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Structural Changes Are Associated with Soluble *N*-Ethylmaleimide-sensitive Fusion Protein Attachment Protein Receptor Complex Formation*

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Dirk Fasshauer‡§¶‖, Henning Otto‡§, William K. Eliason‡ ‡‡, Reinhard Jahn‡§‖, and Axel T. Brünger‡§§**

From the ‡Howard Hughes Medical Institute, §Department of Pharmacology, Yale University School of Medicine, and ‡‡Department of Molecular Biochemistry and Biophysics, Yale University, New Haven, Connecticut 06510

SNAP-25, syntaxin, and synaptobrevin play a key role in the regulated exocytosis of synaptic vesicles, but their mechanism of action is not understood. *In vitro*, the proteins spontaneously assemble into a ternary complex that can be dissociated by the ATPase *N*-ethylmaleimide-sensitive fusion protein and the cofactors α-, β-, and γ-SNAP. Since the structural changes associated with these reactions probably form the basis of membrane fusion, we have embarked on biophysical studies aimed at elucidating such changes *in vitro* using recombinant proteins. All proteins were purified in a monomeric form. Syntaxin showed significant α-helicity, whereas SNAP-25 and synaptobrevin exhibited characteristics of largely unstructured proteins. Formation of the ternary complex induced dramatic increases in α-helicity and in thermal stability. This suggests that structure is induced in SNAP-25 and synaptobrevin upon complex formation. In addition, the stoichiometry changed from 2:1 in the syntaxin-SNAP-25 complex to 1:1:1 in the ternary complex. We propose that the transition from largely unstructured monomers to a tightly packed, energetically favored ternary complex connecting two membranes is a key step in overcoming energy barriers for membrane fusion.

Neurons release their neurotransmitters by the Ca^{2+}-dependent exocytosis of synaptic vesicles. In recent years, several membrane proteins have been identified which are required for exocytotic membrane fusion. These proteins include the synaptic vesicle protein synaptobrevin (also referred to as VAMP)[1] and the synaptic membrane proteins syntaxin and SNAP-25, collectively referred to as SNAREs. Synaptobrevin and syntaxin both contain a single transmembrane domain at the C terminus (1, 2). SNAP-25 does not contain a transmembrane domain but carries palmitoyl side chains attached to cysteine residues in the middle of the sequence (3, 4). Homologues of these proteins have been identified in many eukaryotic cells including yeast, suggesting that fusion of trafficking vesicles with their respective target membranes is mediated by a conserved mechanism (2, 5–8).

While the evidence linking synaptobrevin, SNAP-25, and syntaxin to exocytosis is compelling, their precise role is unknown. In detergent extracts of brain membranes, the three proteins form a tight complex (9, 10). A ternary complex with properties similar to the native complex can be formed using recombinant proteins lacking their transmembrane anchors (11). Both native and recombinant complexes can be disassembled by the concerted action of the ATPase NSF and the protein α-SNAP (9, 10, 12, 13). The latter two proteins are soluble, abundant, and highly conserved through evolution. They are essential for the fusion of trafficking vesicles with their target membranes (9, 10).

The assembly and disassembly of the SNARE proteins has not yet been integrated into a coherent picture of exocytosis. However, any interference with these reactions seriously inhibits membrane fusion (6–8). To further understand these essential reactions, one needs to learn more about the structural and energetic properties of assembly and disassembly. Therefore, we have begun intensive biophysical and biochemical studies of the SNAREs and of their complexes.

Nondenaturing gel electrophoresis, multiangle laser light scattering (MALLS), and CD spectroscopy were used to investigate structural properties of the individual proteins and their complexes. Dramatic changes in structure, oligomerization, and thermal stability upon complex formation were observed.

MATERIALS AND METHODS

Cloning—All recombinant proteins were expressed as His_6-tagged fusion proteins. Subcloning was performed using standard techniques (14). For all polymerase chain reactions (PCR) *Pfu* DNA polymerase was used.

For synaptobrevin 2, the coding sequence for the cytoplasmic domain (*i.e.* residues 1–96) was amplified by PCR using the primers 5′-CCCGGATCCATATGTCGGCTACCGCTGCCACCGTC-3′ and 5′-CGCGGGATCCCTCGAGTTACATCATCTTGAGGTTTTTCCA-3′ and subsequently subcloned into the pET-15b vector (Novagen) using the *Nde*I and *Xho*I restriction sites. This resulted in a fusion protein with an N-terminal His_6 tag that is cleavable with thrombin. The cDNA encoding for rat synaptobrevin 2 (15) was kindly provided by R. H. Scheller (Stanford University).

