Synaptobrevin Transmembrane Domain Dimerization—Revisited†

Rana Roy,‡ Rico Laage,§,⊥ and Dieter Langosch*,‡,§

Lehrstuhl Chemie der Biopolymere, Technische Universität München, Weihenstephaner Berg 3, 85354 Freising, Germany, and Institut für Neurobiologie, Universität Heidelberg, INF 364, 69120 Heidelberg, Germany

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ABSTRACT: Synaptobrevin is a membrane-spanning soluble *N*-ethyl maleimid-sensitive factor (NSF) attachment protein receptor (SNARE) protein of synaptic vesicles that is essential for neurotransmitter release. Various lines of evidence indicate that it exists alternatively as a monomer, as a homodimer, as a heterodimer with synaptophysin, or as a ternary complex with other SNAREs at the various stages of the synaptic vesicle cycle. Homodimerization of synaptobrevin was previously shown by different authors to depend on its single transmembrane segment, and the crucial residues forming the helix−helix interface have been mapped. Since another recent study challenged these results, we reinvestigated this issue. Here, we show that native synaptobrevin can be cross-linked in synaptic vesicle membranes to a homodimer by disulfide bond formation between cysteine residues of the transmembrane segment. Further, we demonstrate that determination of synaptobrevin transmembrane segment interactions in membranes or in detergent solution requires careful control of experimental conditions. Thus, our present results corroborate that homodimerization of synaptobrevin is mediated by its transmembrane segment.

SNARE[1] (soluble *N*-ethyl maleimid-sensitive factor (NSF) attachment protein receptor) proteins form an evolutionarily conserved family of proteins that are essential for all types of intracellular membrane fusion events. The best characterized SNAREs are those mediating neurotransmitter release from presynaptic vesicles, that is, the single-span integral membrane proteins synaptobrevin II (also referred to as VAMP II) and syntaxin 1A, as well as the peripheral membrane protein SNAP-25 (*1*−*3*). These proteins form a stable ternary complex that appears to bridge opposing vesicular and plasma membranes prior to actual membrane fusion (*4*) and to mediate membrane fusion in vitro upon reconstitution in liposomes (*5*). While crystallographic analysis (*6*) has shown that a soluble version of the complex formed from recombinant SNARE fragments corresponds to a coiled-coil structure, the crystal structure of the complete SNARE complex including the transmembrane segments (TMSs) is not known. In vivo, synaptobrevin II molecules that do not participate in SNARE complex formation bind to the synaptic vesicle protein synaptophysin or form a homodimer as revealed by cross-linking experiments performed on brain fractions (*7*−*9*) or visualization of fluorescently tagged molecules (*10*). A homodimer also formed from recombinant synaptobrevin II in vitro, that is, in detergent solution (*11*) or in liposomes (*12*), but only if the full-length version including the TMS was used. Alanine-scanning mutagenesis has indicated that synaptobrevin homodimerization depends on sequence-specific self-interaction of its TMS that involves at least six residues (L99, I102, C103, L107, I110, and I111). Synaptobrevin TMS−TMS interaction has also been demonstrated in membranes in the absence of the cytoplasmic domain using the ToxR system (*13*). Grafting the six crucial amino acid residues onto an inert oligo-alanine host sequence restored homodimerization in detergent solution; in membranes two additional residues (I98 and I106) were required for wild-type-level homodimerization (*13*). These amino acids form a contiguous patch on the synaptobrevin transmembrane helix and are thus supposed to constitute the TMS−TMS interface (Figure 1). The pattern of these interfacial residues suggests that the self-interacting TMSs adopt a negative crossing angle (*11*, *13*). These experimental results are supported by a computational study revealing that the critical residues form a tightly packed interface between α-helices that approach each other most closely at cysteine 103 and cross each other at a packing angle of −38° (*14*). Interestingly, the interfacial residues are almost completely conserved within the TMS of syntaxin 1A, the natural binding partner of synaptobrevin II (*13*). This has predicted that the TMS also mediates self-assembly of syntaxin or its heterophilic association with synaptobrevin, and this prediction was experimentally confirmed (*12*, *13*). In the heterodimeric complex, a negative crossing angle between the TM-helices would contrast the positive angle of the cytoplasmic coiled-coil domain; this suggested that the sequence linking both domains is unstructured (*13*). This conjecture is experimentally supported for syntaxin by electron paramagnetic resonance studies and molecular dynamics simulations, and it was proposed that flexibility

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* Corresponding author. Tel: + 49 8161 71-3500. Fax: +49 8161 71-44 04. E-mail: langosch@bl.tum.de.

