2 Materials and Methods

2.1 Materials

Spin-label (1-oxy-2,2,5,5-tetramethylpyrrolinyl-3methyl)methanthiosulfonate) was a kind gift from Kalman Hideg (University of Pecs, Hungary). Alexa488-maleimide and Alexa594-maleimide were purchased from Molecular Probes. All lipids were from Avanti Polar Lipids.

cDNAs encoding for rat synaptobrevin 2 and rat syntaxin 1a were kindly provided by R. Scheller (Stanford University, CA). cDNA for SNAP-25A was a kind gift from T. Südhof (University of Texas Southwestern Medical Center, TX). The NSF and α-SNAP-constructs in pQE-9 vectors were kindly provided by S. Whiteheart and J.E. Rothman (Rockefeller University, NY). The munc-18 and SyxL165A/E166A(1-262) constructs in pET28a were a generous gift of D. Schütz (MPIBPC, Göttingen). Fab fragments of Cl. 71.1 and Cl. 71.2 were kindly provided by Burkhard Rammner. The recombinant light chain of tetanus neurotoxin (TeNT) was a generous gift of H. Niemann. All enzymes used were either from Stratagene or Boehringer.

Ni-NTA was purchased from Qiagen. All materials used for chromatography were from Amersham Pharmacia. Ingredients for media were purchased at Gibco. All other chemicals were from Sigma.

2.2 Plasmid Construction

In general, all constructs were generated by PCR using the appropriate primers and P*fu* DNA polymerase for amplification. SNARE constructs that were generated by a single PCR reaction are listed in table 1. cDNAs encoding for rat synaptobrevin 2, syntaxin 1a and SNAP-25a served as templates. The PCR products were cloned into their target vectors via an *Nde*1/*Xho*1 cleavage site. Only the fragment coding for SNAP-25 was cloned via a *Nhe*1/*Xho*1 site. The vector pHO4d was built by inserting DNA coding for a C-terminal His$_6$-tag followed by a c-myc epitope and a stop codon into the *Eco*R1/*Bam*H1 sites of pHO2d. The sequence of this insert was: gcgaattcgggccaccatcacc accatcacggcgaacagaaactg atcagcgaagaagatctgaactaggatccg.

Before introducing single cysteine substitutions into the cytosolic SNARE constructs all naturally occurring cysteines had to be removed. The four cysteines in SNAP-25a (positions 84, 85, 90, 92) and the single cysteine in syntaxin 1a (position 145) were

Construct	Vector	Primer	
Syb(1-76)	pET15a	start:	gggattccatatgtcggctaccgctgcc
		stop:	gcgctcgagttactgggaggcccctgcctg
Syb(1-94)	pHO2d	start:	gggattccatatgtcggctaccgctgcc
		stop:	gcgaattccccttgaggttttccacca
Syb(1-96)	pET28a	start:	gggattccatatgtcggctaccgctgcc
		stop:	gcgctcgagttacatcatcttgaggtttttc
Syb(1-116)	pHO2d	start:	gggattccatatgtcggctaccgctgcc
		stop:	gcgaattcccagtgctgaagtaaacgat
Syx(180-253)	pET15a	start:	gcgcatatgggggatcatcatggactcc
		stop:	gcgccctcgagtattatcttggtgtcagacacg
Syx(183-262)	pET28a	start:	gcgcatatggactccagcatctcg
		stop:	gcgctcgagattgcgtgccttgctctggta
Syx(1-179)	pET28a	start:	ggaattccatatgaaggaccgaacccagg
		stop:	gcgccctcgagtattatagaggcaaagatggcg
Syx(1-262)	pET28a	start:	ggaattccatatgaaggaccgaacccagg
		stop:	gcgctcgagattgcgtgccttgctctggta
Syx(254-288)	pHO4d	start:	ccacgccatggccgtcaagtaccagagc
		stop:	gcgaattccctccaaagatgcccccgat
Syx(1-288)	pHO2d	start:	gggattccatatgaaggaccgaacccag
		stop:	gcgaattccctccaaagatgcccccgat
SNAP-25 (1-206)	pET28a	start:	ccatatggctagcatggccgaagacgcgga
		stop:	gtggtgctcgagtcactattattacccactgcccagcatc

Table 1. DNA constructs that were synthesized by a single PCR reaction and corresponding start and stop primers. Target vectors are also listed.

