1 Introduction

The human brain consists of at least 10^{11} neurons, each forming an average of 1000 connections, called synapses (Kandel, Schwartz & Jessell, 2000). These are sites where information is relayed from one cell to the other. When an action potential arrives at the presynaptic site, Ca^{2+}-ions enter the cytosol and trigger the release of neurotransmitters. These transmitters diffuse through the synaptic cleft and bind to receptors in the target membrane eliciting a specific cellular response. The chemical nature of this process was first described by Otto Loewi who showed that acetylcholine is responsible for the transmission of signals between the vagus nerve and the heart (Loewi, 1921). Transmitters are initially stored in synaptic vesicles and then released into the synaptic cleft in the form of defined quanta (Del Castillo & Katz, 1956). The first aqueous connection between the lumen of the vesicle and the extracellular space - the fusion pore - was initially shown to exist by both freeze fracture electron microscopy (Chandler & Heuser, 1980) and patch clamp electrophysiological recordings (Breckenridge & Almers, 1987). Upon arrival of the action potential the local calcium concentration rises from ~100 nM at rest to over 100 μM near the open mouth of the individual calcium channel (Adler *et al.*, 1991). Recent studies using Ca^{2+}-ion uncaging in the large synaptic terminal of the calyx of Held indicated that Ca^{2+} concentrations of 10 μM are sufficient to drive transmitter release (Schneggenburger & Neher, 2000), (Bollmann, Sakmann & Borst, 2000). This process is extremely rapid and can occur within 100 microseconds (Zucker, 1996). Vesicle fusion at the nerve terminal is a highly specialized event that has evolved to facilitate fast transmitter release and is stringently controlled. Membrane fusion reactions, though, occur throughout the cell and are likely to share common molecular mechanisms.

1.1 Historical Outline

Proteins that are synthesized at the endoplasmic reticulum are sorted and transported according to their cellular destinations. Secretory proteins pass through the Golgi apparatus and are packaged into vesicles that fuse with the plasma membrane (Palade, 1975).

In 1984, Rothman and coworkers reconstituted the transport of the vesicular stomatitis virus encoded glycoprotein (G-protein) between successive compartments of the Golgi in a cell-free system (Balch *et al.*, 1984). This assay advanced the field substantially because it

allowed isolating factors that are essential for transport. It was observed that the process requires ATP and could be inhibited by N-ethylmaleimide (NEM) (Glick & Rothman, 1987). NEM treatment caused the accumulation of uncoated vesicles on acceptor Golgi cisternae ((Malhotra *et al.*, 1988), and (Orci *et al.*, 1989)). Subsequently, the NEM sensitive fusion protein (NSF) was isolated. NSF could only bind to Golgi membranes in the presence of soluble NSF attachment proteins (SNAPs). Three isoforms (α, β, and γ) were identified ((Clary & Rothman, 1990), (Clary, Griff & Rothman, 1990), and (Whiteheart *et al.*, 1993)). These proteins themselves were attached to the membrane by receptors. The finding that NSF could release itself from this complex upon hydrolysis of ATP (Wilson *et al.*, 1992) and was trapped on the membrane when non-hydrolysable ATP-γ-S was added to the assay set the stage for the isolation of the SNAP receptors (Söllner *et al.*, 1993).

Use of a myc-tagged NSF protein in the presence of ATP-γ-S, recombinant SNAP proteins and a Triton extract of bovine brain allowed assembling protein complexes that sedimented at 20 S. Bovine brain was chosen because the brain is highly specialized in secretion and receptor proteins are enriched. Addition of ATP and its subsequent hydrolysis caused the release of three membrane proteins that had been cloned and localized before:

1.) Synaptobrevin or VAMP (for Vesicle associated membrane protein) is a type 2 membrane protein with a molecular weight of 12 kDa and is confined to synaptic vesicles ((Trimble, Cowan & Scheller, 1988), and (Baumert *et al.*, 1989)).

2.) Syntaxin is also a type 2 membrane protein, has a molecular weight of 35 kDa, and is localized to the presynaptic plasma membrane (Bennett, Calakos & Scheller, 1992).

3.) SNAP-25 (synaptosome-associated protein of 25 kDa) (Oyler *et al.*, 1989) is associated with the presynaptic plasma membrane via its palmitoyl chains (Hess *et al.*, 1992).

Another important step toward understanding the function of SNARE proteins came from a detailed study of clostridial neurotoxins ((Schiavo *et al.*, 1992), (Link *et al.*, 1992), (Blasi *et al.*, 1993a), (Blasi *et al.*, 1993b), (Binz *et al.*, 1994), (Schiavo *et al.*, 1994), and (Schiavo *et al.*, 1995)). These toxins are the most poisonous substances known. Eight different clostridial neurotoxins have been identified (for a detailed review see (Schiavo, Matteoli &

Montecucco, 2000) and references therein): one tetanus toxin (TeNT) and seven botulinal toxins (BoNT/ A, B, C1, D, E, F, and G). They are homologous heterodimeric proteins. The heavy chains mediate targeting of the toxin to the presynaptic nerve terminal. The light chains, as first observed for tetanus toxin, contain a highly conserved 20-residue-long segment with a zinc binding motif (His-Glu-Xaa-Xaa-His) found in a variety of zinc dependent endopeptidases. A search for the molecular targets revealed that it is the neuronal SNARE proteins synaptobrevin, syntaxin and SNAP-25 that are selectively and specifically proteolyzed by clostridial neurotoxins.
Tetanus toxin and botulinal neurotoxins type B, D, F, and G cleave synaptobrevin; botulinal toxin type C1 cleaves syntaxin, and botulinal toxins type A, E, and C1 cleave SNAP-25. Cleavage of the neuronal SNARE proteins by these toxins causes a complete block of exocytosis.