‡ Technische Universität München.

§ Universität Heidelberg.

⊥ Present address: Axaron Bioscience AG, INF 515, 69120 Heidelberg, Germany.

[1] Abbreviations: CD, circular dichroism; DSS, disuccinimidyl suberate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MU, Miller units; PMSF, phenylmethylsulfonyl fluoride; SNARE, soluble NSF attachment protein receptor; syb, synaptobrevin; TFE, trifluoroethanol, TMS, transmembrane segment.

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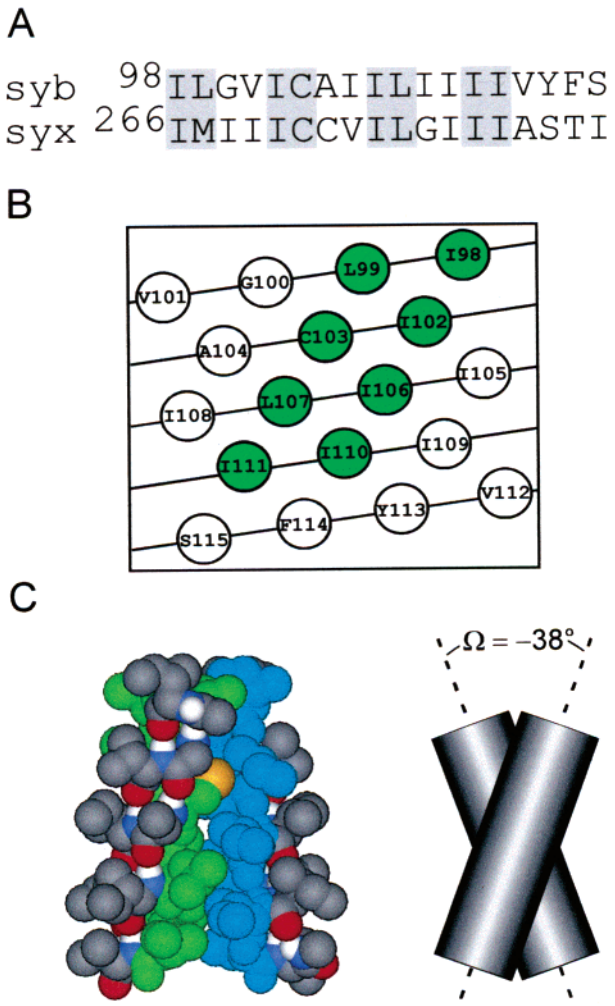

FIGURE 1: The amino acid interface of dimerizing SNARE protein TMSs. Panel A shows the alignment of synaptobrevin II and syntaxin 1A TMSs. Those residues previously (*11*, *13*) shown to be required for homodimerization or for heterodimerization of both proteins in membranes are shaded. Note that seven out of eight interfacial residues are conserved between both TMSs. Panel B shows a helical net representation of the synaptobrevin II TMS where interfacial residue positions are in green. Panel C shows a model of the synaptobrevin II TMS homodimer. The structural model (left) was previously calculated (*14*) on the basis of our (*11*, *13*) mutagenesis data. For easier visualization of TMS−TMS packing, the interfacial residues are colored green and blue (the sulfur atom of C103 is in yellow). The schematic (right) visualizes the right-handed nature of the homodimer of which the crossing angle, Ω, was calculated to be −38° (*14*).

of this region might be required for mechanical coupling between both interaction domains in SNARE protein function (*15*−*17*). The functional relevance of SNARE TMS−TMS interactions is currently not clear. It has been reported previously that substitution of SNARE TMSs by long isoprenoid chains preserves fusogenicity of SNAREs in an in vitro liposome−liposome fusion assay (*18*). On the other hand, a proteinacious TMS appears to be essential for SNARE function in vivo since replacement of the TMS by isoprenoid moieties abolished the function of exocytotic (*19*) and vacuolar (*20*) SNAREs in yeast cells.

Recently, Bowen et al. (*21*) challenged the validity and potential functional importance of the previously obtained results on synaptobrevin homodimerization (*11*−*13*) by reporting that this TMS−TMS interaction would be of only low affinity. Further, they referred to another study that showed that TMS residue cysteine 103 is partially palmitoylated in native synaptobrevin (*22*) and argued that this residue could therefore not be part of the proposed TMS−TMS interface.

