4 Discussion

Syntaxin, discovered as a marker of early amacrine cell development in rat retina (Barnstable *et al.*, 1985) and later found to be localized in the plasma membrane of synaptic vesicle-containing varicosities (Bennett *et al.*, 1992) has been the subject of intense research ever since. It is a key protein in regulated exocytosis at the synapse. Syntaxin participates in multiple interactions its most prominent ones being those with SNAP-25 and synaptobrevin. The formation of a complex between these three proteins is central to membrane fusion. Association is mediated by an interaction of the SNARE motifs. The availability of these domains is likely to be stringently controlled. Syntaxin's SNARE motif, the H3-domain, appears to be of central importance in this respect. Not only does it form homooligomers but also does it interact with its independently folded N-terminal domain. Furthermore, a stable complex between two syntaxin and one SNAP-25 molecules has been observed with the SNARE motifs being responsible for binding. Understanding the molecular details of the interactions between SNARE proteins and the H3-domain in particular is mandatory to deduce refined mechanistic models including intermediates in the pathway of membrane fusion.

This thesis focused on revealing new structural aspects of the neuronal SNARE proteins and dynamic behavior of syntaxin using electron paramagnetic resonance- and single molecule fluorescence spectroscopy as key experimental approaches. Our data strengthen an emerging view according to which SNARE proteins can switch back and forth between random and helical conformations. This switching can either involve the entire SNARE motif or only parts of it in a highly adaptable manner. A binary interaction between SNAP-25 and syntaxin is characterized and shown to have a conformation suitable for an intermediate in the cascade of protein-protein interactions leading to membrane fusion.
Single molecule measurements reveal dynamic fluctuations of syntaxin's intramolecular association of the N-terminus and the H3-domain. A distribution of structural states is observed indicative for a mostly open conformation, and finally new interactions between the transmembrane domains of syntaxin and synaptobrevin are detected.

4.1 Structural Aspects of the SNARE Motif

The EPR data presented show that the monomers of both synaptobrevin and SNAP-25 are unstructured over the entire length of their SNARE motifs. Such lack of secondary structure has already been deduced from CD spectroscopy (Fasshauer *et al.*, 1997b), and (Fasshauer *et al.*, 1997a) and in the case of synaptobrevin by two-dimensional NMR spectroscopy (Hazzard *et al.*, 1999). Similarly, the SNARE motif of syntaxin is also unstructured at low micromolar concentrations but oligomerizes into helical bundles with at least some of its helices arranged in parallel at higher concentrations. Self-association of this domain has been reported previously, but it remained unclear whether it forms defined oligomers or aggregates nonspecifically ((Poirier *et al.*, 1998a), (Fasshauer *et al.*, 1998a), (Lerman *et al.*, 2000), and (Dulubova *et al.*, 1999)). The recently published crystal structure of the H3 domain (Misura, Scheller & Weis, 2001) lends additional support to our view of syntaxin being an oligomer. It is composed of a tetramer in which two pairs of parallel α-helices are aligned in an antiparallel manner (Fig. 1B). It was argued that steric constraints imposed by phenylalanines at position 216 that would point into the interior prevented a parallel alignment of all four helices like in the SNARE complex. Whether this oligomer has a biological function is questionable. Yet, a dimer between syntaxins might be relevant.

A comparison of the EPR spectra of the monomers with those of the ternary complex highlights again the dramatic conformational changes the SNARE proteins undergo upon assembly into complexes. Such transitions appear to be essential features of all SNARE proteins as exemplified by the assembly of SNAREs operating in yeast exocytosis ((Rice, Brennwald & Brunger, 1997), and (Nicholson *et al.*, 1998)) or in the fusion of late endosomes (Antonin *et al.*, 2000). In the fully assembled ternary complex, the structural predictions derived from the spectra of many different labeling positions are in excellent agreement with the crystal structure, which is represented by an elongated four-helix bundle. Furthermore, the EPR spectra confirm that the complex has a tendency to form oligomers. The loop region connecting the two SNARE motifs of SNAP-25 is unstructured, explaining its sensitivity to protease digestion (Fasshauer *et al.*, 1998a), and (Poirier *et al.*, 1998a). Moreover, the high mobilities of the side chains in the loop rule out significant contacts between the loop and the surface of the helix bundle. It should be noted, however, that the loop region adjacent to the transmembrane domains contains four cysteines that are, at least in part, palmitoylated (residues 84, 85, 90, and 92) in the native

protein. These palmitoyl side chains serve as membrane anchors and thus may induce structure in part of the loop.

