The Four-helix Bundle of the Neuronal Target Membrane SNARE Complex Is Neither Disordered in the Middle nor Uncoiled at the C-terminal Region*

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Assembly of the SNARE complex is an essential step for membrane fusion and neurotransmitter release in neurons. The plasma membrane SNAREs syntaxin 1A and SNAP-25 (t-SNAREs) and the delivery-vesicle SNARE VAMP2 (or v-SNARE) contain the “SNARE regions” that essentially mediate SNARE pairing. Using site-directed spin labeling and EPR distance measurement we show that two identical copies of the SNARE region from syntaxin 1A intertwine as a coiled coil near the “ionic layer” region. The structure of the v-SNARE complex appears to be virtually identical to that of the ternary SNARE complex, except that VAMP2 is substituted to the second copy of syntaxin 1A. Furthermore, it appears that the coiled coil structure is maintained up to residue 259 of syntaxin 1A, identical to that of the ternary complex. These results are somewhat contradictory to the previous reports, suggesting that the t-SNARE complex has the disordered midsection (Xiao, W. Z., Poirier, M. A., Bennett, M. K., and Shin, Y. K. (2001) Nat. Struct. Biol. 8, 308–311) and the uncoiled C-terminal region (Margittai, M., Passhauer, D., Pabst, S., Jahn, R., and Langen, R. (2001) J. Biol. Chem. 276, 13169–13177). The newly refined structure of the t-SNARE complex provides a basis for the better understanding of the SNARE assembly process. It also provides possible structural-functional clues to the membrane fusion in the v-SNARE deleted fusion models.

Neurotransmitter release at synapses requires fusion of synaptic vesicles with the target presynaptic plasma membrane. Synaptic membrane fusion is directly or indirectly mediated by a set of highly conserved proteins referred to as SNARE1 proteins (1–3). Target (t-) SNAREs syntaxin 1A and SNAP-25 are plasma membrane associate proteins. Syntaxin 1A is a transmembrane protein in which the soluble domain protrudes from the plasma membrane, whereas SNAP-25 is a soluble protein that is bound to the membrane by lipid chains. Two t-SNAREs spontaneously form the binary complex that might serve as an intermediate for the SNARE complex assembly (4). Upon docking of the vesicle to the plasma membrane vesicle (v-) SNARE VAMP2 interacts with the t-SNARE complex to form a stable SNARE complex that perhaps leads to, or catalyzes, the fusion of two membranes.

The highlight of the SNARE assembly is the formation of a parallel four-helix bundle (5, 6). The SNARE regions, which are the H3 domain of syntaxin 1A, two coiled-coil domains of SNAP-25, and a VAMP2 helical domain (7–10), assemble into a 110-Å-long parallel four-stranded coiled coil (5, 6). The parallel arrangement of syntaxin 1A and VAMP2 would bring two transmembrane domains into close proximity, setting up the stage for membrane apposition (11, 12). Based on this structure, one can envision that the SNARE assembly forces the two membranes into apposition, promoting membrane fusion. The high stability of the complex perhaps implies that the energy released from the complex formation might be used to overcome the fusion energy barrier. A similar mechanism has been proposed for viral-cellular membrane fusion systems such as flu hemagglutinin and human immunodeficiency virus gp41 (13, 14). It is, however, still controversial whether the SNARE complex is actively involved in fusion (1) or whether it plays a mere catalytic role (15) or a different role (16). Nevertheless, slow but measurable membrane fusion has been observed for cell-free SNARE-reconstituted vesicles (17, 18).

On the other hand, the structure of the t-SNARE complex would provide valuable insights into the SNARE assembly process. Recently, the crude alignment of the SNARE regions in the t-SNARE complex has been determined using spin labeling EPR (19, 20). The t-SNARE complex appears to be structurally similar to the ternary complex; syntaxin 1A and SNAP-25 assemble into a parallel four-helix bundle that consists of two copies of the syntaxin 1A H3 domain and the two helical domains of SNAP-25. The data from the EPR study, however, were not sufficient to tell whether the t-SNARE complex actually forms a coiled-coil structure. Furthermore, this study indicated that the middle section of the four-helix bundle is perhaps disordered (19). It is also suggested that the C-terminal region of the t-SNARE complex is partially uncoiled when compared with the structure of the ternary SNARE complex (20). Thus, more data are required to establish its structural relationship to the ternary SNARE complex.