Since we believe in intersubjective reproducibility of experimental results, we carefully reexamined this issue and show here (i) that synaptobrevin can be cross-linked to a homodimer in native synaptic vesicles via TMS residue cysteine 103, (ii) that assaying synaptobrevin TMS−TMS interactions in membranes critically depends on how the TMS sequence is inserted into the ToxR proteins, and (iii) that dimerization assays in detergent depend on the conditions of SDS−PAGE analysis.

EXPERIMENTAL PROCEDURES

Synaptic Vesicle Preparation. Preparation of synaptic vesicles was performed as described by Huttner (*23*) omitting the final glass bead chromatography. Whole brain was prepared from 14 week old male rats (Wistar), homogenized in a glass/Teflon homogenizer, and then subjected to further fractionation steps. The final fraction enriched in small synaptic vesicles was kept in 4 mM Hepes, pH 7.4, 250 mM sucrose at 4 °C and directly used for cross-linking experiments.

Cross-Linking. For disulfide cross-linking with iodine, 50 μl of a fresh synaptic vesicle preparation was mixed with 50 μl of an aqueous solution of elementary iodine, 1 mM, for 15 s. To stop the reaction, the proteins were precipitated with methanol (*24*), washed, subjected to SDS−PAGE using standard procedures, and visualized by Western blot analysis after denaturation for 5 min at 100 °C. Blots were probed with a polyclonal rabbit anti-rat synabtobrevin II antiserum (WAKO Bioproducts, Japan) recognizing the N-terminus of the protein. For cross-linking with the homobifunctional cross-linking reagent DSS (disuccinimidyl suberate, Pierce), DSS was applied to synaptic vesicles according to the manufacturer's protocol at a final concentration of 150 μM for 5 min. The reaction was quenched by addition of 10 mM Tris, pH 7.4, to a final concentration of 1 mM for 10 min. Proteins were precipitated with methanol (*24*) and analyzed as above.

Plasmids. Plasmids ToxR-GpA13, ToxR-GpA13 G83A, and ToxR-syb15 (previously termed syb-wt) were described previously (*13*, *25*). Plasmids ToxR-syb13, ToxR-sybBC, and ToxR-sybTMg were constructed by ligating synthetic oligonucleotide cassettes encoding the desired sequences into the plasmid pHKToxR(TM^{II4})MalE (*26*) previously cut with *Nhe*I and *Bam*HI. All constructs were verified by dideoxy sequencing.

ToxR Activity Assays. Plasmid-transformed FHK12 cells were grown in the presence of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and assayed for β-galactosidase activity as described (*27*). β-Galactosidase activity of cell-free extracts is given in Miller units (MU).

Expression and Solubilization of Recombinant Synaptobrevin. The fusion protein of *Staph* nuclease A and synaptobrevin, cloned in pSNiR vector (*28*), was expressed in *Escherichia coli* BL21(DE3) as described (*11*). The proteins were solubilized in buffer containing 25 mM Hepes, pH 7.6, 1 M NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl

fluoride (PMSF), and 2% (v/v) Thesit (Sigma) for all applications.

Electrophoresis and Western Blot. In one type of experiment, SDS−PAGE was performed as described by Bowen et al. (*21*), that is, the proteins were incubated at 37 °C for 30 min with sample buffer containing 50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.2 M DTT, and 10% (w/v) sucrose. The gel (12.5% polyacrylamide) was prepared without SDS. Alternatively, the solubilized protein was first precipitated with methanol (*24*) and then solubilized with sample buffer containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 1% (w/v) SDS, 0.2 M DTT, and 10% (w/v) sucrose as described by Laage et al. (*11*). The gel (12.5% polyacrylamide) was prepared with SDS (0.1% w/v) and also contained 6 M urea. Electrophoresis was performed at 4 °C. Proteins were visualized by Western blot with a primary antibody against the *myc* epitope of the fusion protein and developed by chemiluminesence. Similar results were obtained when urea was introduced by dialyzing the protein samples against buffer containing 25 mM Hepes, pH 7.6, 1 M NaCl, 1 mM EDTA, 1 mM DTT, and 6 M urea overnight, rather than by precipitating and resolubilizing.