Further important insights into vesicular transport were gained from the molecular dissection of membrane trafficking in yeast, which began with the identification of temperature sensitive sec mutants defective in secretion ((Novick, Field & Schekman, 1980), and (Novick, Ferro & Schekman, 1981)). Two of the factors that were found Sec17p and Sec18p are homologous to α-SNAP (Clary *et al.*, 1990) and NSF (Wilson *et al.*, 1989), respectively. Proteins homologous to SNAREs were identified later for reason of genetic redundancy. It turned out that Sso1p/Sso2p are the yeast equivalents to syntaxin 1 and are localized predominantly to the plasma membrane (Aalto, Ronne & Keranen, 1993). Snc1p/Snc2p are homologous to synaptobrevin and are components of post Golgi vesicles (Protopopov *et al.*, 1993). The C-terminal part of Sec9p is homologous to SNAP-25 (Brennwald *et al.*, 1994). Unlike most SNAREs, Sec9p is a peripheral membrane protein that lacks a plausible membrane anchor. It is also not palmitoylated like its neuronal homologue SNAP-25 (Veit, Söllner & Rothman, 1996) because it lacks cysteines. All three yeast SNAREs form a complex that resembles the neuronal one ((Brennwald *et al.*, 1994), and (Rossi *et al.*, 1997)).

Subsequent studies corroborated the finding that SNARE proteins are involved in all cellular transport processes and that they are conserved from yeast to man ((Bennett & Scheller, 1993), (Ferro-Novick & Jahn, 1994), and (Jahn & Südhof, 1999) for a more recent review).

It is these three major lines of converging evidence: 1.) the identification of SNARE proteins in the context of a cell free transport system, 2.) the pinpointing of SNARE proteins as molecular targets for clostridial neurotoxins, and 3.) the identification of SNARE proteins in the yeast secretory pathway that established the basis and the fundamental importance of SNARE proteins in membrane fusion. These findings have motivated an extensive structural characterization of SNARE proteins.

1.2 Towards a Molecular Understanding of SNARE Proteins

SNARE proteins represent a superfamily of small and mostly membrane-bound proteins ((Rothman, 1994), (Jahn & Südhof, 1999), (Hughson, 1999), (Brunger, 2001), and (Chen & Scheller, 2001)). The SNAREs, functioning in neuronal exocytosis have served as paradigms for the other members of the protein family. They include the plasma membrane proteins syntaxin 1, SNAP-25, and the synaptic vesicle protein synaptobrevin (for an overview of currently available high resolution structures see Fig. 1). These proteins assemble spontaneously into a heat stable ternary complex that is both resistant to SDS-denaturation and to clostridial toxin cleavage. As outlined above the complex can be disassembled by the ATPase chaperone NSF in conjunction with SNAP-proteins as cofactors. It is currently thought that assembly of the SNARE proteins ties the fusing membranes together and thus initiates the fusion reaction (Hanson, Heuser & Jahn, 1997a).

As a common feature, all SNAREs contain a homologous stretch of 60 amino acids that is composed of conserved heptad repeat sequences and referred to as the SNARE motif ((Terrian & White, 1997), (Weimbs *et al.*, 1997), and (Weimbs *et al.*, 1998)). Both, syntaxin and synaptobrevin contain a single SNARE motif adjacent to the C-terminal transmembrane domain. SNAP-25 is composed of two SNARE motifs one at the C- and one at the N-terminal end that are connected by a loop containing palmitoylated cysteines. Although the fatty acids serve to anchor the protein in the membrane their function is not completely understood because homologues proteins exist (Sec9p and SNAP-29) which lack posttranlational hydrophobic modifications.

Binary interactions between synaptobrevin and syntaxin or synaptobrevin and SNAP-25 are weak. The only stable binary complex is formed between syntaxin and SNAP-25. This complex can be isolated by gel filtration and has a 2:1 stoichiometry (Fasshauer *et al.*, 1997b). Interestingly, the homologues complex in yeast between Sso1p and Sec9p exists in