4.2 The N-terminus of Syntaxin

It is commonly thought that in isolated syntaxin the SNARE-motif folds back onto the N-terminus to form a closed conformation (Carr, 2001). Only upon dissociation of these domains is the SNARE motif free to enter ternary complex formation. Interactions between the N-terminus and the H3 domain have been observed directly ((Calakos *et al.*, 1994), and (Dulubova *et al.*, 1999)). Indirect evidence for such intramolecular interactions has been obtained from the disassembly reaction of ternary complexes.

Hayashi at al. found that SNARE complexes containing the complete cytosolic part of syntaxin were more efficiently disassembled than those lacking the N-terminal domain (Hayashi *et al.*, 1995). A likely interpretation of these results is that the N-terminus folds back onto the H3 domain and by this interferes with the reassociation of the SNARE proteins. Furthermore, the data suggest that under the given conditions spontaneous assembly and NSF driven disassembly can occur on similar time scales and that backfolding of the N-terminus is sufficient to shift the equilibrium towards a disassembled state.

Since only the open conformation of syntaxin can form SNARE complexes and thus mediate membrane fusion, it is essential to understand the nature and the dynamics of the conformational switch.

4.2.1 A Weak Interaction between Syntaxin's SNARE Motif and its N-terminus

In Sso1p (syntaxin's homologue at the plasma membrane in yeast) the interaction between the Habc domain and the H3 domain is strong. Isolated fragments comprising these domains form complexes that withstand dilution upon gel filtration (Nicholson *et al.*, 1998). The recently solved crystal structure of Sso1p (Munson *et al.*, 2000) shows that the H3 domain fits snugly into a hydrophobic groove formed by the Hb and Hc helices. The crystal structure of syntaxin in complex with munc-18, in contrast, reveals that the N-terminal part of the H3 domain is composed of three helices that are separated by short loops (Fig. 27). The second helix (H3b) veers out of the groove to form a complex with munc-18. The initial bending of H3b away from Habc was attributed to polar residues in the Habc domain that are incompatible with the hydrophobic surface of the H3 helix

(Misura *et al.*, 2000). Whether the closed conformation we observe in the single molecule measurements resembles syntaxin in complex with munc-18 or whether the H3 domain extends along the complete groove as it does in isolated Sso1p is not conclusively solved. Possibly the abrogation of the canonical four helix bundle between Habc and H3 in syntaxin at this point explains the weaker interaction between both domains and the