Functionally, homotypic membrane fusion has been observed in v-SNARE-deleted yeasts (21, 22), implying that the t-SNARE complex can mediate membrane fusion. It is also shown that t-SNARE proteins are sufficient for the fusion of ER membranes (23). Thus, the structure of the t-SNARE complex might provide some important clues to the t-SNARE induced membrane fusion.

In this study, we investigated the structure of the neuronal t-SNARE complex using site-directed spin labeling (24, 25) and
EPR spectroscopy. In particular, we have used the nitroxide scanning strategy in the middle section and the C-terminal region of the core complex. Because there are two copies of syntaxin 1A in the complex, single spin labeling on syntaxin 1A renders two nitroxide spin labels, and the distance between the pairs can be determined (26, 27). From EPR studies we found that the middle section of the t-SNARE bundle is a coiled coil that is identical to that of the ternary complex. We also found that the C-terminal end of the coiled coil matches with that of the ternary SNARE complex (6), contradicting to the assertion that the C-terminal region of the t-SNARE complex is uncoiled (20). Our results establish the close structural resemblance between the t-SNARE complex and the ternary SNARE complex.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction and Mutagenesis—* Recombinant gluthatione S-transferase (GST) or His₁₆ fusion proteins were expressed in *Escherichia coli* from the pGEX-KG vector (28) or pQE-30 vector (Qiagen), respectively. Plasmids encoding GST-syntaxin 1A (amino acids 191–230), GST-SNAP-25 (amino acids 1–206), His₆-SNAP-25[N], and GST-SNAP-25[C] were gifts from Dr. Michelle Poirier. The four native cysteines in the SNAP-25 loop were changed to alanines. All cysteine mutants were generated by QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and they were confirmed by DNA sequencing (Iowa State University DNA Sequencing Facility).

*Protein Expression, Purification, and Spin Labeling—* GST fusion proteins were expressed in *E. coli* BL21(DE3) Codon Plus RIL (Stratagene) and purified using glutathione-agarose chromatography. The cells were grown at 37°C in LB with 2 g liter glucose, 100 µg/ml ampicillin, and 50 µg/ml chloramphenicol until A₆₀₀ reached 0.6–0.8. After adding isopropylthio-β-D-galactopyranoside (0.3 mM) the cells were further grown for 6 more h at 30°C for GST-SNAP-25, and GST-SNAP-25[C] but at 16°C for GST-syntaxin 1A. The cysteine mutants were labeled with spin labeled, while the protein was bound to the beads. The beads were first treated with 2 mM dithiothreitol. After washing with an excess volume of PBST (phosphate-buffered saline with Tween 20 (0.5%))-Met buffer a 10-fold molar excess of MTSL was added, and the sample was reacted with MTSL for 4 h at the room temperature. After this the sample was left to stand at 4°C overnight. Free MTSL was removed by washing PBST-Met buffer, and the spin-labeled protein was cleaved off from the resin with thrombin. Spin labeling was nearly quantitative for all cysteine mutants. His₆-SNAP-25[N] was expressed in *E. coli* strain M15[pREP4] and purified using Ni-NTA affinity chromatography. The cells were grown at 37°C in LB with 2 g liter glucose, 100 µg/ml ampicillin, and 25 µg/ml kanamycin until A₆₀₀ reached 0.6–0.8. Protein expression was induced by isopropylthio-β-D-galactopyranoside (1 mM), and the cells were grown for 6 h at 30°C. The protein was bound to the Ni-NTA resin and washed with buffer A (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8.0).