Syntaxin (183-262)	SNAP-25 (1-206)	Synaptobrevin (1-96)	Syntaxin (1-262)
L192C	Q20C	S28C	S59C/S91C
T197C	L33C	S61C	S59C/G105C
N207C	L47C	T79C	S59C/D167C
E224C	V48C		S59C/T197C
S225C	M49C		S59C/N207C
Q226C	H66C		S59C/S225C
E238C	K79C		S91C/D167C
H239C	S84C		S91C/S193C
A240C	S92C		S91C/T197C
V248C	A100C		S91C/N207C
S249C	S115C		S91C/S225C
D250C	S130C		G105C/T197C
Y257C	V153C		G105C/N207C
Q258C	G155C		G105C/S225C
S259C	R161C		T197C/D167C
	T173C		S225C/R151C
	T200C		

Table 2. Cysteine mutants that were used in this study

replaced by serines. In the case of synaptobrevin 2 no changes were necessary since the cytoplasmic domain is free of cysteines. Site directed mutagenesis was performed according to the overlapping primer method of Higuchi (Higuchi, 1990). Cysteine double mutants of syntaxin were generated in two consecutive substitution reactions.

Start and stop primers that were used to synthesize the final PCR constructs are listed in table 1. The overlapping PCR primers that introduced the mutations were 30-40 base pairs long with the cysteine codon positioned in the middle. All cysteine mutants used in this study are listed in table 2. For each of the mutants, correctness of the DNA sequences was confirmed by DNA sequencing.

2.3 Protein Expression and Purification

All proteins that were used in the fluorescence and EPR studies including the cysteine free constructs were expressed from pET28a in the BL21(DE3) strain of *Escherichia coli*. The proteins contained N-terminal His$_6$-tags that served to affinity purify them on nickel-nitrilotriacetic acid-agarose (Quiagen).

Bacteria were picked from single colonies and grown overnight in 100 ml LB (20 μg/ml Kanamycin) at 37°C. Overnight cultures were diluted 1:100 in 1 l LB (20 μg/ml Kanamycin) and grown at 37°C until their optical density at 600 nm reached about 0.8. The cells were induced with 0.8 mM IPTG and incubated at 37°C for 3-4 hours. Then, the bacteria were harvested by centrifugation in a Beckman TY.JS 4.2 rotor for 30 min at 3,800 rpm. The pellets were resuspended in 20 ml resuspension buffer (100 mM Tris, pH 7.4, 500 mM NaCl, 8 mM imidazole) and frozen at −50°C. Frozen bacteria were thawed at 37°C and incubated with lysozyme, DNAse and PMSF for 20 min on ice. Complete lysis occurred by the addition of 6 M urea and subsequent sonification. Bacterial debris was removed by centrifugation in an SLA-1500 rotor (30 min at 12,000 rpm) from Sorvall. Ni-beads were added to the supernatant and incubated for 1 hour at 8°C. The beads were washed with ½ x resuspension buffer, followed by elution with two volumes of elution buffer (500 mM imidazole, pH 7.4, ½ x resuspension buffer). At this point 5 mM DTT and 2 mM EDTA were added. The tags were cleaved off by thrombin. Cleavage occurred over night during concomitant dialysis (50 mM NaCl, 20 mM Tris, pH 7.4, 1 mM DTT). The vector-derived residues GSH (syntaxin, synaptobrevin) and GSHMAS (SNAP-25) remained attached to the N-termini of the fragments. Subsequently, the proteins were purified on a MonoQ (syntaxin and SNAP-25) or MonoS column (synaptobrevin) using the

Äkta Explorer (Amersham Pharmacia Biotech). After loading, the proteins were eluted with a linear gradient of NaCl (Buffer A: 50 mM NaCl, 20 mM Tris, pH 7.4, 1 mM DTT, Buffer B: 1 M NaCl, 20 mM Tris, pH 7.4, 1 mM DTT. Then 3 mM DTT were added. The fractions were analyzed by SDS-PAGE and Coomassie staining. The peak fractions were pooled. Proteins were determined to be at least 95% pure.

