The highly conserved proteins syntaxin and SNAP-25 are part of a protein complex that is thought to play a key role in exocytosis of synaptic vesicles. Previous work demonstrated that syntaxin and SNAP-25 bind to each other with high affinity and that their binding regions are predicted to form coiled coils.Circular dichroism spectroscopy was used here to study the a-helicity of the individual proteins and to gain insight into structural changes associated with complex formation. Syntaxin displayed approximately 43% a-helical content. In contrast, the a-helical content of SNAP-25 was low under physiological conditions. Formation of the SNAP-25-syntaxin complex was associated with a dramatic increase in a-helicity. Interaction of a 90-residue NH2-terminal fragment of SNAP-25 comprising the minimal syntaxin binding domain lead to a similar but less pronounced increase in a-helicity. Single amino acid replacements in the putative hydrophobic core of this fragment with hydrophilic amino acids abolished the induced structural change and disrupted the interaction monitored by binding assays. Replacements with hydrophobic residues had no effect. Our findings are consistent with induced coiled coil formation upon binding of syntaxin and SNAP-25.

Neurotransmitters are released from presynaptic nerve endings by Ca2+-triggered exocytosis of synaptic vesicles. Several lines of evidence suggest that exocytotic membrane fusion is mediated by a complex of conserved proteins which includes the synaptic vesicle protein synaptobrevin (also referred to as vesicle-associated membrane protein or VAMP) and the synaptic membrane proteins syntaxin and SNAP-25. Homologs of these neuronal proteins have been identified in many non-neuronal cell types including the yeast Saccharomyces cerevisiae, suggesting that the mechanism of exocytotic membrane fusion is conserved in all eukaryotic cells (for review, see Refs. 1–4).

Although the evidence linking these proteins to membrane fusion is quite compelling, very little is known about their mechanism of action. Rothman and colleagues (5) found that the three membrane proteins form a complex that interacts with additional soluble proteins known to support membrane fusion in cell-free extracts (5). These soluble proteins include the SNAPs1 (soluble NSF attachment proteins) with three isoforms (α-, β- (brain-specific), and γ-SNAP), and the ATPase NSF (N-ethylmaleimide-sensitive fusion protein). SNAPs and NSF apparently operate on all relatives of the synaptobrevin/syntaxin/SNAP-25 protein families, which are therefore commonly referred to as SNAREs (SNAP receptors). ATP hydrolysis by NSF leads to disassembly of the synaptobrevin-syntaxin-SNAP-25 complex (6), an effect that is associated with a different state of syntaxin (7, 8).

Ultimately, vesicle docking and membrane fusion can be viewed as a series of sequential protein assembly and disassembly steps which may involve structural changes and regulated interactions of at least some of the proteins with the participating phospholipid bilayers. It is therefore of interest to understand the structural basis of these processes.

The three proteins synaptobrevin, syntaxin, and SNAP-25 assemble spontaneously into a complex that sediments at 7 S (5) and which is resistant to mild treatment by SDS (9, 10). A complex with very similar properties can be assembled in vitro from recombinant proteins that lack their transmembrane domains (syntaxin, synaptobrevin) or lack their posttranslationally added palmitoyl side chains (SNAP-25), respectively. The in vitro complex is also resistant to mild SDS treatment and can be disassembled by NSF in the presence of ATP (9). Each of these three proteins can bind to one of its two partners, forming binary complexes. SNAP-25 binds syntaxin with high affinity (EC50 of about 0.4 μM for SNAP-25) (11), whereas the binding affinity between syntaxin and synaptobrevin is weakest (12).

Truncation-, deletion-, and site-directed mutagenesis have revealed the minimal essential domains of each of the proteins which participates in the formation of the binary and the ternary complexes (9, 12–14). For the interaction between SNAP-25 and syntaxin, the NH2-terminal half of SNAP-25 (amino acids 2–82 of SNAP-25) and the COOH-terminal domain of syntaxin (amino acids 199–243, also referred to as the H3 domain (14)) are required (9, 13). The H3 domain of syntaxin is also sufficient to bind synaptobrevin (12, 14). Interestingly, the interaction of SNAP-25 with synaptobrevin requires both the NH2- and COOH-terminal domain of the SNAP-25 molecule (13). The binding of synaptobrevin to either syntaxin or SNAP-25 requires most of the conserved part of synaptobrevin (amino acids 27–96, excluding the transmembrane region) (9).

