained well below the published estimate (16). Additionally, the ratio of monomer to dimer was not sensitive to increased detergent concentration (data not shown).

As we failed to observe the reported levels of dimerization for synaptobrevin, we attempted to force synaptobrevin dimerization using the same interface by mutating the transmembrane cysteine to asparagine. Asparagine has been previously shown to be sufficient to induce oligomerization of transmembrane domains independently of the rest of the

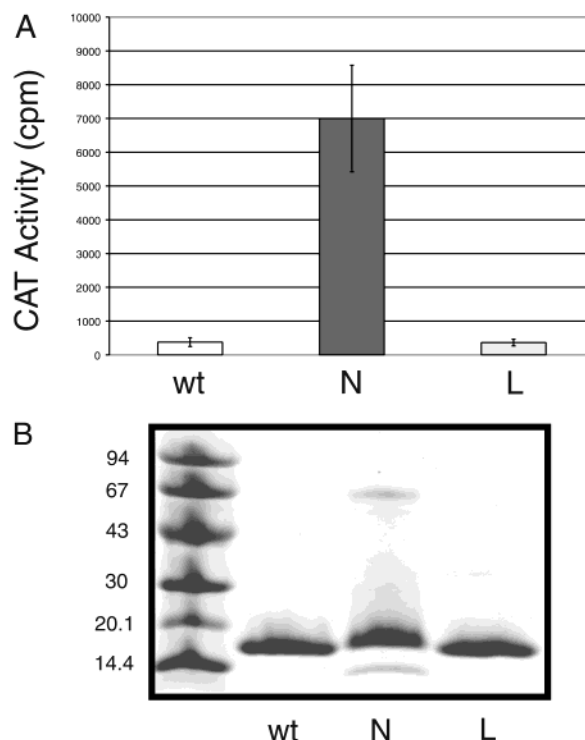


FIGURE 3: (A) Quantitative analysis of dimerization of synaptobrevin cysteine 103 mutants. CAT activity (cpm) for cultures expressing TOXCAT chimeras containing the transmembrane sequence indicated. All mutants were created using the synaptobrevin construct BC (Figure 1). The wt chimera contained the wild-type synaptobrevin sequence. L refers to the mutation of cysteine 103 to leucine. N refers to the mutation of cysteine 103 to asparagine. (B) SDS-PAGE analysis of full-length recombinant synaptobrevin containing transmembrane cysteine mutations. All proteins were purified under identical conditions using 2% Thesit. A total of 4 μL of protein at 100 μM in SDS loading buffer was loaded in each lane. Samples were run in an 8–25% gradient gel. wt refers to full-length rat synaptobrevin 2. N refers to the point mutation of cysteine 103 to asparagine. L refers to the point mutation of cysteine 103 to leucine.

sequence (22). As a negative control, we mutated the cysteine to leucine. The mutations were first examined in the 15-residue synaptobrevin transmembrane construct using TOXCAT. The results are shown in Figure 3A. The leucine mutation had no effect on dimerization in the *E. coli* inner membrane, which is not surprising given the low levels of dimerization of the wild-type sequence in TOXCAT. In contrast, the mutation to asparagine gave a 20-fold increase in CAT activity. This is 2-fold higher than the CAT activity seen for the GpA wild-type sequence. This confirms that CAT activity is indeed sensitive to synaptobrevin when there is sufficient driving force for an association.

The transmembrane cysteine mutations were also examined in the context of the full-length protein (Figure 3B). Both mutants were expressed and purified under identical conditions in 2% Thesit with β -mercaptoethanol. All proteins were adjusted to 100 μM and analyzed by SDS-PAGE with Coomassie staining. The mutation to leucine (C103L) did not significantly affect dimerization relative to the wild type. In contrast, the mutation to asparagine (C103N) was largely monomeric with a small amount of an apparent tetramer. The effect of the asparagine mutation on synaptobrevin oligomerization was similar to that reported previously. In model transmembrane sequences, asparagine gave an in-

creased CAT activity relative to glycoporphin A, while in detergent micelles the protein retained a large fraction of monomer with higher order species indicating a lack of oligomerization specificity (22). The difference between TOXCAT and SDS-PAGE in the effect of polar residues on oligomerization has been noted previously (19, 22). The detergent micelle provides a different environment from a biological membrane in that polar side chains, which would be buried in a membrane, may protrude from the micelle and interact with the solvent (19).