For the expression of SNAP-25 and syntaxin, expression vectors (referred to as pHO vectors) with versatile polylinker and a C-terminal His_6 tag were constructed. First, a short linker consisting of the oligonucleotides 5′-AATTGGTCGAGCC-3′ and 5′-AGCTGGCTCGACC-3′ was inserted between the *Eco*RI and *Hin*dIII site of pET-11c and pET-11d (kindly provided by F. W. Studier and A. H. Rosenberg (16)),

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‖ Present address: Max-Planck-Institut für Biophysikalische Chemie, Abteilung Neurobiologie, Am Fassberg II, 37077 Göttingen, Germany.

** Present address: Freie Universität Berlin, Institut für Biochemie, Thielallee 63, 14195 Berlin, Germany.

§§ To whom correspondence should be addressed.

[1] The abbreviations used are: VAMP, vesicle-associated membrane protein; NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; SNAP-25, synaptosomal associated protein of 25 kDa; PAGE, polyacrylamide gel electrophoresis, PCR, polymerase chain reaction; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride); MALLS, multiangle laser light scattering.

resulting in the deletion of both cleavage sites. The vector pHO2d was constructed by inserting DNA sequence containing a new multiple cloning site followed by bases coding for a His_6 tag and a stop codon. This insert was generated by annealing the partially complementary primers p1 (5′-CGCCATATGGCCATGGTACCCGGGTCGACAAGCTTGAATTCGCAC-3′) and p2 (5′-GGCGGATCCTATCAGTGATGGTGGTGATGGTGCGAATTCAAGCTTGT-3′) and filling the missing 3′ ends by *Pfu* DNA polymerase activity. The product was cut with *Nco*I and *Bam*HI, gel-purified, and inserted into the corresponding sites of the modified pET-11d vector. In analogy, the vector pHO2c was constructed from the modified pET-11c vector using the primer p3 (5′-GGGATTCCATATGGTACCCGGGTCGACAAGCTTGAATTCGCAC-3′) instead of primer p1 for the construction of the insert.

Rat SNAP-25B (1–206, entire coding sequence) was subcloned via *Nco*I and *Eco*RI into the vector pHO2d. The SNAP-25B sequence was first amplified by PCR from the SNAP-25B (rat) cDNA (kindly provided by T. C. Südhof, University of Texas Southwestern Medical Center) (17, 18) using the PCR primers 5′-CATGCCATGGCCGAAGACGCGGAT-3′ and 5′-CGAATTCCCCCCACTGCCCAGCATCTTTGTTGC-3′ and subcloned into the *Nco*I and *Eco*RI sites, resulting in the additional C-terminal sequence GNSHHHHHH in the expressed protein.

The cDNA encoding for rat syntaxin 1A (19) was kindly provided by R. H. Scheller. Rat syntaxin 1A-(1–265) (*i.e.* without the transmembrane region) was subcloned either into TrcHisA (Invitrogen) with an N-terminal His_6 tag as described previously (18) or into the vector pHO2c with a C-terminal His_6 tag. For subcloning into the vector pHO2c, the coding region corresponding to amino acid residues 1–265 was amplified using the primers 5′-GGGATTCCATATGAAGGACCGAACCCAG-3′ and 5′-GCGAATTCCCCTTCTTCCTGCGTGCCTT-3′. The resulting product was subcloned into the *Nde*I and *Eco*RI sites of the vector pHO2c resulting in the additional C-terminal sequence GNSHHHHHH. This syntaxin construct was used for nondenaturing gel electrophoresis (Figs. 1 and 2). However, the location of the His_6 tag showed little effect on the properties of syntaxin.

Protein Purification—His_6-tagged fusion proteins were first purified by Ni^{2+}-Sepharose chromatography (18). Proteins were eluted by increasing the imidazole concentration stepwise to 40, 80, 120, or 240 mM (in 20 mM Tris, pH 7.4, 500 mM NaCl). Fractions were analyzed for purity by SDS-PAGE (20) and staining with Coomassie Blue. Fractions containing recombinant proteins were dialyzed against standard buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). SNAP-25 and syntaxin were further purified by anion exchange chromatography on a Mono-Q column and synaptobrevin by cation exchange chromatography on a Mono-S column (Pharmacia Biotech Inc.). After loading, the proteins were eluted with a linear gradient of NaCl in standard buffer. The peak fractions were pooled and dialyzed against standard buffer. The His_6 tag of synaptobrevin was cleaved with thrombin followed by another chromatographic step on a Mono-S column. The eluted protein was 95% pure as determined by gel electrophoresis and mass spectrometry. The syntaxin-SNAP-25 and ternary complexes were purified using a Mono-Q column (Pharmacia). The ternary complex was disassembled when incubated with NSF, α-SNAP, and ATP (not shown), demonstrating that the proteins are functional with respect to NSF-mediated disassembly (12, 13). After purification, the proteins and the protein complexes were dialyzed against standard buffer and concentrated by ultrafiltration to final concentrations of 1–10 mg/ml. Protein concentrations, measured at 280 nm, were calibrated by internally standardized amino acid analysis following acid hydrolysis (carried out by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University).