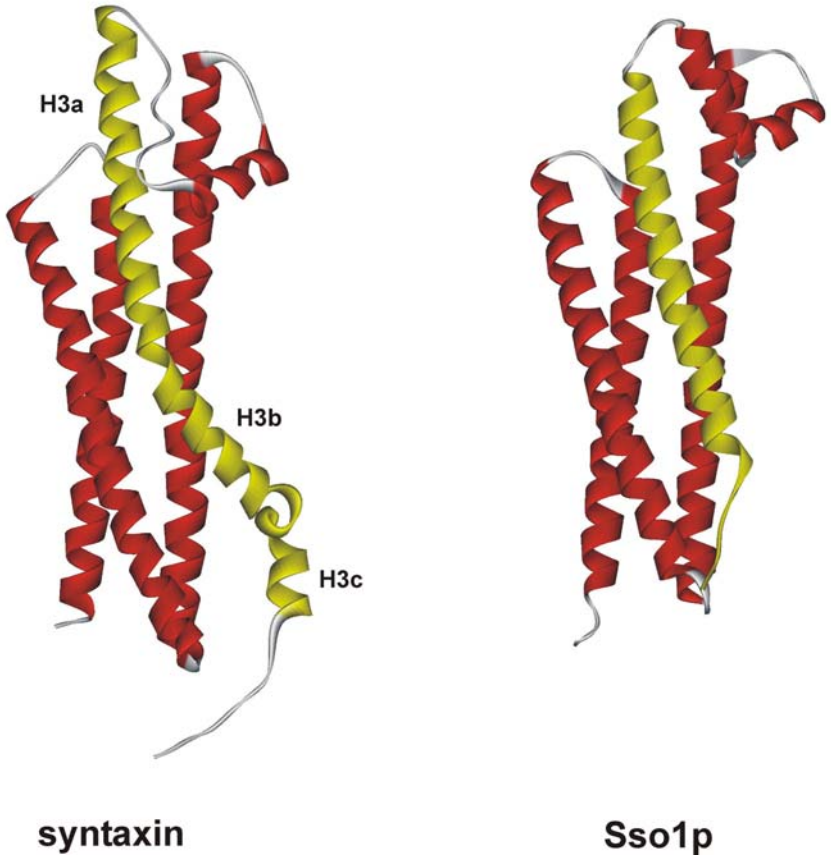

Fig. 27. Comparison of syntaxin 1a and Sso1p structures
The crystal structures of syntaxin 1A in complex with munc-18 (left) (munc-18 not shown) and Sso1p in isolation (right) show significant similarity. The Habc domain and amino acids up to the core region are depicted in red. The H3 domain (yellow) between both proteins differs. It is extended and lying in a groove in the case of Sso1p while it is interrupted twice by short loops and veers out of the groove starting with the second helix in the case of syntaxin 1A. Another difference is the presence of an additional helix in Sso1p in the region connecting the SNARE motif with the Habc domain.

existence of a mainly open conformation. Interestingly, it was observed that at low concentrations the protease subtisilin cleaves syntaxin between residues 220 and 221 (Lerman *et al.*, 2000). In the closed conformation of syntaxin in complex with munc-18 this portion is in direct contact with the Habc domain (Misura *et al.*, 2000). Therefore, cleavage at this site further strengthens the view that this part of syntaxin is less firmly packed and in a dynamic equilibrium with an open conformation.

The kinetic data on ternary complex formation are also in line with a weak interaction. Assuming that the dissociation of the ternary complex is slow compared with its formation (paper in preparation, Dirk Fasshauer), the second order rate constants determined in "3.2.2" can serve to compute free energy differences for the various assembly reactions. According to $\Delta G = -RT\ln K$, binding of syntaxin's N-terminus to the H3-domain involves the release of about 0.9-1.2 kcal/mol. This value again can be used to estimate the ratio between open and closed states. Computing the equilibrium constant for the backfolding of syntaxin (according to $e^{-(\Delta G/RT)} = K$) reveals that only around 15-18% of the protein are closed. A comparative analysis of the kinetic data obtained from yeast Sso1p (Nicholson *et al.*, 1998) demonstrates that here at least 4.5 kcal/mol are released upon interaction of both domains (Habc and H3). This means that over 99.9% of all Sso1p proteins exist in a closed conformation. Note that the distribution of open and closed states of syntaxin derived from the kinetic analysis agrees well with the single molecule data. According to the distribution of bursts in Syx91/225, Syx105/225, and Syx59/207 15-30% of the proteins are closed.

The question remains why Sso1p and syntaxin, both proteins that mediate equivalent fusion steps at the plasma membrane, differ in their apparent affinities between the core region and the N-terminus?

The strengths of the interactions are likely to have evolved to facilitate the specific needs of each particular fusion reaction. While Sso1p is involved in constitutive exocytosis at the yeast budding tip syntaxin mediates fast, regulated transmitter release at the synapse.