 In the case of munc-18 bacteria were induced for 4 hours at 25°C and 0.5% Triton-x100 was used instead of urea.

Proteins that were used for reconstitution into membranes were purified by Ni^{2+}-Sepharose chromatography only. 1.5 % cholate was included in all buffers. Extraction occurred in the absence of urea.

α-SNAP and NSF were expressed and purified according to (Hanson *et al.*, 1995).

2.4 Complex Formation

Binary and ternary complexes used in the EPR studies were formed overnight with one of the SNARE proteins containing a single cysteine substitution. The nonsubstituted mutants were used in 1.4-2-fold molar excess over the single cysteine proteins. Complex formation was verified by nondenaturing page (binary complexes) or SDS-PAGE (ternary complexes). All single cysteine mutants were quantitatively bound into complexes. Excess protein was separated from complexes by anion exchange chromatography (MonoQ, Amersham Pharmacia Biotech).

Truncated complexes were formed using a 2-fold molar excess of the purified cysteine-free SNAREs over the single cysteine mutant. Complexes were allowed to form overnight on ice and were not further purified.

2.5 Spin Labeling and EPR Measurements

DTT was removed by size exclusion chromatography using PD-10 columns (Amersham Pharmacia Biotech) (equilibration buffer: 20 mM HEPES, pH 7.4, 400 mM NaCl). Immediately thereafter a 10- to 20-fold excess of the cysteine specific spin label (1-oxy-2,2,5,5-tetramethylpyrrolinyl-3-methyl)methanethiosulfonate) was added and allowed to react for at least 90 min at 25°C. In the case of buried residues, incubations took place for up to 5 h. Unreacted label was removed by size exclusion chromatography (PD-10 columns). Proteins were concentrated in Microcons (Amicon) and subsequently diluted with sucrose (final buffer: 30% sucrose (w/w), 240 mM NaCl. 20 mM Tris, pH 7.4). The

protein concentrations ranged from 50 to 100 µM. 30% sucrose was used to reduce the rotational correlation time of the monomeric and oligomeric proteins.

EPR spectra were obtained using a Bruker EMX spectrometer. All spectra were recorded at 2 mW incident microwave power using a field modulation of 1.5 G at 100 kHz. Unless noted otherwise, the spectral breadth was 100 G. for the determination of spin-spin interactions of syntaxins in binary complexes labeled and unlabeled syntaxin (1:1 molar ratio) were combined and subsequently mixed with an equal molar amount of SNAP-25 (due to the 2 (syntaxin): 1 (SNAP-25) stoichiometry of the binary complex SNAP-25 was used in 2-fold excess). Complex formation occurred overnight on ice.

2.6 Multiangle Laser Light Scattering

Size exclusion chromatography was performed on a Superdex 200 HR-10/30 column (Pharmacia) in 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM DTT, and 1 mM EDTA at a flow rate of 0.5 ml/min. The elution profiles were monitored by UV absorption at 280 nm, light scattering at 633 nm, and differential refractometry. Light scattering and refractometry were carried out using the Dawn and Optilab instruments from Wyatt Technology Corp. Analysis was carried out using the Astra software (Wyatt, 1993). For each sample 200 µl of protein solution were loaded. The dn/dc value (change of refractive index with respect to a change in concentration of the molecules being investigated) is fairly constant for proteins and was set to 0.185 for the analysis of the light scattering data.