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded for a synaptobrevin TMS peptide (sequence KKKWILGVICAIILIIIIVYKKK) synthesized by standard solid-phase methods (F-moc) by PSL, Heidelberg, Germany. Mass spectrometry showed that the peptide was >90% pure. All CD spectra were recorded on a JASCO J-715 model CD spectrometer with a scan speed of 50 nm/min and an average of 20 scans per sample. The spectra were recorded at 20 °C. Samples were dissolved in TFE or in 10 mM Tris-HCl, pH 6.8, 1% (v/v) Thesit, and 6 M urea or in the same buffer without urea. Samples dissolved in Thesit buffer were incubated for 2 h to equilibrate and centrifuged for 30 min at 19.500 × *g* before recording spectra. Concentrations of the peptides were determined by UV-absorption spectroscopy of the supernatants using molar extinction coefficients calculated with the peptide property calculator program (http://www.basic.nwu.edu/biotools/proteincalc.html). The spectra of the peptide dissolved in TFE or in detergent solution without urea were measured in a 0.1 cm quartz cuvette at a concentration of 40 μM, whereas those of the peptide dissolved in detergent buffer with 6 M urea were measured in a 0.02 cm quartz cuvette at a concentration of 300 μM. Similar data were obtained when CD spectra in the absence or presence of urea were recorded at the same peptide concentration (40 μM). Spectra were fitted using standard software (CDNN, ref *29*, available at http://www.imtech.res.in/pub/pspt/cdnn/win/) for secondary structure calculations.

RESULTS

Cross-Linking of Synaptobrevin in Synaptic Vesicle Membranes. Previously, work from different laboratories had shown that synaptobrevin present in rat brain detergent solubilizates, synaptic vesicle membranes (*7*, *8*), or synaptosomes (*9*) can be partially cross-linked with amine-reactive reagents to a homodimer or to a heterodimer with synaptophysin. Here, we extended these earlier findings by comparing the effects of disuccinimidyl suberate (DSS), a homobifunctional amine-reactive cross-linking reagent, with the effect of iodine treatment that oxidizes a pair of neighboring cysteine residues to a disulfide bridge (*30*).

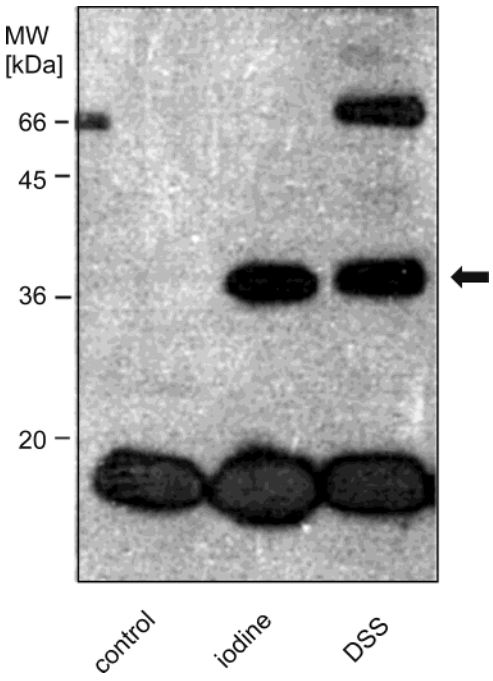

FIGURE 2: Synaptobrevin cross-linking in synaptic vesicles. Purified vesicles were treated with the cross-linking reagents iodine or DSS and analyzed for synaptobrevin by 15% SDS−PAGE followed by Western blotting. The 18 kDa band seen with untreated vesicles (control) corresponds to monomeric synaptobrevin, whereas the bands at 37 kDa seen upon cross-linking indicate formation of synaptobrevin homodimers (arrow). DSS cross-linking also produced a 68 kDa band that corresponds most likely to a synaptobrevin/synaptophysin heterodimer (*9*).

Small synaptic microvesicles were prepared from rat brains (*23*), incubated with DSS or iodine, and subjected to SDS−PAGE followed by immunostaining for synaptobrevin. In agreement with the earlier findings (*7*−*9*), DSS produced a 37 kDa synaptobrevin homodimer plus a 68 kDa adduct that is likely to correspond to a synaptobrevin−synaptophysin heterodimer (Figure 2). Upon iodine treatment, only a synaptobrevin homodimer was produced the intensity of which was similar to that seen upon DSS cross-linking (Figure 2). Since the sole cysteine residue present in the synaptobrevin molecule (C103) is localized within the predicted TMS, the TMSs of membrane-embedded synaptobrevin must be closely juxtaposed prior to cross-linking. Further, this result revealed that the cysteine residue at least of the cross-linkable fraction of native synaptobrevin is not covalently modified by fatty acids.