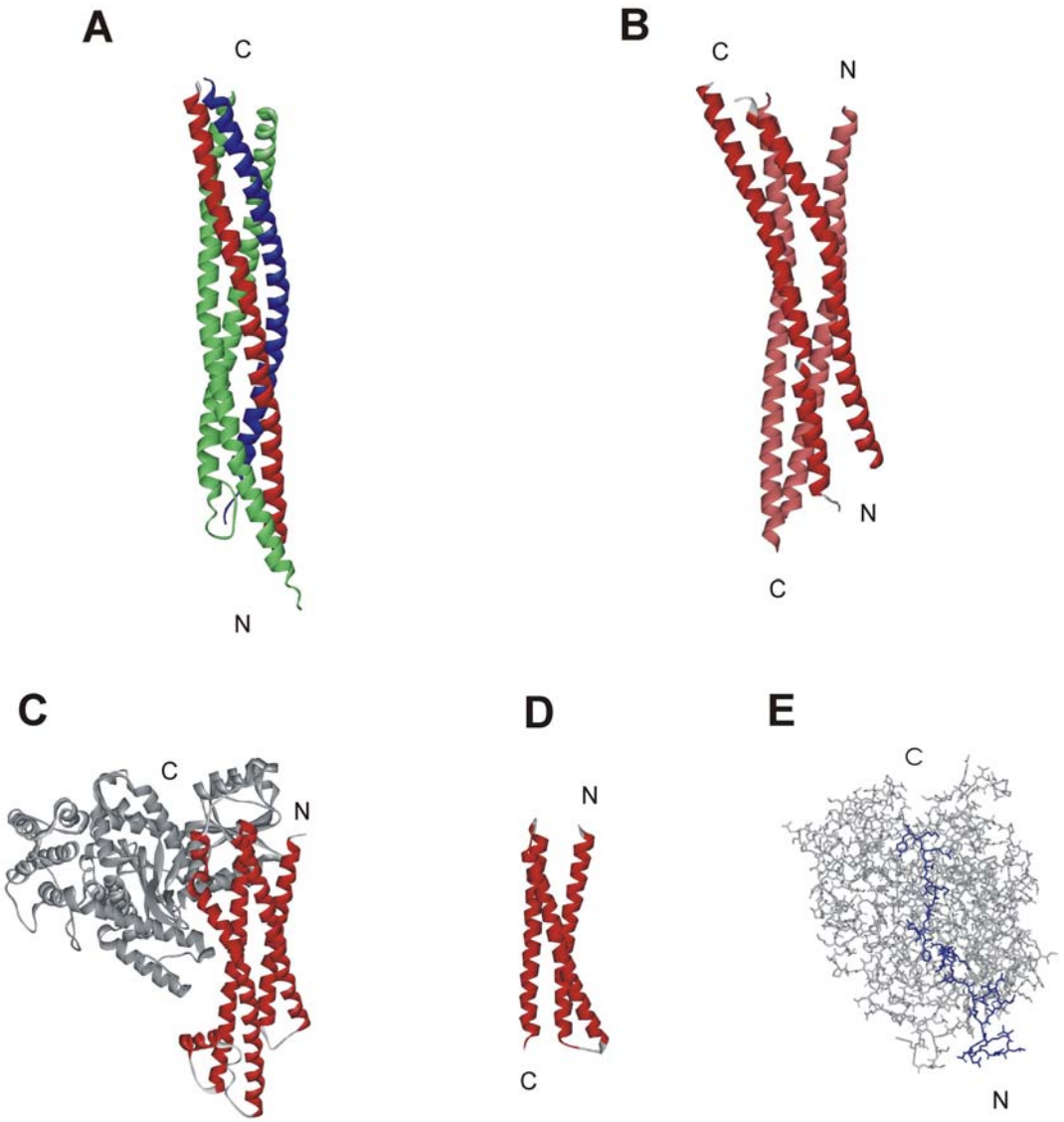

Fig. 1. X-ray crystal structures of neuronal SNARE proteins
Syntaxin (red), synaptobrevin (blue), and SNAP-25 (green) are depicted as ribbons. In (E) a stick model is used because when in complex with BoNT/B synaptobrevin has no identifiable secondary structure.
(A) Ternary complex (Sutton *et al.*, 1998, PDB coordinates: 1SFC)
(B) H3 tetramer (antiparallel dimers, light and dark red) (Misura *et al.*, 2001, PDB coordinates: 1HVV)
(C) Syntaxin/munc-18 complex (Misura *et al.,* 2000, PDB coordinates: 1DN1)
(D) Habc domain of syntaxin (Lerman *et al.,* 2000, PDB coordinates: 1EZ3) (see also Fernandez *et al.*, 1998 for an NMR structure, PDB coordinates: 1BRO)
(E) Synaptobrevin fragments (residues 53-76, and residues 77-88) in complex with BoNT/B (Hanson *et al.*, 2000, PDB coordinates: 1F83).

a 1:1 stoichiometry.

Limited proteolysis of ternary complexes and site directed mutagenesis revealed that the SNARE motifs form a tight complex whereas other regions of the SNAREs do not participate in complex formation ((Hayashi *et al.*, 1994), (Pevsner *et al.*, 1994), (Hayashi *et*

al., 1995), (Calakos *et al.*, 1994), (Kee *et al.*, 1995), (Chapman *et al.*, 1994), (Zhong *et al.*, 1997), (Poirier *et al.*, 1998a), (Hao *et al.*, 1997), and (Fasshauer *et al.*, 1998a)). SNARE domains that are not part of this core complex include the amino-terminal domain of syntaxin, the central palmitoylated region of SNAP-25, the amino-terminal proline rich region of synaptobrevin, and the transmembrane domains of syntaxin and synaptobrevin.