Recently, the NMR structure of Vam3p has been solved (Dulubova *et al.*, 2001). This protein is a homologue of syntaxin and is located on the yeast vacuolar membrane. The N-terminal domain, which had no detectable sequence homology to syntaxin1a and Sso1p, proved to be a three-helix bundle as well. The helices though are shorter and a hydrophobic patch that exists between helices b and c in syntaxin and Sso1p is absent. This patch is thought to form an interaction surface for the H3 domain. Interestingly, the H3 domain in Vam3p neither folds back, nor forms an identifiable secondary structure. This further strengthens the emerging view that syntaxin and its homologues can occur in different conformations depending on the specific regulatory requirements for each fusion step: 1.) closed (Sso1p) at the bud tip, 2.) partially open (syntaxin 1a) at the synapse, and 3.) completely open (Vam3p) at the vacuole.

4.2.2 Kinetic Analysis of the Back-Folding

A kinetic analysis of syntaxin's intramolecular interactions revealed that conformational fluctuations between the open and closed states have a time scale of approximately 0.7 ms. Furthermore, no significant conformational changes take place for time ranges down to µs. This argues strongly for the existence of two major conformations: namely open and closed.

It is known that α-helices can form and collapse in a time range of a few hundred nanoseconds (Lazaridis & Karplus, 1997). The time resolution of single molecule measurements does not yield information in this temporal regime. While, the Habc domain is an extremely stable unit, the H3 domain may be characterized by multiple conformational states. CD and MALLS measurements of the isolated H3 domain indicated that at low micromolar concentrations the protein is unstructured (Fig. 8) and monomeric (Table 1). Secondary structure formation is concomitant with a homooligomeric interaction and vice versa. It is likely that the same is true for a heterotypic intramolecular interaction between the H3 domain and the Habc domain, i.e. the detachment from the three helix bundle results in a fraying (collapse) of the H3-helix. EPR measurements of various spin labeled positions in the H3 domain of full-length syntaxin were carried out to give average structural information on the protein ensembles. Since syntaxin had a tendency to oligomerize in the sensitivity range of EPR detection ((Lerman *et al.*, 2000), our data not shown) the results were not conclusive.

4.2.3 Implications for Munc-18-Binding

The data presented above indicate that syntaxin exists in a mainly open conformation and that fluctuations between open and closed states occur on a millisecond time scale. The protein thus does not need to be activated to switch into an open state. This is an important finding because it has been suggested before that munc-18 binding to syntaxin destabilizes the closed conformation and thereby activates SNARE complex formation (Misura *et al.*, 2000). Furthermore, it was asserted that munc-18 binds only to a closed conformation of syntaxin. A double mutant of syntaxin (165/166), which lacked back folding completely, failed to bind to munc-18 in a GST pull down experiment (Dulubova *et al.*, 1999). In the light of the crystal structure of syntaxin in complex with munc-18 an alternative interpretation can be envisioned. Glu166 in the loop-helix of syntaxin not only interacts with Arg142 in the Habc-domain but also with Arg315 in

munc-18. Therefore it might significantly stabilize the complex itself. When this amino acid is exchanged for alanine as in the double mutant 165/166 a decreased affinity might be the consequence. Even more, the two positive charges, unshielded in the absence of glutamate might repel each other (Fig. 28) and thus drive the two proteins apart.

Whether munc-18 binds the open conformation of syntaxin and induces secondary structure upon binding or whether the closed conformation already exists upon contact is not clear.