**Preparation of the t-SNARE Complex without the SNAP-25 Loop** —To prepare the t-SNARE complex purified syntaxin 1A and SNAP-25(C) were added to the Ni-NTA resin solution that was bound to His₆-SNAP-25[N] with the molar ratio of 2:1:1, and the solution was incubated at 4°C overnight. After washing with buffer A, the t-SNARE complex was eluted from the Ni-NTA resin with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). Protein samples were concentrated using centrifuges with a 5000 molecular weight cutoff (Millipore, Bedford, MA) to perform EPR spectroscopy. The protein concentrations were approximately in the range of 50–100 µM.

**Preparation of the t-SNARE Complex with the SNAP-25 Loop** —Purified syntaxin 1A and SNAP-25 were mixed with a molar ratio of 2:1, and the mixture was left to stand at 4°C overnight. The complex was then purified with Bio-Rad UONO Q1 perfusion column, equipped in the Bio-Rad Duoflow system, in 15 mM Tris-HCl, pH 8.4, buffer using a NaCl gradient from 15 mM to 0.5 M. All protein concentrations were estimated by Bio-Rad protein assay using bovine serum albumin as a standard.

**EPR Data Collection** —EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker, Germany) equipped with a low noise microwave amplifier (Miteq, Hauppauge, NY) and a loop-gap resonator (Medical Advances, Milwaukee, WI). The modulation amplitude was set at no greater than one-fourth of the line width. Spectra were collected at either room temperature or 130 K in first-derivative mode. At room temperature the microwave power was kept at 1 milliwatt, but it was 8 microwatts at 130 K to avoid the saturation of EPR lines. All protein samples contain 10–15% glycerol as a cryoprotectant.

**RESULTS**

Since the previous EPR study (19) indicated that the middle section of the neuronal t-SNARE complex might be uncoiled or disordered, our investigation was focused on the structural determination of the region neighboring the evolutionally conserved Gln-226 in syntaxin 1A (Fig. 1). The nitroxide scanning strategy (29, 30) should be an effective way to examine the secondary and the tertiary structure of the region. We have generated 10 consecutive single cysteine mutants of syntaxin 1A in the region of amino acids 221–230 for spin labeling (Fig. 1a). The cysteine mutants were reacted with thiol specific MTSL for EPR measurements. In this experiment we have chosen to use SNAP-25[N] and SNAP-25[C] for the complex formation. Thus, the complex does not contain the SNAP-25 loop. For individual mutants we analyzed the SNARE complex formation using gel electrophoresis under non-denaturing conditions and SDS-PAGE (data not shown). We found that all spin-labeled syntaxin 1A mutants were capable of forming the binary t-SNARE complex (data not shown).

Because the t-SNARE complex contains two copies of syntaxin 1A, this naturally leads to two nitroxides per complex for singly labeled syntaxin 1A. When two nitroxides are located close to one another, the interaction between them leads to the broadening of the EPR spectrum. The spin-spin interaction is a function of the distance, and the spectral broadening can be analyzed to determine the inter-spin distance (26, 31, 32).
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With the room temperature EPR spectra, however, the weak spin-spin interactions that might be occurring for the other seven positions are not obvious due to the coexistence of motional broadening effects.

The spectral broadening caused exclusively by the spin-spin interaction is better represented in the EPR spectra taken at low temperature where the side chain motion is completely frozen. For the same spin-labeled samples EPR spectra taken at 130 K are also shown in Fig. 2. EPR spectra here are plotted in the absorbance mode. Spectral broadening is conspicuous for positions 223, 226, and 230, as it was at the room temperature. In contrast, for seven other positions, we observe some, but much less, EPR line broadening, indicating the weaker spin-spin interaction and the longer distances between spin pairs. Thus, the EPR data are in a good qualitative agreement with the coiled-coil model in Fig. 1b.

To examine the structure of two syntaxin helices quantitatively, the low temperature EPR spectra were analyzed to determine the interspin distances. We used the Fourier deconvolution analysis (26) that has proven to be effective in the range of 7–25 Å (33–36). The Fourier analysis revealed that the distances for 223, 226, and 230 are 9, 11, and 10 Å, respectively, while the distances for other spin pairs range from 15 to 22 Å.