Electron microscopy ((Hanson *et al.*, 1997b), and (Katz *et al.*, 1998)), fluorescence resonance energy transfer studies (Lin & Scheller, 1997), and EPR measurements (Poirier *et al.*, 1998b) indicated a parallel alignment of all four helices.

A high-resolution structure of the core complex was obtained by x-ray-crystallography (Sutton *et al.*, 1998). The complex is a highly twisted right-handed four-helix bundle with an overall length of 12 nm (Fig. 1A). The center of the bundle contains 16 layers of interacting and mostly hydrophobic side chains, stacked perpendicular to the axis of the helix bundle. These amino acids are highly conserved throughout the SNARE superfamily suggesting that the four helix bundle structure is also conserved. Single amino acid substitutions of SNAREs from yeast and lower animals which map to the core of the bundle generally result in loss, or at least severe impairment of SNARE function (Fasshauer *et al.*, 1998b). In the middle of the bundle an unusual "0" layer was found that is composed of three Gln residues (contributed by syntaxin and SNAP-25) and one Arg residue (contributed by synaptobrevin). These residues are highly conserved throughout the entire SNARE superfamily, leading to their classification into Q-SNAREs and R-SNAREs, respectively (Fasshauer *et al.*, 1998b). The finding that in yeast Gln to Arg mutations resulted in drastically reduced secretion (Ossig *et al.*, 2000), (Katz & Brennwald, 2000) substantiated the significance of this central layer although Arg to Gln substitutions did not cause any abnormality (four Gln are allowed in coiled coils). The amino acids in the zero layer might enforce the right register during SNARE pairing (Sutton *et al.*, 1998).

Whereas SNAP-25 and synaptobrevin do not possess additional structured regions outside the SNARE motifs the N-terminus of syntaxin constitutes an independently folded domain (see below).

Assembly of the core complex is associated with major conformational changes (Fasshauer *et al.*, 1997b). Circular dichroism- and NMR-spectroscopy showed that monomeric synaptobrevin and monomeric SNAP-25 exhibit no significant secondary structure ((Fasshauer *et al.*, 1997b), (Fasshauer *et al.*, 1997a), and (Hazzard, Südhof & Rizo, 1999)).

Recently, the structure of synaptobrevin in complex with botulinum neurotoxin B has been determined by X-ray crystallography (Hanson & Stevens, 2000). In this complex synaptobrevin does not contain identifiable secondary structure elements (Fig. 1E) and is thus structured very differently from the conformation in the core complex.

Most of the structural information on SNARE proteins was obtained from cytosolic versions lacking their membrane anchors. The membrane anchors, though, may have additional functions.

The transmembrane domain of synaptobrevin mediates dimerization that depends on sequence specific residues in the hydrophobic domain (Laage & Langosch, 1997).

Furthermore, binding of synaptobrevin and its close homologue cellubrevin to synaptophysin and BAP-31, respectively, depends on the presence of the transmembrane domains (Edelmann *et al.*, 1995), (Annaert *et al.*, 1997).

1.3 A Model for Membrane Fusion

The structural information obtained during the last several years was integrated into a new model of membrane fusion ((Jahn & Südhof, 1999) for review). The process can be divided into 5 different phases (Fig. 2):

A. SNARE proteins located on the synaptic vesicle membrane and trapped as cis-complexes (Otto, Hanson & Jahn, 1997) are disassembled by the combined action of α-SNAP and NSF
B. Then, synaptic vesicles are attached to the plasma membrane.
   Accurate vesicle targeting requires multiple layers of regulation. It is likely that an interaction of the vesicle with the cytoskeleton is crucial for correct guidance to the plasma membrane. SNARE proteins, although at the hub of membrane fusion, do not mediate specificity ((Fasshauer *et al.*, 1999), (Yang *et al.*, 1999), and (Tsui & Banfield, 2000)). Several lines of evidence indicate that vesicles attach to the membrane, even though SNARE proteins have been cleaved by neurotoxins (Hunt *et al.*, 1994).
C. Syntaxin, synaptobrevin and SNAP-25 form trans-SNARE complexes and lead to an approximation of the opposing lipid bilayers. At this stage the proximal monolayers may fuse to form a hemifusion stalk. The geometry of the trans-SNARE complex bears

some similarity to that of the fusion proteins of enveloped viruses including influenza, ebola, and HIV-1 (jacknife mechanism of fusion) ((Skehel & Wiley, 1998) for review).

