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Single Molecule Measurements of Interaction Free Energies Between the Proteins Within Binary and Ternary SNARE Complexes

W. Liu^{1,4,†,‡}, Vedrana Montana^{2,3,†,‡}, Vladimir Papura^{1,2,3,4,*†}, and U. Mohideen^{1,4,*}

¹Department of Physics and Astronomy, University of California, Riverside, CA 92521, USA

²Department of Cell Biology and Neuroscience, University of California, Riverside, CA 92521, USA

³Centers for Glial $\leftrightarrow$ Neuronal Interactions, University of California, Riverside, CA 92521, USA

⁴Nanoscale Science and Engineering, University of California, Riverside, CA 92521, USA

Abstract

We use an Atomic Force Microscope based single molecule measurements to evaluate the activation free energy in the interaction of SNARE proteins syntaxin 1A, SNAP25B and synaptobrevin 2 which regulate intracellular fusion of vesicles with target membranes. The dissociation rate of the binary syntaxin-synaptobrevin and the ternary syntaxin-SNAP25B-synaptobrevin complex was measured from the rupture force distribution as a function of the rate of applied force. The temperature dependence of the spontaneous dissociation rate was used to obtain the activation energy to the transition state of 19.8 ± 3.5 kcal/mol = $33 \pm 6 k_B T$ and 25.7 ± 3.0 kcal/mol = $43 \pm 5 k_B T$ for the binary and ternary complex, respectively. They are consistent with those measured previously for the ternary complex in lipid membranes and are of order expected for bilayer fusion and pore formation. The ΔG was 12.4–16.6 kcal/mol = 21 – $28 k_B T$ and 13.8–18.0 kcal/mol = 23 – $30 k_B T$ for the binary and ternary complex, respectively. The ternary complex was more stable by 1.4 kcal/mol = $2.3 k_B T$, consistent with the spontaneous dissociation rates. The higher adhesion energies and smaller molecular extensions measured with SNAP25B point to its possible unique and important physiological role in tethering/docking the vesicle in closer proximity to the plasma membrane and increasing the probability for fusion completion.

Keywords

AFM (Atomic Force Microscope); Free Energy; Single Molecule; SNARE Proteins

Introduction

Intracellular fusion of vesicular and target membranes is facilitated by a complex assembly of proteins in which the SNAREs are thought to play the central role (Brunger, 2005; Fasshauer et al., 2002; Jahn et al., 2003; Rothman, 2002; Sollner et al., 1993). The best studied are the neuronal fusion processes, which are mediated by v- and t-SNAREs corresponding to the vesicle and target membrane, respectively. The v-SNARE is a single membrane spanning polypeptide chain called Synaptobrevin (Sb) which is also referred to as VAMP for Vesicle

*Authors to whom correspondence should be addressed.

[†]Present address: Department of Neurobiology, University of Alabama, Birmingham, AL 35294, USA.

[‡]W. Liu and Vedrana Montana contributed equally to this work.

Associated Membrane Protein. The t-SNAREs consist of two proteins, a membrane spanning protein Syntaxin (Sx) and SNAP25 (synaptosome associated protein of 25 kDa) which is attached to the membrane by multiple palmytoylations. The formation of the SNARE complex, which is thought to precede vesicle-membrane fusion, is conjectured to happen in the following sequence: First Sx and SNAP25 are assumed to form an intermediate binary complex at the plasma membrane which then interacts with Sb located on the vesicle (Sudhof et al., 1993). X-ray structural studies (Sutton et al., 1998) of the ternary SNARE complex reveal that it is arranged as a 12 nm long cylinder with all four α -helices (one each from Sx 1A and Sb 2 and two from SNAP25) arranged in parallel with all the N-termini at one end and all the C-termini at the other. The complex is held together by primarily hydrophobic bonds and an ionic interaction in the interior of the core (Fig. 1). The hydrophobic residues (leucine, isoleucine and valine) interact in a plane almost perpendicular to the axis of the cylinder, similar to that observed for parallel, tetrameric leucine-zipper proteins (Sutton et al., 1998). Sandwiched between the hydrophobic leucine zipper layers (8 preceding and 7 following) there is a highly conserved ionic bond in the "0" layer composed of Arg 56 from Sb2, Gln226 from Sx 1A, Gln 53 and Gln 174 from SNAP25. This ionic layer provides registry for the alignment of the complex. The surrounding leucine zipper layers provide a water tight seal and increase the electrostatic interaction. Thus the exposure to water from breaking the preceding hydrophobic seal will cause the disassembly of the ternary complex. Consistent with this model we have previously measured an extension of 12.5 ± 0.4 nm for the rupture of the ternary SNARE complex (Liu et al., 2006).