2.7 Protein Labeling with Fluorescent Dyes

DTT was removed from the proteins by gel filtration using PD-10 columns and PBS as elution buffer (10.4 mM Na_2HPO_4, 3.2 mM KH_2PO_4, 123 mM NaCl). Then, a 10-fold excess of Alexa 488- or Alexa 594-maleimide was added. When both dyes were used at the same time acceptor was added in 30-fold excess and donor in 10-fold excess. This ratio of acceptor versus donor was necessary to compensate for the different binding rates of the dyes. After 3-5 hours on ice, the reactions were stopped by adding 10 mM DTT. The proteins were concentrated in Microcons (YM-10) and again applied on PD-10 columns to remove excess dye.

The proteins were dialyzed overnight against PBS buffer containing 1 mM DTT and SM-2 Biobeads (BioRad) to bind any residual label. The dialyzing tubes had a molecular weight cut off of 12,000-16,000 (Spectra/Por). The proteins were run on 15% SDS gels and

documented with an LAS-1000 reader (Fujifilm) using the appropriate filter sets (HQ470/40x (Cy2) Exiter / HQ525/50m (Cy2) Emitter, and   HQ545/30x (Cy3) Exiter / HQ610/75m (Cy3) Emitter). All labeled proteins were more than 95% pure and free of unincorporated label.

2.8 Absorption Spectroscopy

The labeling efficiencies were determined from the absorption spectra, according to Lambert Beer. The extinction coefficients were used as given by the provider (ε(Alexa 488)$_{493nm}$ = 72,000 M^{-1}cm^{-1}, ε(Alexa 594)$_{588nm}$ = 96,000 M^{-1}cm^{-1})).

All measurements were carried out with a Shimadzu (UV-2401 PC) spectrophotometer.

For the orientation study of binary complexes 1.32 µM labeled binary complex (position 197) was reacted for 1 h with 13.2 µM synaptobrevin. Absorption spectra were recorded of either binary complex alone, or after adding synaptobrevin.

2.9 Fluorescence Anisotropy

All measurements were carried out in a Fluoromax fluorometer (Jobin Yvon) with automated polarizers (Model: 1971). Excitation and emission wavelengths were 488 nm and 522 nm, respectively. The slitwidths were set at 7 nm and the integration time took 1 sec. The G-factor was determined according to $G = I_{HV}/I_{HH}$, where I_{HV} and I_{HH} are the fluorescence intensities of the vertically and horizontally polarized emissions when the sample is excited with horizontally polarized light. After measuring the intensities of the vertically and horizontally polarized emissions upon vertically polarized excitation (I_{VV} and $I_{VH,}$ respectively) the anisotropy values were computed automatically by the software according to following relationship: $r = (I_{VV}-GI_{VH})/(I_{VV}+2GI_{VH})$ (Lakowicz, 2000). The temperature was kept at 25°C throughout the experiment. All assembly reactions took place in polystyrol cuvettes from Sarstedt in a final volume of 3 ml.

SNAP-25, labeled at position 84 (SN84) with Alexa488-maleimide was added to preheated (25°C) buffer (PBS, 0.1 mg κ-Casein/ml, 0.1 %Triton-x100). After 10-20 measuring points were taken (~ 5-10 min) synaptobrevin and either syntaxin (1-262), syntaxin (180-262) or syntaxin/L165A/E166A (1-262) were added.

The final concentration of SNAP-25 was always 5 nM. Synaptobrevin and syntaxin were used at equal concentrations that increased from 250 nM up to 4 µM, depending on the reaction studied.

In a second set of experiments 5 nM syntaxin (183-262) or syntaxin (1-262), both labeled with Alexa488-maleimide at position 249, were incubated with increasing concentrations of synaptobrevin and SNAP-25. Otherwise, the conditions were as above.

Evaluation:

The anisotropy values taken prior to complex formation were averaged and set to zero. Each curve was fitted by a monoexponential rise function, according to pseudo first order reaction kinetics. The individual pseudo first order rate constants of each set of experiments were plotted against the concentration of syntaxin/synaptobrevin in the case of SN84 or synaptobrevin/SNAP-25 in the case of Syx249. The rise of the curves yielded the second order rate constants.

2.10 Single Molecule Measurements

Complex Formation:

In all measurements PBS buffer was used that was stored on charcoal.