D. The membrane bilayers merge creating a lipid continuum and the opening of a fusion pore.

E. Fusion pore expansion leads to completion of the reaction.

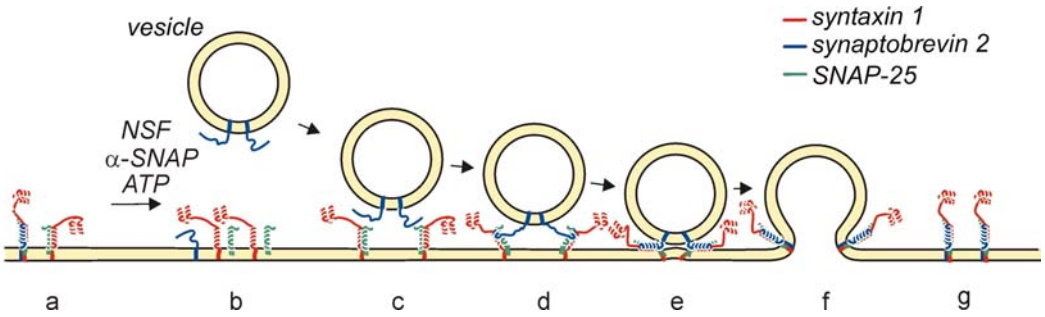

Fig. 2. SNARE mediated membrane fusion at the synapse (model)
(a) cis-SNARE complexes in the plasma membrane and the vesicle membrane (not shown) are disassembled by NSF and α-SNAP and thus activated for fusion. Once vesicles attach to the plasma membrane (b-c) SNARE proteins of the apposing bilayers get into contact (d) and form trans-SNARE complexes (e). The two membranes merge to form a continuous bilayer (f). At the same time transmitters are released from the vesicle interior and diffuse to the postsynaptic membrane. (g) During fusion trans-SNARE complexes convert into cis complexes, and are thus localized in the same membrane.

1.4 Syntaxin at the Center Stage of Regulation

Syntaxin 1 differs from the other SNARE proteins in that it contains an independently folded N-terminal domain. This domain is conserved among syntaxin isoforms that are operating at the plasma membrane. Amongst these are syntaxin 2, 3 and 4, and the yeast isoforms Sso1p and Sso2p (Fernandez *et al.*, 1998). The sequence conservation suggests that the N-terminal domain has a function specific for exocytosis. The three dimensional structure of this domain was first determined by NMR spectroscopy (Fernandez *et al.*, 1998) and later confirmed by x-ray crystallography (Lerman *et al.*, 2000) (Fig. 1D). The only difference detected was a pronounced curvature that was present in the NMR but not in the X-ray structure. The domain consists of an up-and down three-helix bundle. The third helix was not anticipated by sequence analysis since it lacks the heptad repeats typical for coiled coils. However, this helix is highly conserved and binds to the other two helices to form a deep groove between the second and third helix (Hb/Hc). Residues within this groove are highly conserved, suggesting that it serves as an interaction surface for another α-helix.

The evolutionarily conserved N-terminal domain of syntaxin hints toward another role, in addition to forming SNARE complexes with synaptobrevin and SNAP-25.

The domain binds to several proteins that are essential to synaptic vesicle exocytosis, including munc18-1 ((Hata, Slaughter & Südhof, 1993), and (Kee *et al.*, 1995)) and munc13-1 (Betz *et al.*, 1997). An intramolecular interaction with the core region was postulated based on binding studies between syntaxin and synaptobrevin (Calakos *et al.*, 1994). The weak association of these proteins was completely abolished in the presence of syntaxin's N-terminus. Further experiments suggested that after disassembly syntaxin exists in a conformation that prevents it from interacting with other SNARE partners (Hanson *et al.*, 1995). The existence of a closed conformation gained additional support by an NMR study that compared the spectral line shifts of the N-terminal domain with that of the full-length protein (Dulubova *et al.*, 1999).

Crystallization of syntaxin in complex with munc-18/n-sec1 revealed the existence of a closed conformation (Misura, Scheller & Weis, 2000) (Fig. 1C). The SNARE-motif of syntaxin has a different structure from that in the core complex. Only the N-terminal portion is α-helical which is followed by few short turns and helices whereas the C-terminal end is unstructured. Another interesting feature is that the α-helix at the C-terminal end of the Habc-domain extends an additional nine amino acids when compared to the NMR and X-ray structures of isolated syntaxin fragments.

Evidence that the N-terminus has the potential to fold back was corroborated by studies of the yeast homologue Sso1p (Nicholson *et al.*, 1998). A kinetic analysis revealed that SNARE complex formation was slowed down by a factor of 2000 depending on whether the N-terminus of Sso1p was present or absent. Furthermore, when both the N-terminal and C-terminal fragments were combined a stable complex formed that could be isolated by gel filtration chromatography.

The recently solved crystal structure of Sso1p (Munson *et al.*, 2000) lends further support for an intramolecular interaction between the two domains.

The three dimensional structures of syntaxin 1a in the core complex and in complex with munc-18 are snapshots of potential intermediates in membrane fusion. The intramolecular interaction between the N-terminal domain and the core region of syntaxin is likely to have evolved to fulfill the special requirements of membrane fusion at the nerve terminal. Control of this interaction by other proteins like munc-18, or munc-13 may be at the heart of SNARE regulation.

1.5 Electron Paramagnetic Resonance Spectroscopy

Site-directed spin labeling has become a powerful tool to determine protein structure at the level of the backbone fold (for review see (Hubbell, Cafiso & Altenbach, 2000), (Hubbell *et al.*, 1996), (Hubbell *et al.*, 1998), and (Feix, 1998)). The most widely used and best characterized spin label is (1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl) methanethiosulfonate (Berliner *et al.*, 1982). The label is introduced at a selected site of the protein via disulfide coupling (Fig. 3A).

