# Chapter 1

# Outline

## 1.1 Intracellular Protein Transport and SNAREs

All eukaryotic cells comprise diverse compartments which are enclosed by membranes. Cellular life and differentiation depends on preserving the integrity of the cells barrier layer and its organelles constantly. However, these compartments act together by exchange of material and neurotransmitters after the transport to the specific organelle. This membrane transport is a basic requirement in order to maintain the identity of the different cell compartments and the inner organization of the cell. Work done by PALADE in 1975 led to the hypothesis that this exchange and transport between organelles is mediated by small transport vesicles.[1] After budding from the prelimanary compartment the vesicles moved to the target compartment, where they fused after overcoming of the energetic barrier between the membranes. Recognition and assembly of the membrane anchored so-called SNARE (Soluble *N*-ethylmaleimide-sensitive factor Attachment protein REceptor) proteins followed by fusion of the membranes are essential for a series of intracellular transport processes reaching from $Ca^{2+}$-dependent neurotransmitter release to exocytosis via the Golgi apparatus.[1]

SNAREs, the catalysts of fusion, appear in all eukaryotic organisms and their participation in various steps of intracellular transport processes was investigated in detail using various experimental assays.[2-6] The neuronal SNARE complex forms a parallel four-helical bundle, composed of the three individual proteins Syntaxin-1, Synaptobrevin-2 and SNAP-25,[7] which anchor each other at their *N*-terminal end. Via a cycle of assembly and disassembly they lead to the fusion of membranes (Figure 1).

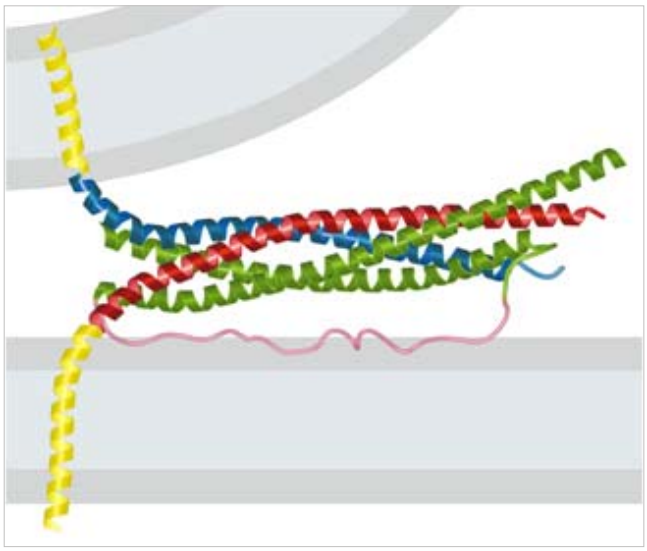

**Figure 1.** *Hypothetical model of the synaptic fusion complex joining two membranes. Synaptobrevin (blue) and Syntaxin 1A (red) are membrane attached via their transmembrane domains (yellow). SNAP-25 (green) is palmitoylated by a cysteine rich interhelical loop (pink) between the helices (picture from R. JAHN, MPI for Biophysical Chemistry, Göttingen).*

The assembly is regulated by special factors, such as Complexins. Meanwhile the mode of binding between Complexin (also called Synaphin) and the SNARE core complex has been investigated. An α-helical central stretch consisting of 58 amino acids was identified to bind the SNARE complex in an anti-parallel fashion.[8]

After fusion the assembled SNARE proteins form a stable complex. They are located in the same membrane and are not able to dissociate autonomously. Hence, it requires the activity of the specialized chaperone-like ATPase NSF (*N*-ethylmaleimide-sensitive factor) in conjunction with its adaptor protein α-SNAP to recover the SNARE motifs via ATP hydrolysis for further rounds of fusion.[9] NSF does not have any binding sites for SNARE complexes. Therefore, it needs its cofactor α-SNAP, which supplies high affinities for both, the SNARE motifs as well as the NSF domains. Without α-SNAP no disassembly occurs.