Sequence-Specific Synaptobrevin TMS−TMS Interaction in Membranes. Synaptobrevin TMS−TMS interaction has previously been investigated with the ToxR transcription activator system. In this method, single-span ToxR chimeric proteins contain the desired TMS that anchors them within the inner *E. coli* membrane and mediates their self-assembly depending on its affinity. Since self-assembled ToxR proteins drive transcription activation of a reporter gene, encoding, for example, β-galactosidase (*31*), reporter enzyme activity reflects TMS−TMS affinity (*25*). Previously, a 15 residue stretch covering the TMS−TMS interface (here termed syb15, Figure 3A) had elicited significant transcription activation indicating its self-interaction in the membrane (*13*).

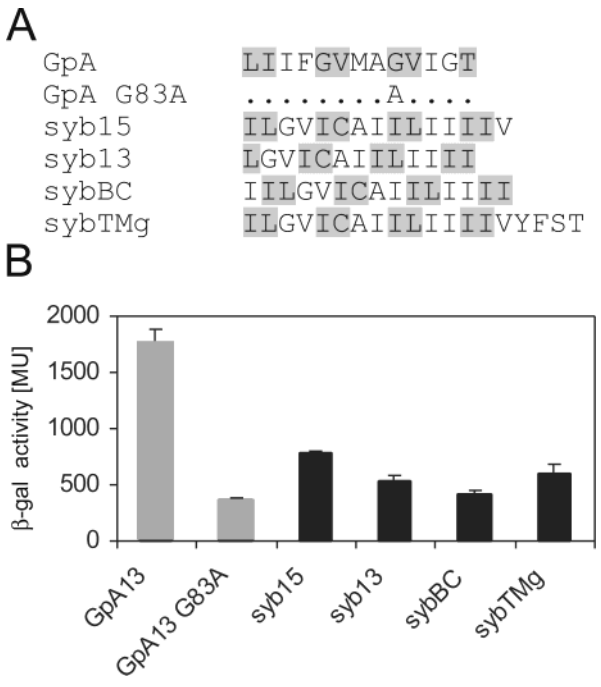

FIGURE 3: Self-assembly of the synaptobrevin TMS in membranes as examined with the ToxR system. Panel A depicts GpA and synaptobrevin II TMS sequences and their mutants inserted into ToxR chimeric proteins. The previously identified (*13*, *25*, *39*) interfacial residues are shaded. Dots represent wild-type residues. Construct syb15 corresponds to syb-wt in ref *13* and sybBC corresponds to Syb BC in ref *21*. Panel B shows degrees of self-assembly of the TMSs shown in panel A as revealed by β-galactosidase activities (in Miller units, MU, means $\pm$ SE, $n \geq 8$ data points) determined upon expression of the respective ToxR chimeric proteins in the reporter strain FHK12. Note that the signal elicited by syb15 (*13*) is above the signals of syb13 or of sybBC and intermediate between the signals of the strongly homodimerizing GpA13 and its mutant G83A.

Bowen et al. (*21*) have tested the activities of a series of ToxR chimeric proteins containing different parts of the synaptobrevin TMS. They found that the signals elicited by their constructs were uniformely low in comparison to a ToxR protein with the TMS of glycophorin A, a well-established model of high-affinity TMS−TMS interaction (*25*, *32*−*34*). Unfortunately, these authors did not test construct syb15 that was previously investigated by us (compare Figure 1B in ref *13*).

We have noted earlier that the orientation of the interacting faces of a TM helix relative to the DNA-binding ToxR domains may influence the coupling of TMS−TMS interaction to transcription activation (*31*). Therefore, we determined the effect of inserting the synaptobrevin TMS at different phases into ToxR chimeric proteins and compared the obtained signals to that seen with construct GpA13 harboring part of the glycophorin A TMS (Figure 3B). We found that the signal elicited by syb15 (*13*) corresponded to 44% of the GpA13 signal and was well above that of the GpA point mutant G83A. In comparison, constructs syb13 and sybTMg, which includes all C-terminal synaptobrevin residues, gave 30% and 34% of the GpA13 signal, respectively. In agreement with the data presented by Bowen et al. (*21*), their construct sybBC elicited only 23% of the GpA13 signal. Syb15 thus gives rise to a significantly stronger transcriptional signal than the other constructs tested in this study or analyzed by Bowen et al. (*21*).