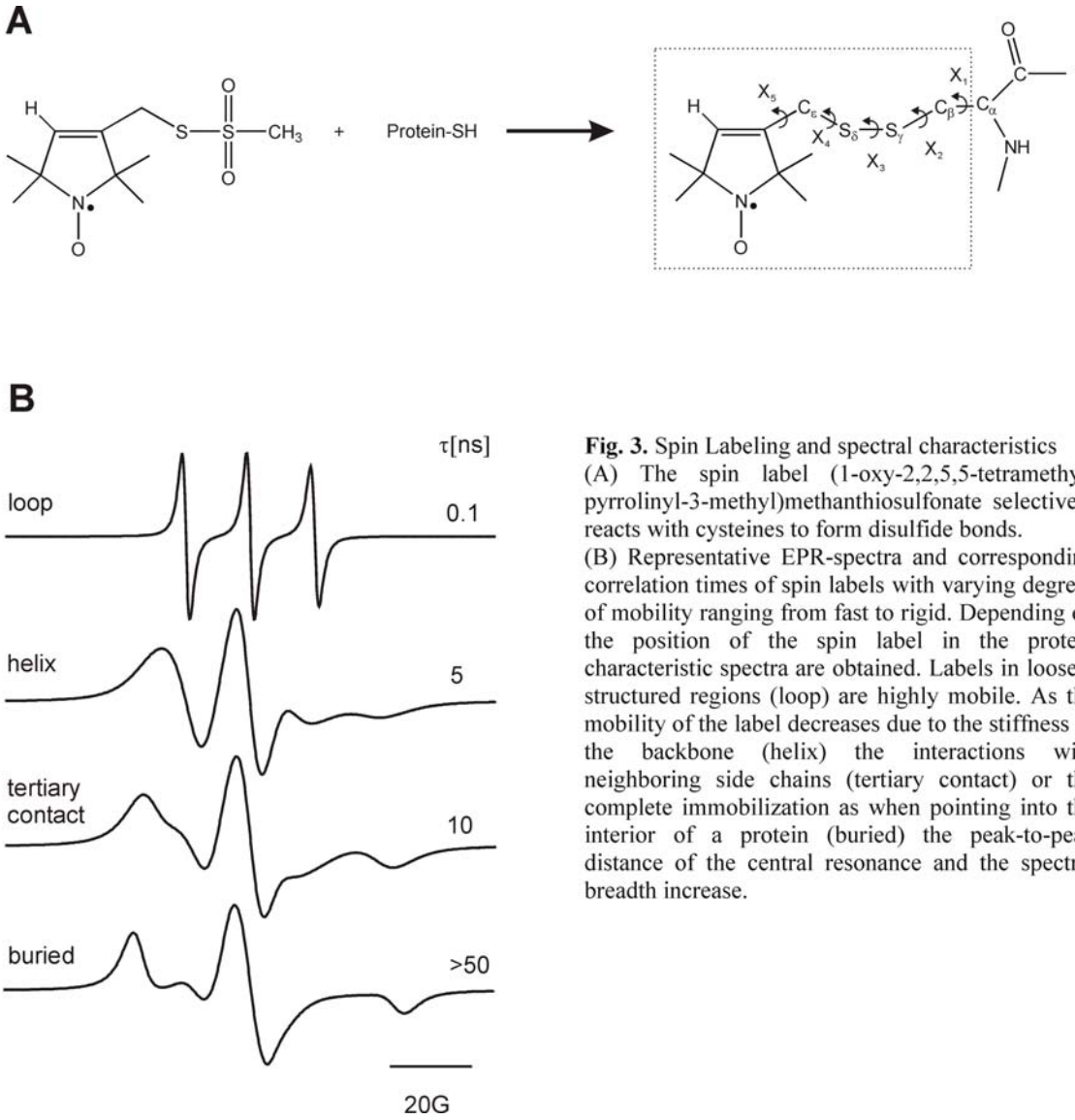

Fig. 3. Spin Labeling and spectral characteristics
(A) The spin label (1-oxy-2,2,5,5-tetramethyl-pyrrolinyl-3-methyl)methanthiosulfonate selectively reacts with cysteines to form disulfide bonds.
(B) Representative EPR-spectra and corresponding correlation times of spin labels with varying degrees of mobility ranging from fast to rigid. Depending on the position of the spin label in the protein characteristic spectra are obtained. Labels in loosely structured regions (loop) are highly mobile. As the mobility of the label decreases due to the stiffness of the backbone (helix) the interactions with neighboring side chains (tertiary contact) or the complete immobilization as when pointing into the interior of a protein (buried) the peak-to-peak distance of the central resonance and the spectral breadth increase.

The EPR spectrum encodes information on the dynamic modes of the nitroxide. Since these modes are directly dependent on the molecular environment of the nitroxide they

reflect information on the structure and dynamics of the protein itself. Three types of motions can be distinguished that are important in this respect:

1. Brownian rotational diffusion
2. Protein backbone fluctuations
3. Bond isomerizations within the side chain

The contribution of Brownian rotary diffusion to the overall spectrum has been reduced by using solutions with increased viscosity such as 30 % sucrose.