We have used circular dichroism (CD) spectroscopy to study the secondary structure of syntaxin and SNAP-25 and that of their binary complex. Although CD spectroscopy is incapable of providing detailed structural information it can be used to assess the approximate a-helical content of a protein. Furthermore, it can be used to assess changes of secondary structure which occur upon modification of the environment or upon complex formation. The SNAP-25 and syntaxin variants from...
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EXPERIMENTAL PROCEDURES

Molecular Cloning of SNAP-25 and Syntaxin from Hirudo medicinalis—As part of our efforts to develop the Retzius cell of the leech (H. medicinalis) as a model system for studying the mechanisms of synaptic transmission (15), all experiments were based on leech syntaxin and leech SNAP-25 isoforms. cDNA clones encoding H. medicinalis syntaxin and SNAP-25 were isolated from a λ-zap library prepared from the nerve cord of the leech. The leech variants of the synaptic proteins SNAP-25 and syntaxin exhibit high homology to their mammalian counterparts. A detailed description of the cloning strategy and the nucleotide sequences will be published elsewhere. For the expression of recombinant proteins, full-length and truncated coding sequences were amplified using the polymerase chain reaction with oligonucleotides containing BamHI and EcoRI restriction sites and subcloned into pGEX4-T (Pharmacia) or pTReHisA (Invitrogen).

For the generation of full-length SNAP-25 (1–212) the sense and antisense primers were 5′-CCGGGATCCGATGACGCTAAGACTAAGC-3′ and 5′-GGGGATCCCTAGAACAATTGCCATC-3′, respectively. For the generation of the 3′ deletion mutant SNAP-25, 253–90, the primer 5′-CCGGGATCCCTATTCACCTATCTGAGGTTG-3′ was used. For the generation of the 5′ deletion mutant SNAP-25, 12–121 the primer 5′-CCGGGATCCGATGACGCTAAGACTAAGC-3′ was used. Leech syntaxin (residues 1–271, comprising the entire cytoplasmic domain) was constructed using primers that were complementary to codons of amino acids 1–6 and 266–271. Site-directed mutagenesis of SNAP-25 251–90 (see Fig. 8) was performed using the overlapping primer method (16). All mutants were confirmed by sequencing the entire coding region.

Purification of Recombinant Proteins—GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose beads essentially as described (13) except that 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT was used as chromatography buffer. Fusion proteins containing His$_6$ tags (expressed in pTReHisA) were purified by Ni$^{2+}$-Sepharose chromatography as described (13). Proteins were eluted by increasing the imidazole concentration stepwise to 40, 80, 120, or 240 (in 20 mM Tris, pH 7.4, 500 mM NaCl). Fractions were analyzed for purity by SDS-polyacrylamide gel electrophoresis (17) and staining with Coomassie Blue. Imidazole-containing fractions with recombinant protein were dialyzed against FPLC-buffer A (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT).

Results

Purification of recombinant proteins was confirmed by SDS-polyacrylamide gel electrophoresis (17) and staining with Coomassie Blue. Imidazole-containing fractions with recombinant protein were dialyzed against FPLC-buffer A (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM DTT). His$_8$-tagged proteins were purified further by anion exchange chromatography on a Mono Q column using an FPLC system (Pharmacia Biotech Inc.). After loading, the proteins were eluted with a linear gradient from 100 to 1,000 mM NaCl, and fractions containing the purified proteins were pooled. For the purification of the binary complex of SNAP-25 and syntaxin, both purified proteins were incubated overnight, dialyzed against FPLC-buffer A, loaded on a Mono Q column, and eluted with a linear gradient from 100 to 1,000 mM NaCl. The peak fractions were pooled, and its homogeneity was verified by size exclusion chromatography on a HR-10/30 Superdex 200 column (Pharmacia).