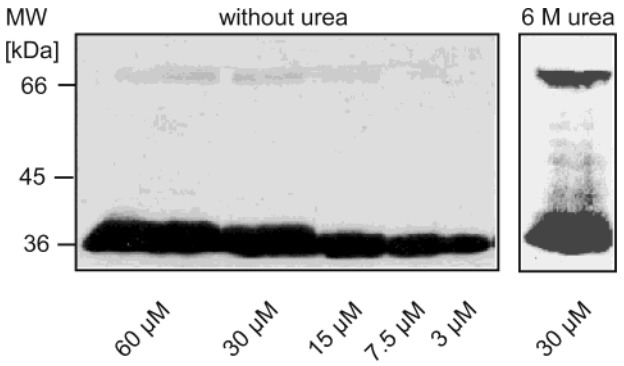

FIGURE 4: Dimerization of solubilized recombinant synaptobrevin 36 kDa fusion protein by SDS−PAGE. In the left panel, the protein was dissolved in sample buffer with 2% (w/v) SDS at the indicated concentrations and incubated at 37 °C as in Bowen et al. (*21*) prior to electrophoresis in a gel without urea. In the right panel, the protein was dissolved in sample buffer with 1% (w/v) SDS plus 6 M urea at a concentration of 30 μM and electrophoresed at 4 °C in a gel containing 6 M urea as described by Laage et al. (*11*). Significant homodimer is visible only in the presence of urea.

We conclude that the orientation of the TMS within construct syb15 elicits optimal ToxR-mediated reporter gene expression and thus reflects the affinity of the TMS−TMS interface more faithfully than the other orientations tested.

Dimerization of Full-Length Synaptobrevin in Detergent Solution. The stoichiometries of protein oligomers based on TMS−TMS interactions can conveniently be monitored by SDS−PAGE provided that the latter are resistant to SDS. Significant noncovalent dimerization had been observed for a 33 kDa recombinant protein of full-length synaptobrevin II fused to *Staphylococcus aureus* nuclease A upon electrophoresis at mild conditions, that is, when a low (1% w/v) concentration of SDS was used in the sample buffer and sample heating was omitted; dimerization was preserved best upon inclusion of 6 M urea in sample buffer and gel (*11*, *13*). Synaptobrevin homodimerization was also seen in the absence of urea when the nuclease A fusion moiety had been removed (*28*) or when synaptobrevin had been reconstituted into liposomes prior to SDS−PAGE (*12*). Bowen et al. detected only marginal synaptobrevin dimerization when they used 2% (w/v) SDS in sample buffer, incubated at 37 °C, and omitted urea (*21*). To test whether these experimental differences could account for the different degrees of dimerization seen by the different authors, we expressed the synaptobrevin fusion protein and solubilized it in Thesit before splitting the sample in two and subjecting the protein to SDS−PAGE at the two different conditions given above followed by immunostaining. In agreement with Bowen et al. (*21*), Figure 4 shows that dimerization was very weak under their conditions at all tested concentrations. On the other hand, significant dimer was preserved under our conditions, that is, in the presence of urea. Because the protein was prepared in the presence of reducing agent, we exclude that homodimerization is due to the formation of a disulfide bond. This is supported by the observation that sample boiling completely disrupts dimerization (data not shown). Since the omission of the 37 °C incubation step in the absence of urea did not enhance dimerization (data not shown), we conclude that the discrepancy between our previous results and those of Bowen et al. are primarily due to the absence or presence of urea in SDS−PAGE analysis.

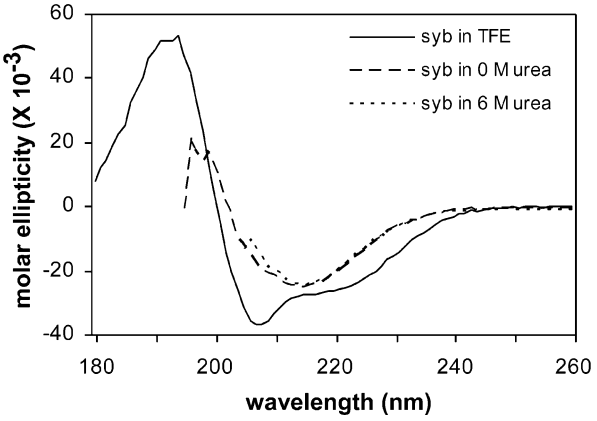

FIGURE 5: CD spectra of a synaptobrevin TMS peptide. The peptide was dissolved in TFE or in Thesit buffer with or without urea. The spectra indicate that α-helicities of both peptides were more pronounced in TFE than in detergent solution and that the presence of 6 M urea had only a minor impact on the secondary structure. Spectra were recorded from 180 to 260 nm (TFE), from 193 to 260 nm (Thesit without urea), or from 205 to 260 nm (Thesit with 6 M urea). The downward deflection of the spectrum recorded in Thesit at wavelengths <200 nm is attributed to an artifact caused by the high optical density of the sample in this region.