In order to analyze EPR spectra two measures of nitroxide mobility have been introduced that reflect both rate and amplitude of motion: the peak-to-peak distance of the central resonance and the second moment (Slichter, 1992), representing the breadth of the spectrum.

The analysis of hundreds of spectra in a broad range of helical proteins including T4 lysozyme (McHaourab *et al.*, 1996), colicin E1 (Todd *et al.*, 1989), annexin XII (Langen *et al.*, 1998), and the T domain of diphtheria toxin (Oh *et al.*, 1996), revealed that in general four types of spectra, can be differentiated depending on whether the label is in a loop position, on the surface of a helix, in tertiary contact with neighboring structural motifs or buried and therefore completely immobile (Fig. 3B).

These studies further revealed that labels on the surface of a helix, although similar in the degree of mobility, are unique in quantitative detail, and give a fingerprint of the specific position. Since neighboring amino acids have little influence on the mobility of the side chain it has been suggested that backbone dynamic modes with correlation times in the nanosecond regime modulate the side chain internal motions. It was observed that the disulfide linkage and its interaction with main chain atoms lock the C_α-C_β-S_χ-S_δ atom group in position (Langen *et al.*, 2000). Therefore, torsional oscillations of X4 and X5 of the side chain are dominant contributors to the motion of the nitroxide. This reduced motional freedom results in an increased sensitivity toward detecting interactions of the nitroxide ring with nearby structures. The disulfide backbone interactions serve to couple backbone fluctuations to nitroxide motion. Recently, this anisotropic motional model has gained further support by spectral simulations and experiments that varied the side chain structure and the geometry of the nitroxide ring (Columbus *et al.*, 2001).

1.6 Single Molecule Fluorescence Spectroscopy

Ensemble measurements yield a mean average of a quantity. If the system under investigation is heterogeneous valuable information about molecular subpopulations in the ensemble are lost. Single molecule measurements can be used to determine the distribution of molecular properties and associated fluctuations (see (Xie & Trautman, 1998), (Ambrose *et al.*, 1999), (Deniz *et al.*, 2001), (Weiss, 1999), and (Weiss, 2000) for review).

A new method termed multi-parameter fluorescence detection monitors single molecules diffusing through the open volume element of an epi-illuminated confocal microscope (Fig. 4) ((Eggeling *et al.*, 1998), (Fries *et al.*, 1998), and (Eggeling *et al.*, 2001b)).

As the fluorescently labeled protein transits the periodic train of laser pulses it continuously cycles between ground and excited states. The result is a burst of emitted photons. Each photon can be analyzed with respect to excited state duration, polarization, spectral position, and the time delay from the previous detected photon.

The accumulated information from all photons within a burst allows determining parameters like fluorescence lifetime, anisotropy, intensity and spectral range.

Analysis of a large number of bursts again is used to identify distributions of fluorescent properties.

Fluctuations in the system can be observed by autocorrelating the fluorescent intensities of the recorded bursts or performing binning time analyses that subdivide bursts into either photon- or time-intervals (Eggeling *et al.*, 1998).

Single molecule detection is not as much a sensitivity issue as it is a background issue. A fluorescence burst characteristic for a single molecule must be detected on top of the background associated with the solvent. Therefore, small volumes in the range of 1-2 fl are used. Raleigh and Raman scattering generate most of the non-specific background. Their contributions to the accumulated signal can be effectively reduced using appropriate sets of filters.

In a “multi channel scaler trace” fluorescent bursts are depicted on a macroscopic time scale. The counts of all photons are binned in certain time intervals (in our case: 1 ms).

Since bursts are characterized by an increased number of consecutive photons, they are easily separated from background photons. Those have comparatively large interphoton times and thus will give only weak amplitudes within the binning interval.

Ensemble fluorescence resonance energy transfer measurements have been used to study