CD Spectroscopy—Far UV-CD spectra were obtained by averaging 5–20 scans with a step size of 0.5 nm on an AVIV model 62DS CD spectrometer at 25 °C. All measurements were performed in a Hellma quartz cuvette with a path length of 0.1 or 0.5 cm. All CD spectra were performed with purified His$_8$-tagged proteins (pTReHisA). After purification the proteins were dialyzed against 10 mM phosphate buffer, pH 7.4, 150 mM NaCl (standard conditions) and concentrated by ultrafiltration to final concentrations of 1–10 mg/ml. Protein concentration of SNAP-25, 253–90 fragments and of the purified SNAP-25-syntaxin complex 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) and subsequently determined by measuring absorbance at 280 nm. Protein concentrations of SNAP-25, 253–90, syntaxin, 1–121, and full-length SNAP-25 were determined by the Coomassie Blue binding method (18). Unless indicated otherwise, all recordings for single proteins were performed in 10 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl.

The CD spectra of SNAP-25-syntaxin complexes were recorded after reaching equilibrium following an overnight incubation at 4 °C in 10 mM phosphate buffer, pH 7.4, 100 mM NaCl, 2 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, comparable to the conditions used for binding assays with GST fusion proteins. To evaluate changes of the CD spectrum attributable to complex formation, the theoretical noninteracting spectrum was calculated from the spectra of the individual proteins using the equation \[\theta_{Q} \text{sum} = c_1 \theta_{A} + c_2 \theta_{B} \] where $c_1$ and $c_2$ are the respective concentrations of peptide molarity, $\theta_1$ and $\theta_2$ are the observed mean residue ellipticities of the two proteins. The CD spectrum of the purified SNAP-25-syntaxin complex was recorded in 10 mM Tris, pH 7.4, 300 mM NaCl, 1 mM EDTA, 1 mM DTT. The molar ellipticity was calculated assuming a 1:1 complex.

The fractional α-helical content for each protein was calculated using the assumption that for 100% α-helix the mean residue ellipticity, [θ]$_222$, at 222 nm is [θ]$_222$ = −36,300 (1–2.57X), where X is the number of amino acids in the protein (19).

Size Exclusion Chromatography—Size exclusion chromatography was performed on an HR-10/30 Superdex 200 column (Pharmacia) in 10 mM sodium phosphate buffer, pH 7.4, containing NaCl concentrations as indicated at a flow rate of 0.5 ml/min at 25 °C. The elution profiles were monitored photometrically at 280 nm. 200 μl of protein solution (10 μM of protein) was loaded. Globular proteins that were used as molecular mass standards were loaded at a concentration of 1.0 mg/ml and included alcohol dehydrogenase, bovine serum albumin, ovalbumin, carbonic anhydrase, and myoglobin (molecular mass = 150, 66, 45, 29.5, and 17 kDa, respectively).

Binding to Glutathione-Sepharose-immobilized Proteins—Soluble proteins were incubated together with indicated amounts of GST fusion protein immobilized on glutathione-Sepharose beads in binding buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM MgCl$_2$, 1 mM DTT). Incubations were carried out overnight at 4 °C. The beads were then washed three times in 1 ml of binding buffer. Proteins bound to the beads were finally solubilized in SDS sample buffer (final concentrations: 80 mM Tris, pH 6.8, 2% SDS, 10% glycerine, 3% β-mercaptoethanol) and heated for 5 min at 95 °C, subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis using T7-tagged monoclonal antibody (Novagen). The blots were stained with an alkaline phosphatase-conjugated secondary antibody using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Prediction of Coiled Coils—For the prediction of coiled coil domains the Lupas (20, 21) and the paircoil algorithm (22) were used. The programs were accessed through the Internet. 3 ulrec3.unil.ch/software/COILS_form.html and http://ostrich.lcs.mit.edu/cgi-bin/ige-bi-bin/score).
oligomeric state of the molecule, samples were analyzed by size exclusion chromatography. In 100 mM NaCl, SNAP-25_1–90wt eluted in two peaks with apparent molecular masses of 50 and 90 kDa. At 500 mM NaCl the molecule eluted in two peaks with apparent molecular masses of 50 and 160 kDa. When the NaCl concentration was raised to 1 M, the protein eluted as a single peak of molecular mass of 160 kDa (Fig. 2C). Apparently, high ionic strength induced the formation of a defined higher order oligomer with apparent molecular mass of 160 kDa, whereas in low salt the protein structure is undefined, and the two peaks may correspond to different oligomeric species. The view that SNAP-25_1–90wt is more structured in high salt was further supported by limited tryptic digestion of the protein. In high salt, only partial digestion was observed, resulting in a defined fragment of reduced size, whereas in 100 mM NaCl the protein was digested into small fragments (not shown). Divalent cations were about 100-fold more potent than