CD Spectroscopy—Far-UV CD spectra were obtained by averaging over 5–20 scans with a step size of 0.5 nm on an AVIV model 62DS CD spectrometer equipped with a thermoelectric temperature controller. All measurements were performed in Hellma quartz cuvettes with a path length of 0.1 or 0.5 cm. For thermal melts, the mean residue ellipticities at 222 nm were measured as a function of temperature with the conditions indicated in the panels. Thermal unfolding experiments were repeated in the presence of 10 mM dithiothreitol. Little or no effect on the CD profiles was observed.

All CD spectra were recorded after reaching equilibrium following an overnight incubation at 4 °C in standard buffer.

To evaluate changes of the CD spectrum attributable to complex formation, the spectra were compared with the theoretically noninteracting sum of the spectrum using the equation $[\Theta]\text{sum} = \Sigma_i c_i n_i [\Theta]_i / \Sigma_i c_i n_i$, where c_i are the respective concentrations of protein molarity, n_i are the respective numbers of amino acids, and $[\Theta]_i$ are the mean residue ellipticities of the individual proteins.

In the case of several species, CD spectra will show the average mean residue ellipticity weighted by the concentration of each species. Thus, in the interpretation of the binary and ternary complexes, the high order complexes will have a negligible effect on the spectra.

Gel Electrophoresis—SDS-PAGE was carried out as described by Laemmli (20). When testing for SDS resistance, samples were solubilized in SDS sample buffer (final concentrations: 60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 3% β-mercaptoethanol) and incubated at room temperature (unboiled) or 95 °C (boiled) for 5 min before being analyzed on a 15% gel.

Nondenaturing gels were prepared and run in an identical manner as SDS-polyacrylamide gels (20) except that SDS was omitted from all buffers. To allow for comparison with the spectroscopic data, the samples were incubated overnight in standard buffer at the concentrations indicated in the panels. After adding sample buffer (final concentrations: 60 mM Tris, pH 6.8, 10% glycerol), the samples were separated on a 3% stacking (Tris, pH 6.5) and a 9% separation gel (Tris, pH 8.8).

Multiangle Laser Light Scattering—Size exclusion chromatography was performed on a HR-10/30 Superdex-200 column (Pharmacia) in standard buffer with the NaCl concentration increased to 300 nm and with 10 mM TCEP at a flow rate of 0.5 ml/min. The elution profiles were monitored by UV absorption at 280 nm, light scattering at 690 nm, and differential refractometry. Light scattering and differential refractometry were carried out using the Mini-Dawn and Optilab instruments of Wyatt Technology Corp. Analysis was carried out as described by Astra software (21). For each sample, 100 μl of protein solution was loaded. The protein concentrations were obtained by amino acid analysis (W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University). The d*n*/d*c* value (change of solution refractive index with respect to a change in concentration of the molecules being investigated) is fairly constant for proteins (22) and was set to 0.185 for the analysis of the light-scattering data.

RESULTS

Stoichiometric Changes—In our initial experiments we noticed that comparison of CD spectra of the purified SNAP-25-syntaxin complex with that of a 1:1 mixture showed a large discrepancy in agreement with earlier CD results (see Fig. 5, *D* and *F*, in Ref. 23). Although it is currently believed that the SNAP-25-syntaxin complex has a 1:1 stoichiometry, we thought that this discrepancy warranted reinvestigation of the oligomerization of the SNAREs and their complexes. Assembly reactions at precisely defined protein concentrations (using amino acid analysis) were performed, and each mixture was analyzed using nondenaturing gel electrophoresis. This technique allows for the separation of the individual proteins from complexes, provided that the latter are of sufficient stability.

In the first set of experiments, the formation of the binary syntaxin-SNAP-25 complex was monitored. Constant amounts of SNAP-25 were incubated with increasing amounts of syntaxin (Fig. 1*A*) and *vice versa* (Fig. 1*B*). Both experiments showed that about 2 molar equivalents of syntaxin were required for complete binding of 1 molar equivalent of SNAP-25. A binary complex of synaptobrevin with either SNAP-25 or syntaxin was not detected by nondenaturing gel electrophoresis (not shown), in agreement with previous observations reporting that these interactions are weaker than the interaction of syntaxin with SNAP-25 (11, 24–27).

Next, the experiment described above was repeated in the presence of an excess of synaptobrevin. Only individual proteins and the ternary complex were observed. This suggests that any binary complex of syntaxin and SNAP-25 participates in the formation of a ternary complex (Fig. 2). Fig. 2 shows that the molar ratio between syntaxin and SNAP-25 changes from 2:1 in the binary complex to 1:1 in the ternary complex.