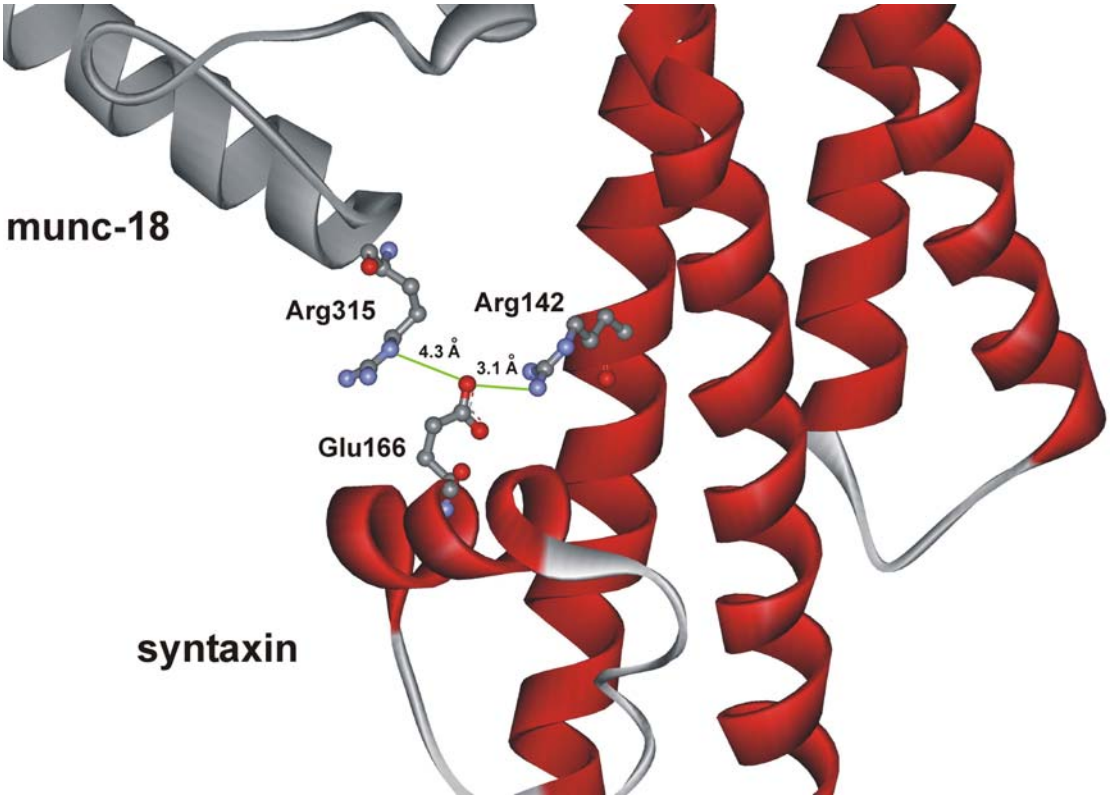

Fig. 28. Glu166 in the loop helix of syntaxin forms salt bridges with Arg315 of munc-18 and Arg142 of the Habc domain.

It seems likely that the stabilization of syntaxin's closed conformation is an important biological function of munc-18. When both proteins are in complex with each other ternary complex formation is perturbed (Yang *et al.*, 2000).

Interestingly, Sso1p, which has a very strong intramolecular affinity between its Habc domain and its H3 domain, does not form a complex with Sec1p (the yeast homologue of munc-18). Here, Sec1p interacts with the yeast SNARE complex at the plasma membrane (Carr *et al.*, 1999). These data are still difficult to integrate into a common model for

munc-18/Sec1p function in membrane fusion, but indicate that they play a central role in this complex cascade of protein-protein interactions.

4.3 Syntaxin in the Membrane

The analysis of syntaxin inserted into artificial lipid bilayers revealed the existence of homodimers (this study). Helix formation in the membrane is favored because only then all hydrogen bonds are saturated (β-sheets being an alternative motif as exemplified by the porins, for a recent review see (Koebnik, Locher & Van Gelder, 2000)). Although the interaction surface of two trans membrane helices is small (~30Å) compared for example with the length of the SNARE complex (120Å), the dimers are very stable. We observe that SDS cannot fully disrupt the interaction between two syntaxin molecules. A similar result was observed for glycophorin (Furthmayr & Marchesi, 1976), a single span membrane protein that forms dimers in the plasma membrane of erythrocytes. The introduction of an α-helix into a membrane results in local perturbation of the lipid structure. This is due to poor packing between lipids and helices. Often α-helices pack with one another better than with lipids (Lemmon & Engelman, 1994). Similar rules apply to detergent molecules. The hydrophobic tail of SDS has to compete with the helix-helix interaction. Interestingly, the interactions between helices in the membrane are similar to those in solution with only the amino acids pointing toward the lipid phase being more hydrophobic. Therefore, coiled-coils like the SNARE complex are in general more prone to withstand SDS denaturation. EEA1 and matrilin-4 both proteins that form coiled coils are also SDS resistant ((Callaghan *et al.*, 1999), and (Klatt *et al.*, 2001)).