It is presently not clear why urea promotes preservation of the synaptobrevin homodimer in the presence of SDS. Because urea normally denatures proteins, we tested whether it influences the secondary structure of the synaptobrevin TMS. CD spectroscopy showed that a synthetic synaptobrevin TMS-peptide was primarily (85%) α-helical in TFE. Under the conditions used for detergent solubilization of the synaptobrevin fusion protein, that is, in Thesit solution, the peptide displayed 69% α-helical structure (Figure 5A). In the presence of 6 M urea, similar helicity was found (65%) suggesting that the effect of urea on self-assembly must be of an indirect nature as discussed below.

DISCUSSION

In the present study, we present new data and compare experimental conditions previously used by us (*11*, *13*) and Bowen et al. (*21*) to clarify whether sequence-specific interactions between the TMSs are responsible for synaptobrevin homodimerization.

First, we cross-linked native synaptobrevin in synaptic vesicle membranes either with a bifunctional reagent (DSS) or with iodine. Both methods confirm that substantial fractions of native synaptobrevin molecules exist as homodimers. In agreement with earlier reports (*7*−*9*), no cross-linking to syntaxin or to SNAP-25 was seen. Whereas DSS primarily reacts with lysine side chains localized within the soluble part of the protein, iodine leads to intermolecular disulfide bridge formation between the side chains of the sole cysteine of the whole protein, that is, to cysteine 103 within the TMS. Since disulfide formation requires spatial proximity of cysteines, our data suggest that homodimerization of synaptobrevin in synaptic vesicles involves the previously identified (*11*, *13*) TMS−TMS interface. This agrees well with the model computed by Fleming and Engelman (*14*) where cysteine 103 represents the site of closest contact within the TMS−TMS interface. Disulfide cross-linking experiments have previously been used to map TMS−TMS interfaces of other membrane proteins, too (*30*, *35*, *36*). Bowen et al. (*21*) considered it unlikely that native synaptobrevin could homodimerize via its TMS since the central cysteine 103 would be palmitoylated as reported by Veit et al. (*22*). Indeed, that study reported that addition of [^{3}H]-palmitic acid to synaptosomes or synaptic vesicles isolated from adult rat brain or to PC12 cells covalently labeled synaptobrevin. However, labeling had occurred at substoichiometric ratios (*22*). Further, synaptobrevin could not be palmitoylated in synaptic vesicles from embryonic brain (*22*). Our data show that DSS and iodine are equally efficient in cross-linking native synaptobrevin within synaptic vesicle membranes. Accordingly, the cross-linkable synaptobrevin molecules must contain an unmodified cysteine residue. Nevertheless, the intriguing possibility remains that part of native synaptobrevin is acylated at cysteine 103 and thus unable to homodimerize via TMS−TMS interaction. One may therefore speculate that synaptobrevin homodimerization in vivo is regulated by this covalent modification that is known to be reversible and frequently regulated in a highly specific way (refs *22* and *37* and references therein).

Second, we reinvestigated synaptobrevin TMS−TMS interaction by the ToxR system to clarify whether the relatively low signals reported by Bowen et al. (*21*) could be due to different orientations of the synaptobrevin TMS within ToxR chimeric proteins. Our present data reveal that our previously (*13*) investigated ToxR construct (syb15) elicits a signal that is about twice as large as that of sybBC used by Bowen et al. (*21*). The distinguishing feature between both constructs is that the interfacial amino acids within sybBC are shifted by one residue toward the C-terminus, that is, they assume a different orientation relative to the ToxR domain as compared to the interfaces of construct GpA13 used as positive control or of syb15 (Figure 3A). Assuming α-helicity of the TMS, this shift would rotate the ToxR domain by 100° relative to the TMS−TMS interface and thus may compromise transcription activation of the reporter gene. We conclude therefore that the magnitudes of the signals critically depend on the phase of the TMS sequences within the ToxR proteins as noted previously for the glycophorin A TMS (*25*) and the erythropoietin receptor TMS (*38*). Bowen et al. (*21*) investigated three other synaptobrevin constructs. In Syb BD, the interface has the same phase as that in sybBC. In Syb AD and Syb AC, they inserted several residues between the ToxR domain and TMS that may be part of the soluble domain (*13*). Insertion of additional residues at this site appears to be detrimental to the coupling of TMS−TMS interaction to transcription activation as found in another study where insertion of a six-residue flexible linker decreased the signal elicited by the erythropoietin receptor TMS by ∼50% (*38*).