![Figure 1](image1.png)

**Fig. 1.** CD spectra of syntaxin, SNAP-25, and SNAP-25 fragments at standard conditions. Panel A, CD spectra of full-length SNAP-25 and syntaxin_1–271. Panel B, CD spectra of SNAP-25_1–90wt and SNAP-25_112–212. Panel C, mean residue ellipticity ([θ]) at 222, 208, and 203 nm for different concentrations of SNAP-25_1–90wt.

![Figure 2](image2.png)

**Fig. 2.** Increase of α-helicity and change of the oligomeric state of SNAP-25_1–90wt by increasing concentrations of NaCl. In the experiments standard conditions were used except that NaCl concentrations were adjusted as indicated. Panel A, effect of increasing NaCl concentrations on the CD spectra of SNAP-25_1–90wt. Panel B, the mean residue ellipticity ([θ]) at 222 nm of SNAP-25_1–90wt spectra is plotted against NaCl concentrations. Panel C, size exclusion chromatography of SNAP-25_1–90wt at different NaCl concentrations. A Superdex-200 column was loaded with 200 μl of SNAP-25_1–90wt (approximately 10 μM) at 25 °C in 10 mM sodium phosphate buffer at the NaCl concentrations indicated and eluted with the same buffer. Column effluent was monitored at an OD of 280 nm. Arrows indicate the positions of globular proteins used as molecular mass standards (see “Experimental Procedures”).
monovalent cations in inducing $\alpha$-helical structure in SNAP-251–90wt (Fig. 3) with both Mg$^{2+}$ and Ca$^{2+}$ being effective. This potency is only partially attributable to the increased ionic strength because similar concentrations of divalent anions were ineffective (not shown). Rather, it may be due to more efficient shielding of charges or formation of salt bridges between negatively charged residues.

As shown in Fig. 4, lowering the pH to 6.0 also increased the $\alpha$-helical content in SNAP-251–90wt. At pH 3 the $\alpha$-helical content increased even further. This may be explained by the fact that SNAP-251–90wt is negatively charged at neutral pH (calculated pI = 4.55 including the His$_6$ tag; note also that the protein was insoluble at pH values around its isoelectric point). Negatively charged groups become protonated at low pH, thus neutralizing potential charge-charge repulsions (24).

As mentioned above, the $\alpha$-helical content of full-length SNAP-25 was somewhat lower than that of the NH$_2$-terminal fragment SNAP-251–90. The increase in its $\alpha$-helical content in high salt or at low pH was also less pronounced (19% in 1 M NaCl; 38% at pH 3) than for the NH$_2$-terminal fragment. When the COOH-terminal fragment of SNAP-25 (SNAP-25112–212) was analyzed at high ionic strength or at low pH, no increase in $\alpha$-helical content was observed (not shown). This suggests that the $\alpha$-helical structure induced by these environmental changes is confined to the NH$_2$-terminal half of the molecule.

**Structural Changes Induced by Complex Formation**—In the next series of experiments we investigated whether the binding of SNAP-25 to syntaxin was associated with changes in secondary structure. For this purpose, CD spectra were obtained after an overnight incubation of approximately equimolar concentrations of syntaxin and full-length SNAP-25. The CD spectrum of this complex was compared with the sum of spectra (i.e. theoretical, noninteracting) that were recorded for each individual protein, corrected for variations of the protein concentrations (see “Experimental Procedures”).