The question arises whether interactions between transmembrane helices are specific or whether the loss in entropy that results from lipid perturbation always drives helix association. Consecutive mutations of the amino acids in the transmembrane region of syntaxin demonstrated that a specific stereochemical fit between both membrane regions is responsible for dimerization (Laage *et al.*, 2000). Similarily, mutations in the transmembrane region of synaptobrevin (that also forms dimers) revealed that alterations in the side-chain volume (not the charge) result in reduced oligomerization. This “knobs-into-holes packing” between neighboring membrane regions has been observed in various other proteins forming dimers in lipid bilayers ((Lemmon & Engelman, 1994), and (Langosch & Heringa, 1998)), glycophorin being the best characterized (e.g. (Lemmon *et al.*, 1992)).

If the transmembrane regions of syntaxin interact it is conceivable that in the membrane protein the cytoplasmic domains interact as well. As discussed above, in solution homotypic oligomerization between the SNARE motifs has been observed ((Lerman *et al.*, 2000), (Misura *et al.*, 2001) and this study). Furthermore, oligomerization was detected for syntaxin homologues, either for the cytoplasmic versions of Pep12 ((Tishgarten *et al.*, 1999) and Ufe1 (Patel *et al.*, 1998)) or between Drosophila Sed5 that contained a membrane anchor (Banfield *et al.*, 1994).

Two dimensional diffusion in the lipid membrane (as opposed to three dimensional diffusion in solution) and the fixed orientation of the TMRs with respect to each other, namely parallel alignment, significantly increase the local concentration of syntaxin and the chance of successful encounter between its SNARE motifs. Interestingly, a recent study revealed that syntaxin in PC12- and BHK-cells is concentrated in cholesterol-enriched patches (Lang *et al.*, 2001). These patches had a significant overlap with the docking and fusion sites of secretory vesicles. Addition of cyclodextrin, a chemical that chelates cholesterol caused a homogeneous distribution of syntaxin over the whole plasma membrane. Therefore, the study demonstrated that microdomains could serve to further restrict the diffusional freedom of proteins. The nature of these domains might be different, but their function might be similar - compartmentalization of the membrane and enrichment of its composing proteins.

Since the interaction between syntaxin's N-terminus and the H3-domain is weaker than previously anticipated the question may be raised as to which is the predominant form of syntaxin in the membrane: open or closed? A delicate equilibrium between free, monomeric syntaxin and homooligomerized syntaxin may exist that has evolved to comply with the demands of fast transmitter release.

Interestingly, syntaxin, like the ternary complex binds α-SNAP and NSF. As for the SNARE complex ATP hydrolysis results in complete dissociation of all participating proteins. It has been suggested that in this process syntaxin undergoes a conformational change that transforms the protein into a closed state (Hanson *et al.*, 1995). Whether NSF and α-SNAP act on a monomer or whether an oligomer is the target of their action is not known. It is conceivable that a helix bundle of syntaxin molecules comprises the actual interaction surface and that hydrolysis of ATP not only causes a switch in syntaxin's conformation but also the preceding dissociation of a syntaxin homooligomer. It is known that the ATPase activity is stimulated over tenfold when α-SNAP and NSF act upon

syntaxin (Matveeva & Whiteheart, 1998). In comparison, only a small change is observed when acting upon the SNARE complex. This difference could reflect the fast dissociation of a syntaxin oligomer as compared to the SNARE complex, which is energetically much more stable.

It is likely that additional proteins including munc-18 and munc-13 have key regulatory roles in the conformational switching of syntaxin.

4.4 Syntaxin in Complex with SNAP-25

Binary complexes between syntaxin and SNAP-25 have been observed in solution but it is not clear whether they resemble possible intermediates in membrane fusion.

Our data shed new light on the structure of the binary syntaxin-SNAP-25 complex. Surprisingly, the complex is folded almost throughout its entire length, with only a few amino acid residues at the C- and N-terminal ends being unstructured. Because the EPR spectra of almost all labeled SNAP-25 variants are superimposable between the binary and the ternary complex, the structures of the SNAP-25 helices must be largely identical. Recently, additional structural information of the binary complex was obtained in an EPR study that aimed at determining exact distances between pairs of spin labels in various helices of the complex (Xiao *et al.*, 2001). These results are in good agreement with the results presented here and confirm the parallel alignment and 2:1 stoichiometry.