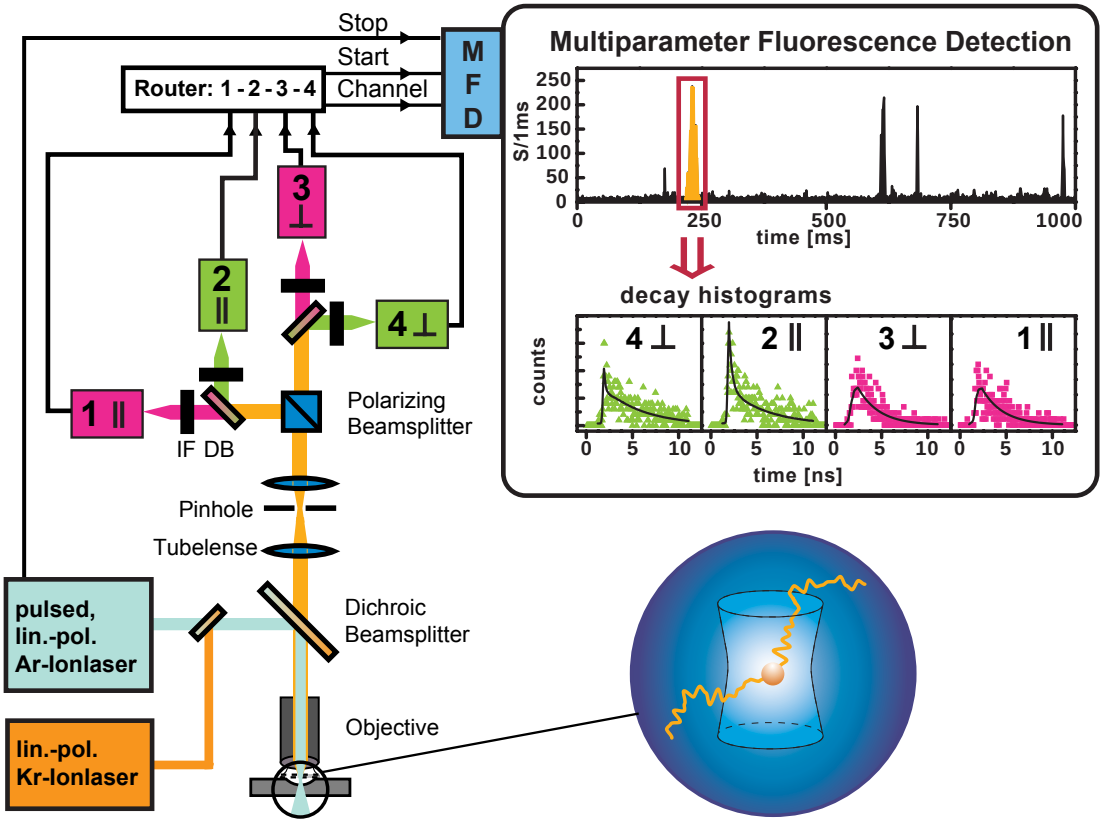

Fig. 4. Setup for multi parameter fluorescenc detection
Molecules diffusing through the open volume element of the confocal microscope are excited by a pulsed Ar-Ionlaser. The emitted photons are collected according to (1) wavelength (red/green) and (2) polarization (VV/VH), where V is vertical and H is horizontal. The inset shows a typical multi channel scaler trace (bin width: 1 ms) of a donor-/acceptor-labeled protein sample. For each photon within the burst the arrival time relative to the exciting laser pulse is measured by time-correlated single photon counting. This information is used to build up arrival time decay histograms for each of the four channels.

static distances in the 2-8 nm range ((Förster, 1948), (Stryer & Haugland, 1967), (Selvin, 1995), and (Selvin, 2000)). Since in those measurements the precise labeling efficiency of each position must be known even average distances between two proteins are difficult to determine with accuracy.

The simultaneous labeling of two cysteines in a protein with donor and acceptor dyes results in a distribution of differently labeled species that can only be separated at the single molecule level. It is also here that labeling efficiency is less relevant because bursts that correspond to both donor and acceptor dyes can be selectively chosen.

Single pair FRET measurements were first carried out on DNA molecules ((Ha *et al.*, 1996), (Deniz *et al.*, 1999), and (Dahan *et al.*, 1999)). Studies on proteins, though, are still in their infancy (see for example (Schutz, Trabesinger & Schmidt, 1998), and (Ha *et al.*, 1999), (Ishii *et al.*, 1999)).

1.7 Aim of this Work

Because formation of trans-SNARE complexes is thought to play a crucial role in membrane fusion, it is essential to understand the details of the assembly reaction, including the structures of intermediate states. For instance it is unknown whether all SNARE proteins assemble simultaneously or whether binary complexes must precede the formation of ternary complexes. Since in the binary complex one of the syntaxins can be replaced by synaptobrevin yielding a ternary complex, binary complexes may serve as precursors in membrane fusion (Fasshauer *et al.*, 1997b). Furthermore, it is currently thought that core complex formation is initiated at the N-terminal ends of the SNARE motifs, but it is not known whether a step-by-step assembly of the helix bundle is possible. Such a reaction pathway would require that intermediates exist in which part of the complex is helical, whereas the remainder of the proteins is unstructured. Loosely assembled intermediates have been postulated (Xu *et al.*, 1999), but so far there is no evidence for the existence of partially assembled complexes.

Syntaxin appears to be at the center stage of regulation because it's independently folded N-terminal domain can interact intramolecularly with the SNARE-motif resulting in a "closed" conformation ((Calakos *et al.*, 1994), (Hanson *et al.*, 1995), and (Dulubova *et al.*, 1999)). Such an interaction competes directly with SNARE complex assembly, because only in an "open" conformation can syntaxin bind synaptobrevin and SNAP-25. Yet, the structural and dynamic details of this molecular switch are mostly unknown.

Information on the behavior of syntaxin in the membrane is scarce. Additional interactions may exist which are mediated by the membrane anchors and may also play a role in ternary complex formation.

Aim of this thesis was to study the structural and dynamic details of SNARE complex assembly using electron paramagnetic resonance- and fluorescence spectroscopy as key methodological tools.