To evaluate the stoichiometric ratio of synaptobrevin to syntaxin and SNAP-25 in the ternary complex, approximately equimolar amounts of syntaxin and SNAP-25 were incubated with increasing amounts of synaptobrevin. In the absence of synaptobrevin, all syntaxin was found in the binary complex, whereas a fraction of SNAP-25 was uncomplexed, in agreement with a 2:1 stoichiometry of the binary complex. When increas-

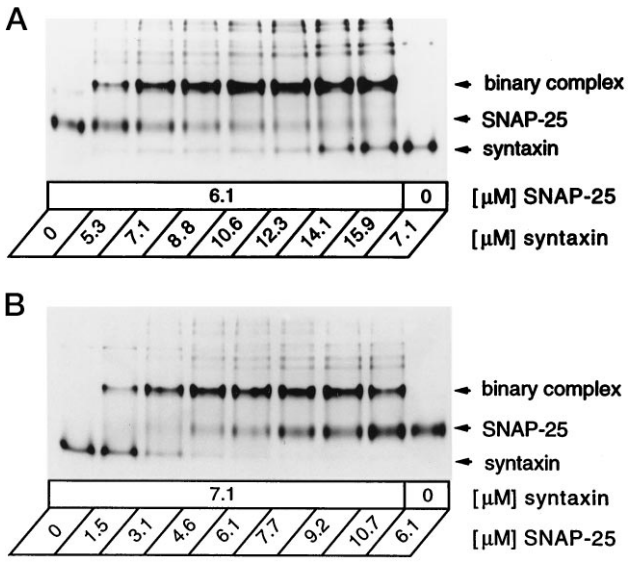

FIG. 1. **Stoichiometry of the binary syntaxin-SNAP-25 complex, monitored by nondenaturing electrophoresis.** SNAP-25 and syntaxin (concentrations as indicated) were incubated overnight in standard buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) prior to separation by nondenaturing gel electrophoresis. *A*, constant amounts of SNAP-25 were incubated with increasing amounts of syntaxin; *B*, constant amounts of syntaxin were incubated with increasing amounts of SNAP-25.

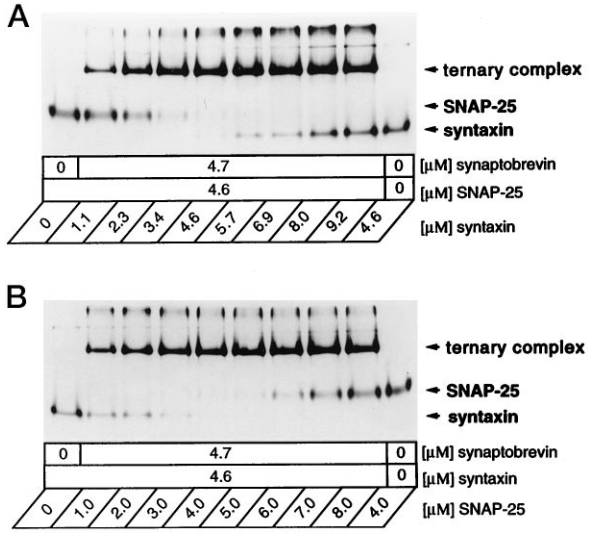

FIG. 2. **Stoichiometry of SNAP-25 and syntaxin in the ternary complex with synaptobrevin, monitored by nondenaturing electrophoresis.** Synaptobrevin, SNAP-25, and syntaxin (concentrations as indicated) were incubated overnight in standard buffer prior to separation by nondenaturing gel electrophoresis. Note that due to an isoelectric point of 8.5, monomeric synaptobrevin is not detectable in the nondenaturing gel. To achieve complete transition to the ternary complex in each case, an excess of synaptobrevin was used. *A*, constant amounts of SNAP-25 were incubated with increasing amounts of syntaxin; *B*, constant amounts of syntaxin were incubated with increasing amounts of SNAP-25.

ing amounts of synaptobrevin were added, the binary complex as well as uncomplexed SNAP-25 gradually disappeared, and ternary complex was formed (data not shown). The three proteins were present in the ternary complex in roughly equimolar amounts (1:1:1 stoichiometry).

The results described so far suggest that in the binary syntaxin-SNAP-25 complex, one of the syntaxin molecules may serve as a "place holder" that occupies the binding site for synaptobrevin and that it is displaced when synaptobrevin is added. To test this hypothesis, synaptobrevin was added to the

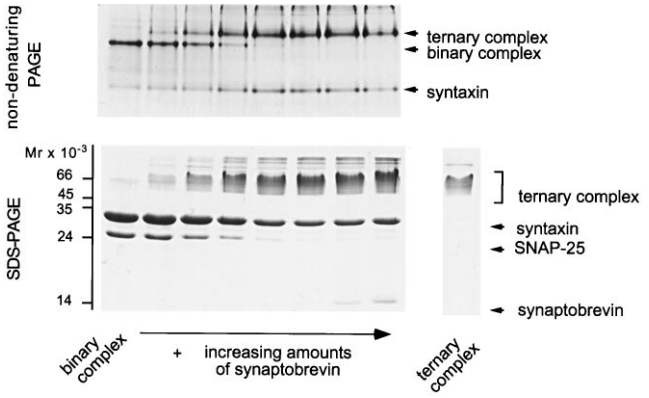

FIG. 3. **Displacement of syntaxin by synaptobrevin that was added to the purified binary syntaxin-SNAP-25 complex.** Purified syntaxin-SNAP-25 complex was incubated with increasing amounts of synaptobrevin in standard buffer. In the *bottom panel*, samples were not boiled before SDS-PAGE to separate the SDS-resistant ternary complex from the monomers. Note that upon separation of the purified ternary complex virtually no individual proteins are visible under these conditions (*right panel*).