For ternary complex formation a 50-fold molar excess of synaptobrevin and SNAP-25 was applied. The proteins were allowed to associate overnight on ice.

For the formation of complexes between munc-18 and syntaxin, munc-18 was used in 10-fold molar excess. Again, complex association occurred overnight on ice. Completion of the assembly reactions was monitored either with SDS-PAGE in the case of ternary complexes, or native gel electrophoresis in the case of syntaxin/munc-18 complexes. The samples were diluted to a final concentration of 10-100 pM. To prevent dissociation of munc-18, the dilution buffer contained 2 µM munc-18 that was dialyzed against PBS/charcoal buffer to reduce contaminating noise.

Measurements:

A drop encompassing 10 µl was placed into the measuring chamber of the confocal microscope. To prevent surface attachment cover slides were pretreated with κ-Casein in PBS-buffer (1 mg protein/ml). The setup in Fig 4 was used in all measurements. The fluorescent molecules were excited by an actively mode locked argon-ion laser operated at 476.5 nm (Sabre, Coherent, Palo Alto, CA, USA; APE, Berlin, Germany). The excitation pulses have pulse widths of 190 ps and a repetition rate of 73 MHz. A dual-band dichroic

mirror separates the incoming excitation wavelength from the outgoing fluorescence signal (488/594PC, AHF Analysentechnik, Tübingen, Germany). The objective is a 60x water immersion lens (Olympus, UplanApo, 60x, 1.2 N.A). The mean excitation intensity within the detection volume is 28 kW/cm2 (at 476.5 nm). The detection volume of approx. 2 fl is calculated from fluorescence correlation spectroscopy (FCS) measurements. It is restricted by pinhole size (diameter = 100 μm) and the beam waist ω_o (= 0.56 μm). Further setup parameters obtained via FCS are the ratio ω_0/z_o of 4, and the characteristic diffusion time τ_D of the labeled proteins (1 ms). Here, ω_o and z_o are the distances from the center of the laser beam focus, in the radial and axial directions respectively, at which the collected fluorescence intensity has dropped by a factor of e^2 compared to its peak value. The fluorescence signal is divided into its parallel and perpendicular components with respect to the linear polarized excitation beam by a polarizing beam splitter cube (VISHT11 Gsaenger, Planegg, Germany), which is then subsequently divided into yellow-green and red fluorescence components by dichroic mirrors. (DB:595 DCRX, AHF) resulting in four signal paths. Band pass filters (IF:HQ535/50 and HQ650/75) are used in the final step to separate the signal in spectral region from background. Four avalanche photodiodes (APD) (AQR 14, EG & G Vandreuil, Quebec, Canada) are used to monitor the photons generating a TLL pulse for each. A PC-BIFL-card (SPC431, Becker & Hickel GmbH Berlin, Germany) registers for each event the arrival time after the laser pulse, the inter-photon time and the detector channel in which the photon arrived (corresponding to spectral range and polarization).

The parameters fluorescence intensity, lifetime, anisotropy and their spectral range are determined according to (Eggeling *et al.*, 2001a).

2.11 Data Analysis of Multi Parameter Single Molecule FRET

The intensities of a single burst measured in kHz are determined by dividing the number of registered events by its individual burst duration time. The fluorescence intensity of the donor, F_D, and the acceptor, F_A, is calculated by

$$\frac{F_D}{F_A} = \frac{g_R F_G}{g_G F_R} = \frac{g_R}{g_G} \frac{S_G - B_G}{\left(S_R - B_R\right) - \alpha\left(S_G - B_G\right)}$$

The net fluorescence signal, F_G and F_R, is obtained by subtraction of the background signal, B_G (= 7.0 kHz) and B_R (= 2.5 kHz) (including direct excitation of the acceptor), from the total signal, S_G and S_R measured by the green and red detectors. Furthermore, the spectral cross talk, α(= 0.035), given by the ratio of the donor signal registered in the red and green detectors, and the different detection efficiencies, g_G (= 0.032) and g_R (= 0.020), of the green and red detectors must be taken into account.