As shown in Fig. 5A, the CD spectrum of the SNAP-25-syntaxin complex was clearly more $\alpha$-helical than the theoretical noninteracting sum of the individual spectra, demonstrating that the complex had a higher $\alpha$-helical content than the
A similar but less pronounced increase was observed when the NH$_2$-terminal fragment of SNAP-25 (SNAP-25$_{1–90wt}$) instead of the full-length protein (Fig. 5B) was used in the binding reaction. In contrast, no increase in $\alpha$-helicity was observed upon mixing of the COOH-terminal half of SNAP-25 (SNAP-25$_{112–212}$) with syntaxin (Fig. 5C).

The observed increase in $\alpha$-helicity during complex formation may be due to a change in SNAP-25 alone, in syntaxin alone, or in both proteins. However, the CD spectrum of syntaxin did not change under any of the environmental conditions tested (high salt, divalent ions, high or low pH; not shown), whereas $\alpha$-helicity could readily be induced in SNAP-25. This suggests that the increase in $\alpha$-helicity upon complex formation is due to a change in SNAP-25.

CD spectra of the complexes were also recorded in high salt (1 M NaCl; Fig. 5, D and E). When the spectra were compared with the theoretical noninteracting sum of the spectra of the individual components under these conditions, the induced $\alpha$-helicity was less pronounced in the SNAP-25-syntaxin complex, and no induction was observed in the SNAP-25$_{1–90wt}$-syntaxin complex. Since the induced $\alpha$-helicity was always larger for the full-length SNAP-25-syntaxin complex than for the SNAP-25$_{1–90wt}$-syntaxin complex, it is likely that the COOH-terminal half of SNAP-25 also becomes more structured upon complex formation.

Although binding experiments have demonstrated that binding of full-length SNAP-25 to syntaxin occurs with high affinity, it cannot be excluded that dynamic equilibria exist which may contribute to the spectrum. For these reasons, the binary complex was purified by ion exchange chromatography, and its homogeneity was verified by size exclusion chromatography. No measurable dissociation occurred during purification (data not shown). The purified complex has a CD spectrum that is very similar to that of the complex formed directly from its constituents without further purification (compare Fig. 5, F).
and D), although the molar ellipticities are somewhat higher. This indicates that in the mixing experiments the resulting CD spectrum is determined mainly by the complex.

No direct binding of a COOH-terminal fragment of SNAP-25 to syntaxin has been described in previous studies (9, 13). This suggests that the COOH-terminal fragment of SNAP-25 might bind only when a complex between syntaxin and the NH₂-terminal fragment of SNAP-25 has formed. To confirm this idea, GST-syntaxin, immobilized on glutathione-Sepharose, was incubated sequentially with SNAP-25₁₋₉₀wt and syntaxin (Fig. 7, Panel B). SNAP-25₁₋₉₀GD reduced the ability of these mutants to bind to GST-syntaxin (Fig. 7B). These differences between the α-helical contents of the binding and nonbinding mutants became more pronounced at high ionic strength or in the presence of divalent cations (not shown). All SNAP-25₁₋₉₀ variants had a high α-helical content at pH 3. Apparently, protonation of the glutamate (SNAP-25₁₋₉₀GD) or the aspartate side chains (SNAP-25₁₋₉₀GD) restores the ability of these mutants to form α-helices.

Upon interaction with syntaxin, the binding mutant SNAP-25₁₋₉₀GD exhibited an increase in α-helicity which was very similar to that of the wild type fragment (Fig. 7C). In contrast, no difference between the measured and calculated CD spectra was observed when the nonbinding mutants (SNAP-25₁₋₉₀KE and SNAP-25₁₋₉₀GD) were mixed with syntaxin (Fig. 7, D and E).