purified syntaxin-SNAP-25 complex. In addition to nondenaturing gel electrophoresis, the incubation mixture was also analyzed by SDS-PAGE without boiling the samples, a procedure known to preserve the ternary but none of the binary complexes (11). As shown in Fig. 3, some syntaxin is released from its complex with SNAP-25 when synaptobrevin is added, confirming that the stoichiometric ratio of syntaxin and SNAP-25 changes upon transition from the binary to the ternary complex.

Molecular Masses—During size exclusion chromatography, synaptobrevin, SNAP-25, and syntaxin and their complexes eluted at positions of high apparent molecular mass when compared with globular molecular mass standards (not shown). To determine the absolute molecular mass, we used MALLS, a technique that is independent of the shape of molecules (21). Synaptobrevin eluted as a monomeric species (Fig. 4*A*) with a molecular mass of 11 kDa, in excellent agreement with the molecular mass determined by mass spectroscopy (11 kDa, not shown). Furthermore, a ^{15}N-^{1}H heteronuclear single-quantum coherence-NMR spectrum of synaptobrevin at 0.5 mM concentration showed narrow line widths, demonstrating that synaptobrevin does not aggregate even at high concentrations.[2] Both SNAP-25 and syntaxin eluted as monomeric species with molecular masses of 26 kDa (Fig. 4*B*) and 37 kDa (Fig. 4*C*), in agreement with the theoretical molecular masses of 25 and 35 kDa.

The syntaxin-SNAP-25 complex eluted as a major peak in the 86-kDa range, which is reasonably close to the theoretical molecular mass of the 2:1 complex (Fig. 4*D*). The purified ternary complex eluted as a major peak in the 90-kDa range, which is within measurement error to the theoretical molecular mass of the ternary complex (Fig. 4*E*).

Structural Changes—The results presented so far demonstrate that both the binary SNAP-25-syntaxin and the ternary complex are defined molecular entities with a fixed stoichiometry that can be purified by chromatographic methods. Furthermore, complex formation is virtually quantitative when the components are mixed in the correct stoichiometric ratio. Therefore, comparison of CD spectra obtained from the individual proteins with those of stoichiometric mixtures or purified complexes should reveal structural changes associated with complex formation.

The CD spectrum of syntaxin showed significant α-helical

[2] G. Warren and A. T. Brünger, unpublished results.