Based on error propagation rules, the standard deviation of the fluorescence ratio F_D/F_A is given by

$$\sigma\left(\frac{F_D}{F_A}\right) = \frac{g_R F_G}{g_G F_R}\left[\left(\frac{1}{F_G} + \frac{\alpha}{F_R}\right)^2 (S_G - B_G) + \left(\frac{1}{F_R}\right)^2 (S_R + B_R)\right]^{1/2}$$

The donor-acceptor distance, R_{DA}, can be calculated directly from F_D/F_A

$$R_{DA} = R_{0r}\left[\frac{F_D}{F_A}\Phi_{FA}\right]^{1/6}$$

Φ_{FA} (= 0.7) is the fluorescence quantum yield of the acceptor.
R_{0r} (= 59.1 A) is the reduced Förster Radius, which in contrast to the traditional Förster radius R_0, is independent of the donor fluorescence quantum yield

$$R_{0r} = \frac{R_0}{\sqrt[6]{\Phi_{FD}}} = 9780\left(J(\lambda)\kappa^2 n_D^{-4}\right)^{1/6}$$

where $J(\lambda)[M^{-1}cm^3]$ is the spectral overlap integral between donor and acceptor, Φ_{FD}(= 0.62) is the fluorescence quantum yield of the donor, n(= 1.33 for water) is the refractive index, and κ^2(=2/3) is an orientation factor that accounts for the relative orientation of the donor emission dipole to the acceptor excitation dipole.

The standard deviation of the distance R_{DA}, referred to as shot noise, is given by

$$\sigma(R_{DA}) = \frac{1}{6} R_{0r} \left[\frac{F_D}{F_A} \Phi_{FA} \right]^{1/6} \left[\left(\frac{1}{F_G} + \frac{\alpha}{F_R} \right)^2 (S_G - B_G) + \left(\frac{1}{F_R} \right)^2 (S_R + B_R) \right]^{1/2}$$

with definitions as above.

2.12 Fluorescence Correlation Spectroscopy

The intensity fluctuations of diffusing particles through the open volume element can be described by the normalized autocorrelation function ($G_D(t_c)$) (Aragon & Pecora, 1976):

$$G(t_c) - 1 = G_D(t_c) = \frac{1}{N} \left(\frac{1}{1 + t_c/\tau_D} \right) \left(\frac{1}{1 + (\omega_0/z_0)^2 \, t_c/\tau_D} \right)^{1/2}$$

where τ_D is the characteristic diffusion time ($\tau_D = \omega_0^2/4D$, with ω_0 being the radius and D the translational diffusion coefficient), N is the average number of fluorescent molecules in the sample volume, $2z_0$ is the effective length of the sample, and t_c is the correlation time.

In single molecule spectroscopy high laser intensities can cause a distortion of the FCS curves. Often, intensity fluctuations are not only due to the diffusion of the fluorescent molecules in and out of the laser beam but also due to triplet state kinetics ((Widengren, Rigler & Mets, 1994), and (Widengren, Mets & Rigler, 1995)), photodestruction (Mathies & Peck, 1997), or other phenomena like bond isomerizations and dye interactions with the protein

For the analysis of the donor/acceptor-island of Syx91/225 three bunching terms were introduced to describe the curves:

$$G(t_c) = 1 + \frac{G_D(t_c)}{(1 - A - B - C)} \times \left[1 - A - B - C + A \, \exp(-t_c/t_{rA}) + B \, \exp(-t_c/t_{rB}) + C \, \exp(-t_c/t_{rC}) \right]$$

with A, B, and C being amplitudes and t_{rA}, t_{rB} and t_{rC} corresponding relaxation times.

First, the diffusion times for green and red autocorrelation were determined with the last term set at zero. Then, the mean diffusion time from both autocorrelations was kept fixed and the anticorrelation term obtained.