**DISCUSSION**

The binding of syntaxin and SNAP-25 is thought to be part of a sequence of protein-protein interactions that leads from vesicle docking to membrane fusion. Although an increasing number of these interactions are known and the participating protein domains have been identified, no biophysical and structural information regarding these interactions has been available up to now. The CD data presented here demonstrate that the formation of a binary complex between syntaxin and SNAP-25 is associated with a dramatic increase in α-helical content which presumably occurs mainly in SNAP-25.

Is the interaction between the two proteins mediated by direct interaction between adjacent α-helices? Several investigators noted earlier that the minimal binding domains of both SNAP-25 and syntaxin have a high propensity for the formation of coiled coils (9, 13, 14, 25) and suggested that binding is mediated by such interactions. The dramatic increase in α-helical content upon complex formation is consistent with an induced coiled coil formation, although alternative structures cannot be excluded at present. Two-stranded coiled coils consist of two amphipathic right-handed α-helices that are twisted around each other, forming a left-handed superhelix (26). The individual helices bury hydrophobic residues along one side of the helix. These side chains form the hydrophobic core of the superhelix and are often flanked by charged side chains that

**Fig. 6. Binding of the COOH-terminal half of SNAP-25 to a complex of SNAP-25₁₋₉₀wt and syntaxin.** Panel A, 10 μg of GST-syntaxin₁₋₂₇₁ immobilized on glutathione-Sepharose was incubated overnight at 4 °C with 500 μl of the indicated His₆-tagged fragments of SNAP-25 (each about 10 μM). Panel B, 10 μg of GST-SNAP-25₁₋₉₀wt immobilized on glutathione-Sepharose was incubated overnight at 4 °C with 500 μl the His₆-tagged proteins as indicated (each about 10 μM). Proteins bound to the beads were analyzed by an immunoblot assay using the monoclonal T7-tag (TrcHisA). The NH₂-terminal half of SNAP-25 only binds to a complex of the NH₂-terminal half of SNAP-25 and syntaxin.
interact electrostatically (24). Coiled coils frequently form the basis of regulated protein-protein interactions, e.g. binding and activation of bZIP transcription factors (27, 28).

We have reanalyzed the binding domains of all hitherto reported isoforms of syntaxin and SNAP-25 using two different algorithms (20–22). The results generally confirm the high scores reported earlier. However, there is considerable variation between different species and between the two algorithms, particularly for the NH2-terminal portion of SNAP-25. In this domain, two sets of heptad repeats were identified (13, see Fig. 8). Although both algorithms yield a probability of 1.0 for coiled coil formation of the second repeat in all species variants, the scores are much more divergent for the first repeat (Lupas (20, 21), using a window size of 27 residues/paircoil (22); rat 1.0/0.68, goldfish 0.99/0.51, Torpedo 0.44/0, Drosophila 0.36/0, leech 0.02/0). Helical wheel projection of the two putative repeats (Fig. 8, B and C) shows that according to the predictions Met-40 and Met-43 of the SNAP-25 molecule would be located in the hydrophobic core of a coiled coil. Their replacement with charged side chains would be expected to destabilize a coiled coil interaction, whereas their replacement with appropriate hydrophobic side chains should have little or no effect. These predictions agree with our experimental findings.

Our data show that not only the NH2-terminal domain of SNAP-25, but also the COOH-terminal domain participates in binding to syntaxin, although, unlike the NH2-terminal domain, it cannot bind on its own. Binding of the NH2-terminal domain results in an increase in $\alpha$-helicity which is similar to but less pronounced than binding of the full-length protein. This difference reflects a true structural difference and not merely a higher relative complex concentration since in 1 M NaCl an increase in $\alpha$-helicity is only observed when full-length
SNAP-25 is used. It is likely that the COOH-terminal domain also undergoes a structural change upon binding, although other explanations cannot be ruled out. Previous work has established that the COOH terminus of SNAP-25 is required for binding of synaptobrevin to SNAP-25 (13) and, furthermore, that formation of the SNAP-25-syntaxin complex greatly increases the affinity for synaptobrevin beyond that of either partner alone (9, 11). It is tempting to speculate that formation of the ternary complex is mediated by a global structural change in SNAP-25 which in turn provides an optimized attachment site for synaptobrevin.

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