Interestingly, SNAP-25 exists in microdomains, which partially overlap with those of syntaxin (Lang *et al.*, 2001). In the membrane, preassembled syntaxin dimers might serve as a binding platform for SNAP-25.

The properties of the neuronal complex differ substantially from the structural properties of the binary complex formed by the SNAREs functioning in yeast exocytosis (Fiebig *et al.*, 1999). Here, the stoichiometry between Sec9p (corresponding to SNAP-25) and Sso1p (corresponding to syntaxin) is 1:1, suggesting that only three SNARE motifs are involved. NMR spectroscopy showed that in this complex Sso1p is helical only up to residue 240 with the C-terminal 24 residues remaining unstructured. Thus it is possible that the presence of an additional syntaxin in the neuronal binary complex is responsible for the extension of helical structure toward the C-terminus. In the yeast complex, it is easy to imagine that the binary three-helix bundle forms a grooved acceptor site to which Snc1/2p can bind. In contrast, in the neuronal binary complex the synaptobrevin binding site is occupied by the second syntaxin molecule. Because one of these syntaxins can easily be

replaced by synaptobrevin, it is conceivable that one of the syntaxins is more loosely bound than the other. The palmitoyl chains of SNAP-25 are likely to have an influence on the lipid packing around the transmembrane helices. Whether this involves a biological function remains to be determined.

4.5 Multiple Conformations of Syntaxin's H3-domain

In order to address the question whether local conformation in the SNARE motifs is dependent on juxtaposing helices in the ternary complex synaptobrevin was truncated at its C-terminal end (after amino acid 76).

The EPR measurements revealed that such a complex appears to be perfectly intact upstream of the cleavage site, whereas the helices that face the stretch removed in the mutant become disordered at their C-terminal ends. Apparently, the formation of interacting layers in the core of the bundle is not dependent on the formation of layers in nearby positions. In other words, the findings support the view that SNARE complexes can assemble only partially in such a way that part of the helical bundle is correctly folded, whereas the remainder of all four participating SNARE motifs is unstructured. This feature agrees well with the proposed zippering mechanism (Hanson *et al.*, 1997a) and provides a structural basis for the hypothesis that defined complexes that are partially assembled form intermediates in the progression toward membrane fusion (Xu *et al.*, 1999). These findings also support the emerging picture that the SNARE motifs are extremely versatile in their ability to undergo conformational changes. Apparently, SNARE motifs can switch between random coils and helical conformations in such a way that either the entire domain or only parts of the domain become α-helical with the rest remaining unstructured. These helices are characterized by the mostly hydrophobic ribbon of "layer" residues that have a tendency to interact with corresponding hydrophobic surfaces. The best evidence for such versatility is available for the SNARE motif of syntaxin. This region can be unstructured (monomer at low concentration), fully helical (core complex) (Sutton *et al.*, 1998), mostly helical except for the C- and N-terminal ends (homooligomer, binary complex) (this study), helical with a disordered C-terminus (truncated complex (this study)), or composed of a consecutive helix-loop-helix structure with a disordered C-terminal end (in complex with munc-18 (Misura *et al.*, 2000). Apparently, the length and the position of the helix formed depend on the nature of the binding partners.

4.6 Syntaxin at the Final Step of Membrane Fusion

The investigations on ternary complexes in solution revealed that they form oligomers, with interaction surfaces being restricted to the C-terminal ends. In binary complexes oligomers were not observed. Interestingly, the homologues SNARE complexes in yeast show similar interaction behavior. While the complex between Sec9p and Sso1p is monomeric the respective ternary complex forms oligomers. The question whether these complexes are biologically significant is not easy to answer. It is attractive to speculate that a ring of SNARE complexes forms a fusion pore and thus mediates membrane merger. In detergent extracts of neuronal membranes oligomers of different sizes have been identified ((Hayashi *et al.*, 1994), and (Otto *et al.*, 1997)). In other fusion systems oligomers are also implicated in the final merging event (see for example (Blumenthal *et al.*, 1996), (Gaudin *et al.*, 1996), and (Plonsky & Zimmerberg, 1996)). Yet, even in the hemagglutinin mediated fusion reaction of influenza virus, the best-studied fusion system of all, the existence of direct interactions between protein clusters is controversial (Skehel & Wiley, 2000).