## 1.2 Investigation of Independently Folding Proteins using Single Molecule Fluorescence Spectroscopy

It is well-known how the primary nucleotide sequence information is stored in the DNA as well as the amino acid sequence in proteins. However, the complexity of their unique three-dimensional structure, a basic requirement for the specific function, has not completely been understood, yet.[10] The development of techniques that are able to monitor dynamics of folding processes, and therefore, help understanding relations between structure and function is a considerable research area. A known fact of the protein folding and unfolding is the high dimension of the structural and conformational heterogeneity to form the final structures. Therefore, investigating the structures of large ensembles of molecules in detail is difficult with classical techniques. In recent years, single molecule fluorescence spectroscopy (smFRET) experiments provide new tools for the study of biological systems, especially the conformational dynamics and interactions of individual peptides and proteins.[11] However, the method was refined within the last decade, but to be able to answer more complex dynamics it is necessary to investigate further and different folding pathways. The combination of protein design and synthesis methods as well as Molecular dynamics simulations these methods will continue to afford an important supplement to other developments. Especially the small HTH- (helix-turn-helix) and the WW motifs (named after two highly conserved tryptophans) demonstrate excellent model systems to improve our appreciation of mechanistic characteristics in protein folding (Figure 2).

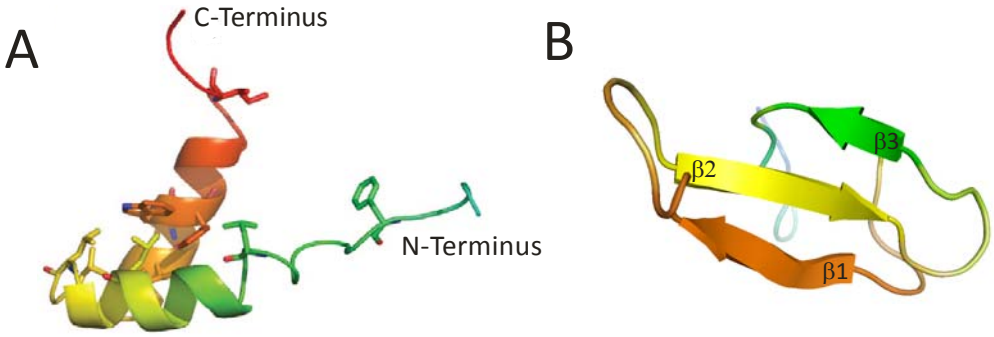


***Figure 2. A*** *Crystal structure from the helix-turn-helix (HTH) motif of the Engrailed Homeodomain (PDB code 2P81).* ***B*** *Crystal structure of the WW domain (pdb code 1I6C).*

## 1.3 Motivation of this Study

Inhibition or at least deceleration of the disassembly event could help to clarify molecular mechanisms. Furthermore, no effective inhibitor for the NSF/α-SNAP mediated disassembly is known to date.

The first aim of this study is to synthesize a new class of SNARE derivatives mimicking the SNARE motif of Synaptobrevin as well as, secondly, synthesizing the recognition unit of Complexin to obtain molecules that can inhibit the α-SNAP/NSF mediated disassembly.

By replacing individual residues in the SNARE motif of Synaptobrevin by amino acids with inversed charge to the *wild type* SNARE motif or by insertion of non-native amino acid building blocks it was aspired to inhibit, or at least to weaken, the binding of α-SNAP. As mentioned before, NSF is not able to bind to SNARE complexes without its cofactor α-SNAP, therefore, if α-SNAP binding is prevented, the disassembly may be unsettled. Requirements for effective disassembly inhibitors are the capability to form stable complexes with the biological counterparts while avoiding α-SNAP binding.

Up to date, molecular details of the disassembly process are less understood. Essential structural characteristics can be identified by systematic integration of synthetic amino acid building blocks and comparison of the resulting binding properties with known parameters. It is not completely understood how the α-SNAP is capable to interact with the SNARE complex, consisting of three different proteins with different characteristics and recognition units. Solely HANSON *et al*. performed a systematic investigation to identify residues in α-SNAP interacting with the SNARE complex.[12] Based on this study, which determined several important residues in the individual SNARE proteins for α-SNAP complex recognition, a series of Synaptobrevin mimics had to be synthesized and their ability to prevent α-SNAP binding was investigated. Additionally, it is of great interest to monitor individual disassembly steps. Furthermore, with the exception of clostridial neurotoxins like botulinum and tetanus toxins, blocking the synaptic-vesicle fusion, no specific SNARE inhibitors are established.

Thus, within this work peptide synthesis and subsequent complex assembly and disassembly studies are performed. The goal of the first part contains:

i) Design and synthesis of an amino acid building block with a bulky side chain.
ii) Design and synthesis of derivatives mimicking the whole SNARE motif (residues 28-89), exhibiting exchanged charges or amino acid building blocks with bulky side chains at selected residues.
iii) SNARE complex assembly studies with the synthesized Synaptobrevin derivatives and their natural counterparts.
iv) Disassembly studies with the α-SNAP/NSF machinery.