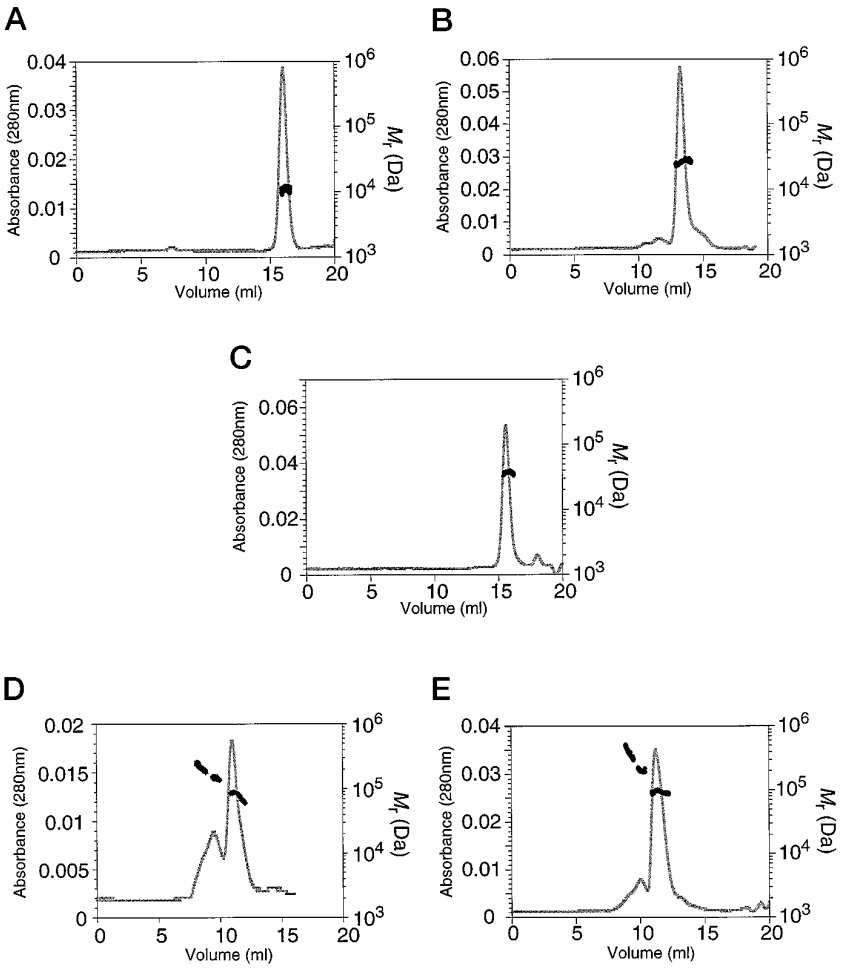

FIG. 4. **Molecular mass determination by MALLS.** Synaptobrevin (*A*), SNAP-25 (*B*), syntaxin (*C*), the binary syntaxin-SNAP-25 complex (*D*), and the ternary complex (*E*) were analyzed by a size exclusion column connected to UV, MALLS, and refractive index detectors. Concentrations of the injected samples were 28 μM for synaptobrevin, 50 μM for SNAP-25, 21.5 μM for syntaxin, 16 μM for the binary complex, and 18 μM for the ternary complex. Standard buffer was used except that 10 mM TCEP was added and the NaCl-concentration was increased to 300 mM. Shown are the UV absorption profiles at 280 nm (*continuous lines*, *left axis labels*) and the molecular masses as determined by MALLS using the protein concentration determined by the refractive index detector (*broken lines*, *right axis labels*).

content, as indicated by the characteristic double minimum at 208 and 222 nm (Fig. 5*A*). In contrast, SNAP-25 had a low secondary structure content (Fig. 5*A*), in agreement with previous results on the SNAP-25 homologue from leech (*Hirudo medicinalis*) (23). Likewise, synaptobrevin showed little secondary structure content (Fig. 5*A*). It also appears to lack tertiary interactions as assessed by two-dimensional ^{1}H nuclear Overhauser effect NMR spectroscopy,[2] suggesting a random coil conformation or an unusual conformation with little secondary structure.

To investigate structural changes associated with binding, CD spectra of each binary combination and of the ternary complex were measured by mixing the proteins at the appropriate stoichiometric ratios. The CD spectra of these mixtures were compared with the theoretically noninteracting sum of the individual mean residue ellipticities (see "Materials and Methods"). For the syntaxin-SNAP-25-complex, the mean residue ellipticity is significantly larger than that expected for a noninteracting mixture (Fig. 5*B*), similar to our previous findings on the leech homologues (23). In contrast, only a small increase in molar ellipticity was observed when SNAP-25 and synaptobrevin were mixed (Fig. 5*C*), and no change was observed when synaptobrevin was mixed with syntaxin (Fig. 5*D*), in agreement with the observation that no complexes were found by nondenaturing gel electrophoresis.

Upon ternary complex formation, an even larger relative increase in mean residue ellipticity was observed than for the syntaxin-SNAP-25 complex (Fig. 5*E*). Little difference was observed between the stoichiometric mixtures of the components and the purified complexes (not shown). Furthermore, the observed CD spectrum has a more pronounced α-helical double minimum profile than the theoretical noninteracting mixture. Thus, the formation of the ternary complex is associated with a dramatic increase in secondary structure. Most likely, this is due to induction of structure in the unstructured proteins synaptobrevin and/or SNAP-25.

Thermal Stabilities—The spontaneous and quantitative binding of SNARE proteins suggests that assembly is an energetically favorable reaction that results in stable complexes. As a first step toward understanding complex stability, thermal stabilities have been measured by monitoring Θ_{222} as a function of temperature.

The thermal unfolding of syntaxin shows a mildly biphasic behavior, with a first transition point at 55 °C and a second, more pronounced transition at 80 °C. In contrast to syntaxin, no significant changes in ellipticity were observable for synaptobrevin and SNAP-25, in agreement with the apparent lack of secondary structure (Fig. 6*A*).

Fig. 6*B* shows the thermal unfolding curve of the purified syntaxin-SNAP-25 complex. The curve shows a more pronounced biphasic behavior but no increase in thermal stability compared with syntaxin. In contrast, formation of the ternary complex resulted in a dramatic increase in thermal stability (Fig. 6*C*). The thermal unfolding curve was monophasic, with a cooperative transition at $T_m \approx 90$ °C. No precipitates were detectable at 95 °C. In addition, thermal denaturation was largely reversible with about 80% recovery (not shown).