According to the zippering model the formation of trans SNARE complexes heralds membrane fusion. A cooperative interaction between various complexes could release substantially more energy than a single one.

The identification of trans SNARE complexes is accompanied with difficulties. This is in part due to the fact that trans SNARE complexes may form short-lived intermediates. Our experiments on artificial lipid bilayers showed that complexes between syntaxin, synaptobrevin and SNAP-25 formed at low temperature where fusion would not be expected. Subsequently, the liposomes were subjected to detergent treatment. In the future it will be necessary to prove the existence of trans SNARE-complexes in an unperturbed system. Single molecule measurements may be a promising approach.

4.7 Syntaxin in Complex with Synaptobrevin

Interestingly, we found an interaction between syntaxin and synaptobrevin that is mediated by their transmembrane domains. Besides the homotypic interaction between synaptobrevin (Laage & Langosch, 1997), and the homotypic interaction between syntaxin ((Laage *et al.*, 2000) and this study), this is the third type of membrane interaction observed between neuronal SNARE proteins.

That this heterotypic complex is characterized by a specific stereochemical fit and therefore likely to be relevant has recently been shown in an extensive mutagenesis analysis of the transmembrane domains (Laage *et al.*, 2000).

The helix bundle of the SNARE complex is very densely packed in the region close to the membranes, and even a small truncation of SNAP-25 as induced by BoNT/A poisoning (Blasi *et al.*, 1993a) blocks exocytosis. Thus it is conceivable that an extension of the helix bundle into the trans membrane domain is promoted by an interaction in this region.

4.8 Outlook

Though the assembly studies clearly demonstrate that removal of the N-terminus speeds up ternary complex formation the reaction is still 100-fold slower than that observed between other coiled coils (Zitzewitz *et al.*, 1995). Furthermore, fast transmitter release occurs within 100 µs. The observed kinetics does not explain the rapid fusion event at the synapse. Studies with SNARE proteins reconstituted into membranes did also fail to significantly speed up the process ((Weber *et al.*, 1998), and (Parlati *et al.*, 1999)). It is possible that SNARE proteins are preassembled in the membrane and that this is an important regulatory step on the pathway to fusion. Ca^{2+} entry into the presynapse triggers an immediate fusion event. The local protein machinery has evolved to fulfill just that task. Although structural information is ample dynamic details are scarce. In order to better understand the mechanism of protein mediated membrane fusion at the synapse, a temporal deconvolution of the various conformational states of SNARE proteins in the context of lipid bilayers will be necessary.

The lipid bilayer entails an additional level of complexity to protein structure due to existing gradients ((Popot & Engelman, 2000), and (White & Wimley, 1999)). The environment in the immediate vicinity of the membrane is very different from that in solution. The hydrophobic core of a membrane has a diameter of about 30 Å and is surrounded by interfacial regions, each of which comprises an additional 15 Å enough space to accommodate unfolded and folded polypeptide chains. If viewed as solutions the head group layers are highly concentrated (500-700 mg/ml for phosphatidylcholine). Most of the water molecules in this region are used to hydrate these polar head groups. The dielectricity constant is in the range of 10-30 (Haltia & Freire, 1995) compared to 80 in free solution. Therefore, formation of secondary structure may be favored. Direct

interactions with the lipid head groups are also likely, and these may be varied dependent on the phospholipid composition.

We are only at the beginning to understand SNARE function in the context of lipid bilayers. Additional conformational states of syntaxin might be unraveled. Also, proteins that interact with the ternary complex or individual SNARE proteins have to be taken into account and studied at the molecular level. Syntaxin is known to have more than 30 interacting partners. Whether these proteins are relevant for the regulation of membrane fusion or important in sorting and transport will have to be studied one by one.