2.13 Preparation of Proteoliposomes

Lyophilized lipids were solubilized in chloroform/methanol, 2:1 (v/v) immediately before use. The following mixture was prepared for reconstitution (molar ratios): cholesterol (1), phosphatidylserine (1), phosphatidylinositol (1), phosphatitylethanolamine (2), phosphatidylcholine (5), and rhodamine phosphatidylethanolamine as tracer (0.05). The lipid mixture was dried on a rotary evaporator and resuspended in cholate buffer (20 mM Tris (pH 7.4), 120 mM NaCl, 1 mM dithiothreitol (DTT), 5 % cholate (w/v)) at a detergent to lipid molar ratio of 8:1. Then, equal volumes of SNARE proteins (containing 1.5 % cholate) were added, with a final lipid:protein molar ratio of approximately 1000:1. When different SNARE proteins were to be co-reconstituted they were used at a protein:protein molar ratio of 1:1. Proteoliposomes were formed by detergent removal using size exclusion chromatography on Sephadex G-50 S (volume ratio sample:column 1:35).

2.14 Detergent Assisted Insertion

Proteoliposomes containing recombinant synaptobrevin were incubated for 45 min at room temperature with constant concentrations of recombinant syntaxin and increasing concentrations of octylglucoside. The approximate molar ratios of synaptobrevin:syntaxin were 2:1. For this experiment we used syntaxin that was purified in the absence of detergent. No precipitation of the protein was observed.

Synaptobrevin proteoliposomes that were cleaved with TeNT light chain were sedimented by ultracentrifugation (85,000rpm for 30 min in a Beckman TlA 100.3-rotor).

2.15 Assembly of SNARE Complexes between Different Membranes

Recombinant SNAP-25 (6 µM) was incubated on ice for 30 min either in the presence of Fab fragments of Cl. 71.1 (12 µM), Cl. 71.2 (12 µM), or buffer (control). A mixture with equivalent amounts of synaptobrevin (1.5 µM) and syntaxin (1 µM) containing liposomes was subsequently added (total volume 196 µl). After 90 min incubation on ice, 0.5 ml buffer were added, and the liposomes were sedimented for 30 min at 200,000 x g_{av} using a Beckman Tla 100.3 rotor. The liposomes were washed once with 1 ml buffer and resedimented. The pellets were resuspended in 32 µl SDS sample buffer (62.5 mM Tris, pH 6.8, 4% SDS, 10% sucrose, 5% β-mercaptoethanol, 0.001% bromophenolblue). Seven microliters of boiled or nonboiled sample were loaded per lane.

Similar results were obtained when the centrifugation steps at the end of the incubation were omitted.

2.16 Disassembly of co-reconstituted cis-SNARE Complexes

SNARE complexes in liposomes (0.7 µM) or in solution without membrane anchors (3 µM) were disassembled for 40 min at 30°C. The buffer (10 mM Tris pH 7.4, 120 mM NaCl) contained 2.5 mM ATP, 2 mM $MgCl_2$, 12 µM α-SNAP, 1 µM NSF. As a control 10 mM EDTA replaced the Mg^{2+}. The reactions were stopped by addition of SDS sample buffer. The samples were then analyzed by SDS-PAGE and immunoblotting.

2.17 Other Methods

SDS-PAGE and immunoblotting was performed according to standard procedures ((Laemmli, 1970), and (Kyhse-Andersen, 1984)).

Circular dichroism measurements were carried out as described (Fasshauer *et al.*, 1999).

Cleavage by TeNT was performed as in (Otto *et al.*, 1997). Monoclonal antibodies used for detection include HPC-1 for syntaxin (Barnstable, Hofstein & Akagawa, 1985), Cl 69.1 for synaptobrevin 2 (Edelmann *et al.*, 1995), commercially available from Synaptic Systems (Göttingen, Germany) and monoclonal antibody for c-myc (cell line obtained from American Tissue Culture Company). Secondary antibodies coupled to horseradish peroxidase and alkaline phosphatase were purchased from Sigma. Immunoblots were developed using either an Enhanced Chemiluminescence Kit from Pierce (Super Signal), or where indicated, a combination of nitro blue tetrazolium (0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/ml). Protein concentrations were determined according to Bradford (Bradford, 1976).