In Fig. 3A EPR-determined distances were compared with two sets of model distances. The first distance set (open circles) is obtained from the corresponding β carbon-to-β carbon distances in the crystal of the ternary complex (7). For this we assumed that the second copy of syntaxin 1A substituted the VAMP2 helix without any structural rearrangement. The second data set (open squares) is obtained from a simple helical wheel model that retains the parallel four-helix bundle geometry, but the extension of the nitroxide side chains is taken into consideration (Fig. 3B). The diameter of the helix near the “0” layer is ~7 Å (7). The tip of the nitroxide is six a-bonds away from the β carbon, and it is shown that the nitroxide is located ~7 Å away from the center of the helices (26, 37). A simple model best representing these conditions are depicted in Fig. 3B.

In Fig. 3A it is shown that EPR-determined distances are in excellent agreement with those from a simple structural model (Fig. 3B). This suggests that the structure of the t-SNARE complex in the middle is virtually indistinguishable from the crystal structure of the core SNARE complex within experimental uncertainty. The uncertainty of the EPR measurement mainly stems from the side chain degree of freedom. It is estimated to be 2–3 Å for a pair of fully exposed nitroxide side chains (38). Tertiary conformations may increase the uncertainty depending on the circumstances.

Since a previous study suggested that the t-SNARE complex is frayed at the C terminus (20), we next directed our investigation to find where the coiled-coil structure ends at the C terminus. We have generated 10 consecutive cysteine mutants for nitroxide scanning experiments in this region (Fig. 1a). For this experiment we have used the SNAP-25 construct containing the long loop. All spin-labeled syntaxin 1A mutants were capable of forming the binary t-SNARE complex, as confirmed by gel electrophoresis under non-denaturing conditions and SDS-PAGE (data not shown).

EPR spectra of the ten spin-labeled mutants collected at room temperature are plotted in Fig. 4. The tendency of spectral narrowing along the sequence is clearly seen. This indicates that the polypeptide chain becomes progressively more mobile toward the C-terminal end. However, it appears that the first four spectra (256 through 259) are significantly different from the next six (260 through 265), and their spectral line widths are much broader than that of the rest. Residue 258...
exhibits the broadest EPR spectra, indicating some extensive tertiary contact (39). We note that residue 258 is predicted to be the α position in the coiled coil. Thus, it is most likely that the coiled coil structure is retained up to residue 259. We determined the interspin distance for individual spin labeled mutants using the Fourier deconvolution method. Although position 258 is predicted to be the α position, the distance is 19 Å, much longer than what is expected for the coiled coil. Instead, we observe a shorter 14 Å for position 257, shorter than what is expected for the g position. Such anomaly perhaps indicates some structural distortion at the end of the coiled coil. Distances for all other positions are over 20 Å, consistent with the divergent C-terminal region (data not shown).

DISCUSSION

Our EPR results strongly suggest that two copies of syntaxin 1A in the t-SNARE complex intertwine as a coiled coil in the middle section around Gln-226, and the coiled coil most likely extends to residue 259. In the four-helix bundle of the ternary SNARE complex residue Gln-226 of syntaxin 1A makes the ionic 0 layer, together with residue Arg-56 of VAMP2, and residues Gln-53 and Gln-175 from the N- and the C-terminal “SNARE regions” of SNAP-25, respectively. Therefore, in the t-SNARE complex Gln-226 from the second copy of syntaxin 1A likely occupies the spot for VAMP2 Arg-56 in the ternary complex. We speculate that four glutamine residues at the 0 layer significantly destabilize the four-stranded coiled-coil structure (40, 41). Yet, we found from our EPR study that this region assembles into a well defined coiled coil in the t-SNARE complex.