The assembly and disassembly studies were realized in cooperation with the laboratory of Prof. R. JAHN, MPI for Biophysical Chemistry, Göttingen.

A different approach to create an effective inhibitor for the SNARE disassembly is to influence the assembly-disassembly cycle of SNARE proteins at an earlier stage. MC MAHON *et al*. reported that α-SNAP is not able to bind to the SNARE complex when Complexin is added in large excess.[13] Complexin interacts only with the Synaptobrevin and Syntaxin helices, but not with SNAP-25. The *N*-terminal part of Complexin, namely amino acids 48-70, is in close contact with the complex. The *C*-terminal end is slightly bended, probably due to its EEE-segment followed by positively charged amino acids. The structure of Complexin bound to the neuronal SNARE complex has been solved (Figure 3).[14] This leads to assumptions that they might have inhibitory properties regarding the SNARE disassembly.[13]

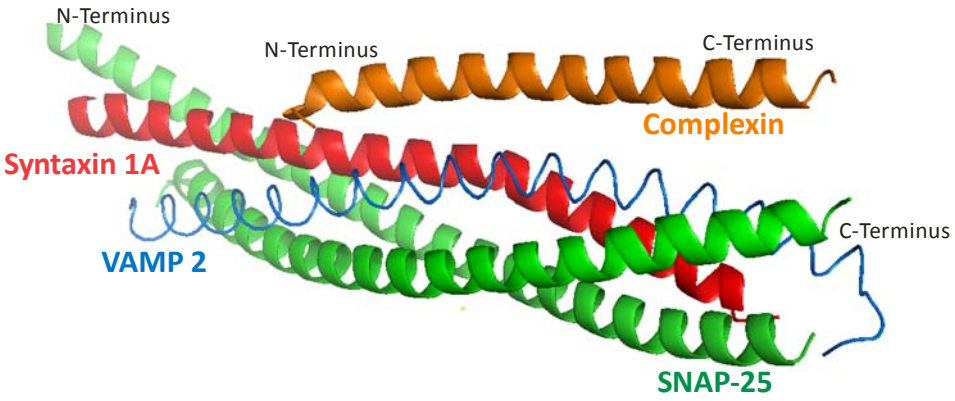


***Figure 3.*** *Crystal structure of the Complexin/SNARE complex. Complexin binds antiparallel to the assembled SNARE complex (pdb code 1KIL).*

Synthesis of Complexin analogues with defined binding sites interacting significantly stronger with the SNARE proteins could also impede the disassembly. Introduction of additional potentially interacting amino acids and elongation of

the recognition area to the SNARE protein helices with further charges should enhance the association. Therefore, truncation of the *C*-terminal end behind the EEE-section and elongation at the *N*-terminus was intended to be realized as an α-peptide, and additionally, as a β-peptide forming a 14-helix.

The α-peptide was synthesized without changes in the sequence, whereas the sequence of the β-peptide is designed promoting a specific 14-helix by incorporation of β-amino acid building blocks. Nevertheless, regarding important side chain orientations and distances to the SNARE complex the use of β-amino acid building blocks known to induce a 14-helix, a Complexin-mimic could maintain its binding to the SNARE complex and prevent interaction with α-SNAP, and therefore, the disassembly.

Purpose of the second part:

i) Design and synthesis of Complexin analogues as α-peptides (residues 48-70), elongated at the *C*-terminal end with the AKRK-motif. Another approach is elongating the *N*-terminal end with amino acids known to stabilize the propensity building of a α-helix.
ii) Synthesis of β-amino acid building blocks.
iii) Design and synthesis of Complexin as a β-peptide.
iv) SNARE complex assembly studies with the Complexin analogues (α-peptide and β-peptide) and the individual *wild type* SNARE proteins.

In a third part the challenge is to investigate polypeptides in single molecule experiments. Hence, labeling with extrinsic fluorophores is unavoidable. For FRET measurements two (or more) chromophores are needed. Short peptides are simple systems, which can designed and synthesized with only one residue with a suitable reactive side chain.[15] With solid phase peptide synthesis natural amino acids can be individually labeled in the natural sequence by using orthogonal side chain protection groups. Applying chemical synthesis also prevents labeling of an undefined number of side chains. For some applications, such as *in vivo* imaging, the amount of labeling is not completely important, but for FRET, specificity it is absolutely essential.