A thermal unfolding curve of the ternary complex was also recorded in 0.1% SDS (Fig. 6*D*). This experiment was suggested by the observation that the ternary complex is resistant to treatment with SDS without boiling (11). Indeed, SDS resistance has been used for evaluating the stability of ternary

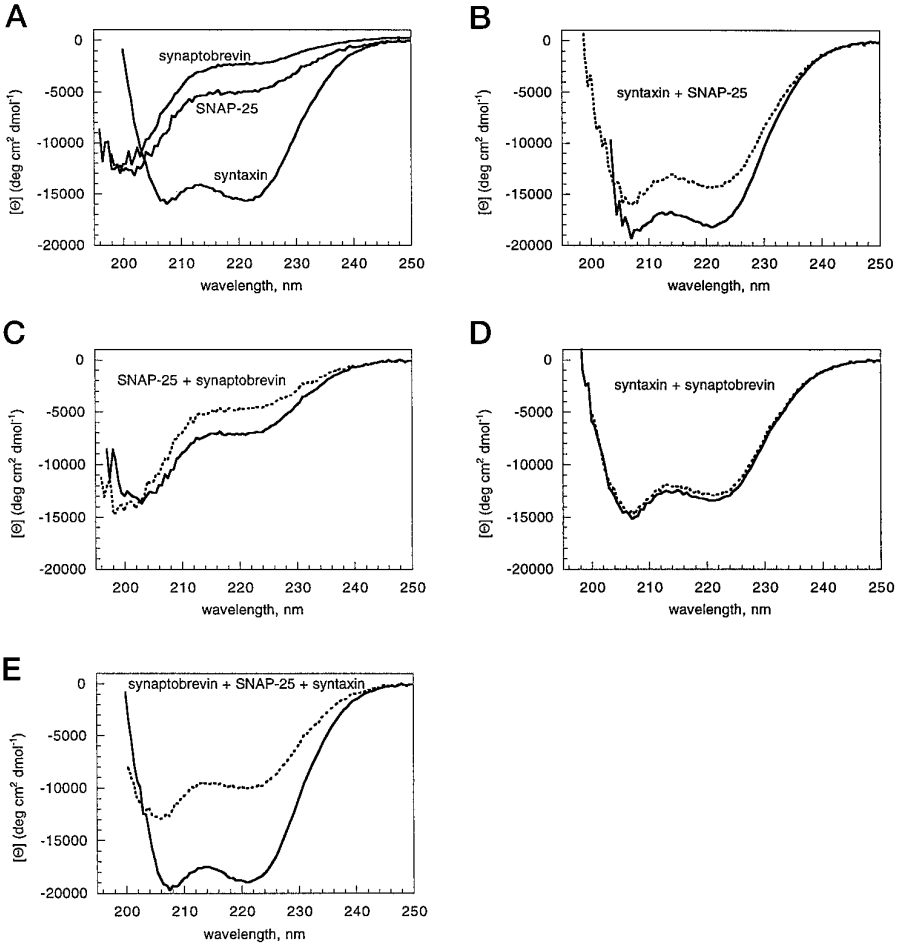

FIG. 5. **Changes in the CD spectra caused by interaction among synaptobrevin.** SNAP-25, and syntaxin CD spectra were recorded in standard buffer at 25 °C. *Dotted lines* represent the theoretically noninteracting mean residue ellipticities calculated from the observed CD spectra of the individual proteins. The spectra of the various combinations (*solid lines*) were recorded after overnight incubation of the proteins at 4 °C. Shown are the CD spectra at 25 °C of synaptobrevin (7.1 μM), SNAP-25 (7.8 μM), and syntaxin (7.4 μM) (*A*); SNAP-25 (8.1 μM) plus syntaxin (14.7 μM) (*B*); SNAP-25 (7.8 μM) plus synaptobrevin (7.1 μM) (*C*); syntaxin (7.4 μM) plus synaptobrevin (7.1 μM) (*D*); syntaxin (7.4 μM) plus SNAP-25 (7.8 μM) plus synaptobrevin (7.1 μM) (*E*). Note that an equimolar theoretical noninteracting mixture between the binary complex and synaptobrevin would have a lower mean residue ellipticity than of the binary complex alone. Thus, the fact that the mean residue ellipticities are roughly equal for both binary and ternary complexes indicates significant induction of structure in synaptobrevin or SNAP-25-syntaxin or both.