A previous EPR study suggested some structural disorder around the 0 layer, although the t-SNARE complex forms a four-stranded coiled coil in the C- and N-terminal regions (19). The explanation for the discrepancy between the previous work and the conclusions from this work could be due to the presence of the mixed complexes. Recently, it has been shown that the t-SNARE complex is actually a mixture of two different kinds: (i) the complex composed of two copies of syntaxins, and N- and C-terminal SNARE regions of SNAP-25, and (ii) the complex composed of two copies of syntaxins and two identical copies of the N-terminal SNARE region of SNAP-25 (42). In the previous EPR work, the nitroxide pairs were mostly attached to SNAP-25. Thus, coexistence of two types of t-SNARE complexes could have resulted in the spin distances that are (i) neither representing those in the complex nor (ii) corresponding to those in the complex, severely impairing the structural interpretation.

Present EPR work suggests that the four-stranded coiled-coil structure of the t-SNARE complex extends to residue 259 of syntaxin 1A. According to the crystal structure the syntaxin in
the ternary complex is also helical up to residue 259. However, an EPR study has shown a dramatic increase of the motional rate at positions 258 and 259 in the t-SNARE complex when compared with those in the ternary complex (20). This result was interpreted as the evidence for the frayed C-terminal region of the four-helix bundle. We argue that this conclusion may not properly represent the structure of the relevant SNARE complex. The discrepancy between the EPR study and our present work could be attributed to the differences in the length of the polypeptide chains; in our study residues up to 266 are included, while a four-residue shorter polypeptide was used in the other EPR study. The extra four residues could have contributed to the stability of the helical structure at the C-terminal end.

The H3 domain of syntaxin 1A spontaneously assembles into an anti-parallel arrangement of two parallel dimers (42). It is conceivable that the two copies of syntaxin in the binary complex are structurally similar to the parallel helices in the syntaxin oligomer. As mentioned above syntaxin is helical up to residue 259 in the t-SNARE complex. In contrast, the last ordered residue in the crystal structure of the syntaxin oligomer is residue 253. The interaction between syntaxins and SNAP-25 helices in the t-SNARE complex perhaps contributes to the further extension of the helical structure rather than that in the syntaxin oligomer.

Spin labeling at Q226C results in the replacement of two glutamine residues by the nitroxide side chain in the t-SNARE complex, perhaps severely hampering the stability around the 0 layer. Surprisingly, we observe that the four-helix bundle is still intact as evidenced by the strong spin-spin interaction (Fig. 2), although we detected some motion in the EPR spectrum, indicating local destabilization.

Membrane apposition, water exclusion, and rearrangement of lipids at the fusion site are a few early steps toward the membrane fusion (43). These are likely to be energetically costly processes. The current fusion model, at least for viral-cellular membrane fusion, postulates that the requisite energy is derived from the conformational change of the fusion proteins (13, 14). It has been speculated that the formation of the ternary complex in the case of SNARE-induced fusion may do the similar function. If so, the assembly of the t-SNAREs with v-SNARE would bring about the juxtaposition of two membranes, and the energy released from the assembly process is perhaps directly funneled to the fusion site for water exclusion and the rearrangement of the lipid. Likewise, although highly speculative, the four-stranded coiled-coil structure of the t-SNARE complex demonstrates its capability as a potential fusogen. The t-SNARE four-stranded coiled coil could play an active role in membrane fusion in v-SNARE-deleted fusion models, although other explanations are possible for membrane fusion in those fusion models.

It appears that the neuronal t-SNARE complex is structurally different from the yeast t-SNARE complex. An NMR investigation (44) revealed that the yeast t-SNARE complex is a 1:1 complex of Sso1 (homologous to syntaxin) and Sec9 (homologous to SNAP-25) for which the C-terminal domain of syntaxin below residue 245 is largely unstructured. Intriguingly, it is recently shown that the yeast t-SNAREs likely form the 2:1 complex in vivo (22), warranting further investigation.

For residue 258, that is predicted to be an a position, we observed a broad slow motional EPR spectrum characteristic of an internal position in the coiled coil. However, we do not observe the strong spin-spin interaction, as it has been the case for the nitroxides attached to the midsection. Instead, we detected a relatively strong spin-spin interaction for position 257. This might indicate that the coiled coil is somewhat distorted near the end so that position 257 moves more toward the core region and to be closer to the nitroxide from the second copy of syntaxin 1A.
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