Nevertheless, the TMS of synaptobrevin apparently self-assembles with a significantly lower affinity than that of glycophorin A although the latter also adopts a negative packing angle as indicated by mutagenesis (*11*, *13*, *25*, *33*, *39*), model building (*14*, *40*), and NMR spectroscopy (*41*, *42*). It should be noted, however, that the glycophorin A TMS−TMS interface is a special case because it harbors a critical GxxxG motif. This motif is thought to drive TMS−TMS packing by formation of a flat helix surface, by limiting the entropy loss that is normally associated with the freezing of side chain conformations upon protein/protein interaction (*43*), or by facilitating hydrogen bond formation between

C_α-hydrogens and the backbone of the partner helix (*44*) or by a combination of these. By comparison, the model of the synaptobrevin TMS−TMS interface comprises a much smaller molecular surface area (396 Å^2) than that of glycophorin A (550 Å^2), its formation is associated with a higher loss of side chain rotamer entropy, and it is not stabilized by intermolecular hydrogen bonds (*14*). It is not surprising, therefore, that the synaptobrevin TMS self-interacts with a lower affinity than that of glycophorin A.

Third, we reinvestigated how the conditions of SDS−PAGE analysis affect preservation of full-length synaptobrevin homodimerization in detergent solution since Bowen et al. (*21*) observed less efficient dimerization than we did previously (*11*, *13*). Here, significant homodimerization in the presence of SDS was only seen when the sample was not heated and electrophoresed in the presence of urea. A similar effect of urea was observed previously with fusion proteins of syntaxin 1A (*13*) or with the glycophorin A TMS (our unpublished data). The fact that homodimerization is maintained in the presence of urea supports specificity of this protein−protein interaction since urea is known to suppress unspecific aggregation of membrane proteins in SDS−PAGE (*45*). Since urea did not affect TMS secondary structure in detergent solution, as assessed by CD spectroscopy, it must influence synaptobrevin dimerization indirectly. Possibly, urea is required to fully denature the nuclease fusion domain of our constructs (*46*, *47*) since low-level homodimerization was also seen in the absence of urea when the nuclease fusion domain was missing (*12*, *21*) or had been proteolytically removed (*28*). In addition to that, urea is known to affect the behavior of detergents by increasing the water solubility of their acyl moieties (*48*) and might thus induce formation of larger and less tightly packed micelles. Conceivably, therefore, TMS−TMS interactions may be promoted indirectly by the effect of urea on the structure of the SDS micelles or by its effect on the ability of SDS to bind to the TMS and thus to compete with TMS−TMS interaction or both.

CONCLUSION

Taken together, our results show that the discrepancies between earlier work on synaptobrevin TMS−TMS interaction and the recent report by Bowen et al. are due to different experimental conditions used and that at least a substantial fraction of the interfacial cysteine 103 is not covalently modified in synaptic vesicles. A number of hypotheses concerning the functional relevance of synaptobrevin TMS−TMS interaction have previously been forwarded including stabilization (*11*) or multimerization (*13*, *49*) of the SNARE complex and propagation of the "zippering up" of the cytoplasmic coiled-coil domains into the membrane (*12*, *13*). Cross-linking of synaptobrevin and synaptophysin in synaptic vesicle membranes (*7*, *8*, and this study), which requires the synaptobrevin TMS (*9*), as well as monitoring dynamic interactions between both molecules in live neurons (*10*), provided yet another possible function of an interacting synaptobrevin TMS. The latter study showed that the synaptobrevin−synaptophysin complex, as well as the synaptobrevin homodimer, dissociated prior to fusion, suggesting that the stability of both complexes may regulate the availability of synaptobrevin for SNARE complex formation (*10*). Thus, synaptobrevin appears to exist in different forms—that is, as a monomer, as a homodimer, or bound to synaptophysin or to other SNAREs—at the various stages of the synaptic vesicle cycle. Synaptobrevin TMS−TMS self-assembly may have to be of sufficiently low affinity to allow for rapid interconversion of these complexes during neurotransmitter release.

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