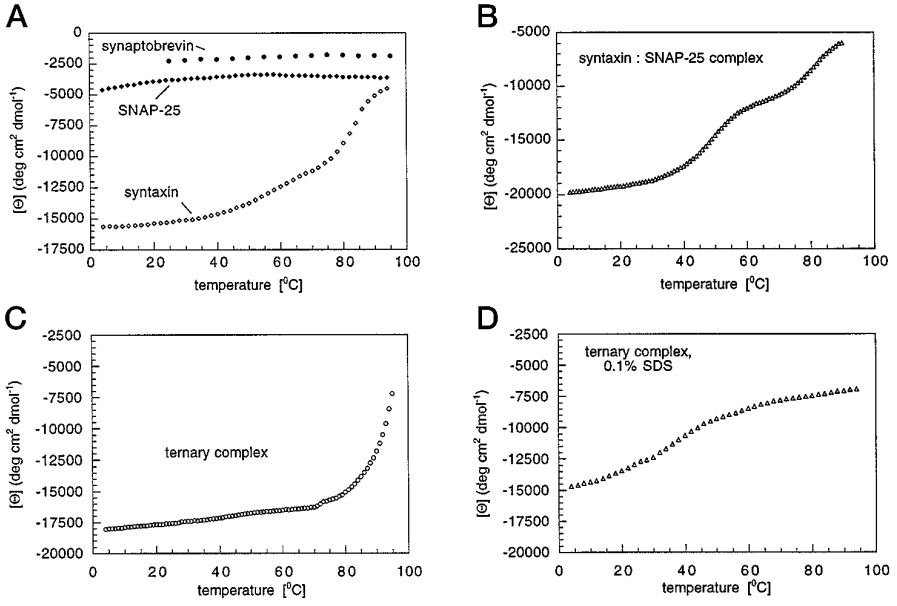

FIG. 6. **Increased thermal stability upon complex formation.** Change in the mean residue ellipticity [Θ] at 222 nm as a function of temperature in standard buffer is shown. The temperature increment was 5 °C for synaptobrevin (44 μM), 2 °C for syntaxin (13.2 μM), SNAP-25 (22 μM) and the ternary complex (5 μM) in the presence of 0.1% SDS, and 1 °C for the syntaxin-SNAP-25 (5.6 μM) and the ternary complex (5 μM). The temperature equilibration time was 6 min for synaptobrevin, 3 min for syntaxin and SNAP-25, 3.5 min for the syntaxin-SNAP-25 complex, 5 min for the ternary complex, and 4 min for the ternary complex in the presence of 0.1% SDS (w/v). The averaging time was 1 min.

complexes formed from mutant proteins (11, 26, 27). As shown in Fig. 6*D*, denaturation occurs gradually as a function of temperature with no cooperative transition, indicating that SDS affects folding and stability of the ternary complex.

DISCUSSION

Synaptobrevin, syntaxin, and SNAP-25 form a stable ternary complex with 1:1:1 stoichiometry and a tendency to self-associate. This complex can be purified by chromatographic procedures and migrates as a single band on a nondenaturing gel. The mean residue ellipticity of the complex is about twice as high as that of the theoretically noninteracting mixture of its components. Interestingly, similar structural changes were observed upon assembly of the yeast homologues Snc1, the SNAP-25-like domain of Sec9 (Sec9c), and Sso1 (28). We conclude that the structural similarities of the assembly reactions between the yeast and neuronal SNARE proteins are far higher than indicated by their sequence identity. A detailed under-

standing of this unique assembly reaction will be essential for elucidating the mechanism by which these proteins drive membrane fusion.

The 2:1 stoichiometric ratio of the "binary" syntaxin-SNAP-25 complex is surprising. However, a close evaluation of our data on the leech homologues (23) yields the same ratio, suggesting that this is common to neuronal complexes. In contrast, the complex formed from the yeast homologues Sso1 and Sec9c shows a 1:1 stoichiometry (28). This represents a potentially interesting structural difference between neuronal and yeast SNAREs.

In contrast to syntaxin, both SNAP-25 and synaptobrevin have very little secondary structure. However, both proteins are active in the sense that they can form the ternary complex with high efficiency (Figs. 2 and 5*E*). Although we cannot distinguish which of the three proteins is responsible for the large increase in ellipticity during assembly, it is likely that both SNAP-25 and synaptobrevin assume a more stable and ordered conformation in the complex.

Unlike syntaxin and the syntaxin-SNAP-25 complex, the ternary complex is remarkably resistant to heat denaturation. In addition, the equilibrium for the assembly reaction appears to be far on the side of complex formation, being essentially irreversible under normal experimental conditions (12, 13). Thus, it is probable that substantial amounts of energy are released upon assembly and that the complex represents a state of lower energy.

How can these findings be integrated into a coherent concept that explains assembly and disassembly of this complex in the context of membrane fusion? Recent studies from our laboratory have shown that a ternary complex from native proteins can assemble with all three proteins residing as neighbors in a single membrane (29). However, the bulk of syntaxin and SNAP-25 is localized to the plasma membrane and synaptobrevin is almost exclusively localized to the membrane of synaptic vesicles (2). Therefore, it is attractive to speculate that complex assembly results in a "zipping up" of the proteins that forces the membrane anchors and thus the two opposing membranes close together (30). This is further supported by results from the yeast homologues suggesting significant interactions between SNAREs close to the transmembrane domain of Snc1 (28). The proteins would achieve their final "low energy conformation" by bringing the membranes close together, and the energy released during assembly would thus be utilized to overcome energy barriers for membrane fusion.

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