DISCUSSION

In this study, we reexamined synaptobrevin homodimerization using TOXCAT. This system assesses dimerization of transmembrane sequences in the inner membrane of *E. coli* without the need for extraction and solubilization (19). We found the synaptobrevin transmembrane domain to interact very weakly, giving only 20% of the CAT activity of the dimeric Glycophorin A transmembrane domain (Figure 1). The measured CAT activity for synaptobrevin was only 2-fold higher than that measured for the nondimerizing G83I mutant of Glycophorin A. Additionally, we found a synaptobrevin mutant (C103N) that resulted in a 20-fold increase in CAT activity relative to the wild-type synaptobrevin transmembrane domain. The mult triple mutant, reported to block dimerization (16), gave consistently less CAT activity than the wild-type sequence, but the difference was always within the error of the measurement. The relative CAT activity of these three sequences helps to set an interaction scale for this sequence. By directly comparing these sequences along with glycoporphin A, we are able to provide the first assessment of the strength of synaptobrevin dimerization.

Early reports using brain-derived synaptobrevin did not report stable oligomers (8, 10, 11, 15) and instead resorted to chemical cross-linkers to stabilize dimers for immunodetection. However, recombinant synaptobrevin readily forms a SDS-resistant dimer. The two reports in the literature disagree on the magnitude of dimerization, but different detergents were used, which makes direct comparison difficult (16–18). The phenomenon of SDS-resistant aggregation of membrane proteins has been described previously and is highly dependent on the choice of solubilization conditions (23), so we attempted to directly compare the two detergents used. We found that the amount of SDS-resistant synaptobrevin dimer was dependent on the detergent used during the extraction and the presence of reducing agents (Figure 2). However, we were unable to reproduce the previously reported dimer-to-monomer ratio of 0.62 in any condition (16). The previous report used chimeras of synaptobrevin and *Staph* Nuclease A in a SDS-PAGE assay run in the presence of urea. Either of these differences could be contributing to their enhanced dimerization.

In our hands, recombinant synaptobrevin was largely monomeric in SDS-PAGE assays at concentrations approaching 100 μ M. Overexposed western blots were necessary to reveal dimeric material in less concentrated samples, which is similar to the approach used by others (18). At this protein concentration, a sample showing only a few percent dimer is indicative of a dissociation constant on the order of 10 mM.

The cysteine at position 103 in the synaptobrevin transmembrane domain was identified as being at the center of the dimerization motif (16), and a molecular model of the synaptobrevin homodimer has recently been published, which also shows that the closest approach between the helices occurs at cysteine 103 (24). By mutating this cysteine to asparagine, we were able to provide sufficient interactions to stabilize the oligomerization (Figure 3). Thus, we were able to force dimerization using the same face of the helix. However, this cysteine has been reported to be palmitoylated in adult brain, which would completely alter the dimer interface *in vivo* (25). In the molecular model, the dimer appears to be stabilized only by van der Waals forces arising from complimentary surfaces. As such, a 16-carbon palmitoyl group at the closest point of approach would be expected to significantly disrupt the interaction. As our results were obtained using unmodified synaptobrevin, the interaction is likely to be even weaker for the palmitoylated protein. We conclude that synaptobrevin dimerization is energetically possible, but such a weak binding is not likely to contribute to biological activity. Additional support for this notion comes from a recent report looking at synaptobrevin dimerization *in vivo* in hippocampal neurons, which concluded that very few oligomers were formed (26).

ACKNOWLEDGMENT

The authors wish to thank William P. Russ, Fang Zhou, Melanie Cocco, R. Bryan Sutton, and James Ernst for technical assistance, stimulating discussions, and critical reading of the manuscript.

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BI0269411