In addition to the ternary complex, the conserved amino acid motifs in the transmembrane domains of Sx and Sb, lead to the formation of heterodimer coiled-coils in detergent micelles and membranes (Laage et al., 2000; Margittai et al., 1999). Studies of SNARE mediated exocytosis using purified recombinant proteins and artificial membranes (Bowen et al., 2004) or large dense core neurosecretory granules (McNally et al., 2004; Woodbury and Rognlien, 2000) found that tethering/docking and fusion occurred due to the interaction of Sx and Sb alone, in the absence of SNAP25. *In vitro* studies using the single vesicle methodology (Liu et al., 2005) have reported that with Sx only bilayers lacking SNAP25, resulted in tethering/docking and fusion of Sb containing vesicles with similar rate constants as those for bilayers containing SNAP25. Additionally, investigations using SNAP25 knockout mice have shown that vesicle tethering/docking and spontaneous fusion persisted although Ca^{2+} triggered exocytosis was abolished (Washbourne et al., 2002). Fluorescence Resonant Energy Transfer (FRET) studies measured (Bowen et al., 2004) the presence of the parallel (N-terminal to N-terminal) aligned Sx-Sb binary complex. Formation of Sx-Sb heterodimers have also been observed in NMR (Hazzard et al., 1999) and single molecule experiments using the AFM (Liu et al., 2006; Yersin et al., 2003). Additionally, based on the above evidence it appears that Sx and Sb alone might be sufficient to bring about tethering/docking and fusion of the vesicle to the membrane. However the X-ray structure of this complex is yet to be obtained and may prove extremely difficult. Thus a measurement of the interaction energy of this heterodimeric complex and its comparison to the heterotrimeric complex is a key step in providing a full understanding of their individual roles in vesicular tethering/docking at the plasma membrane.

The assembly of the SNARE complex (zippering of the coiled-coils) is thought to be the key step in tethering/docking of the vesicle at a critical distance from the plasma membrane, and providing the energy to overcome the initial repulsion between the two membranes, which eventually leads to fusion (Brunger, 2005; Dennison et al., 2006; Giraudo et al., 2005; Hanson et al., 1997; Hayashi et al., 1994; Hu et al., 2003; Jahn et al., 2003; Lin and Scheller, 1997; Lu et al., 2005; Reese et al., 2005; Rothman, 2002; Schaub et al., 2006; Weber et al., 1998). This conclusion was based on investigations of the stability of neuronal SNARE complexes (Chernomordik and Kozlov, 2005; Giraudo et al., 2005; Hanson et al., 1997; Hayashi et al., 1994; Hu et al., 2003; Lin and Scheller, 1997; Lu et al., 2005; Reese et al., 2005; Schaub et al.,

2006; Weber et al., 1998) and also on studies, where the SNARE proteins were shown to be sufficient to drive fusion when they were expressed in synthetic liposomes or on the surface of cells (Dennison et al., 2006; Hu et al., 2003; Weber et al., 1998). The vesicle tethering and docking to within 1 nm of the plasma membrane (Niles et al., 1996) requires that the energy supplied by the SNARE assembly be used to overcome the net effect of electrostatic repulsion, van der Waals attraction and, entropic and water based repulsive hydration forces (Marra and Israelachvili, 1985; Niles et al., 1996). Based on typical vesicle contact diameters of 10 nm the required energy for tethering the vesicle can be estimated to be of order 5–10 $k_B T$ (Marra and Israelachvili, 1985; Niles et al., 1996).

Theoretical modeling of membrane fusion suggests that it advances in 3 stages (Markin and Albanesi, 2002). The role of the SNARE proteins is not incorporated into these models. First, the *cis* monolayers on the opposing membranes form an hour glass shaped connection called a stalk. Due to opposite curvatures along the two axis of the saddle shaped stalk, it is stress free and the energy for its creation is thought to be minimal (Markin and Albanesi, 2002). In the next step often referred to as hemifusion, the trans monolayers form curved surfaces around the stalk with a flat diaphragm like single bilayer segment in the middle, bounded by the curved regions of the stalk at the edges (Chernomordik and Kozlov, 2005). For typical membrane curvatures of 20 nm radius lead to energy estimates of 13 $k_B T$ for the formation of the stalk and hemifusion stages (Markin and Albanesi, 2002). Finally, the fusion process is completed by pore formation, requiring an energy barrier estimated to be about 46 $k_B T$ (Markin and Albanesi, 2002). Larger energy barriers >50 $k_B T$ for this last step of growth of the pore have also been estimated (Cohen and Melikyan, 2004). Thus, studies of intermediate states of SNARE complex assembly are valuable because they are central to models of the regulation of membrane fusion.

In the last few years three groups (Giraudo et al., 2005; Lu et al., 2005; Reese et al., 2005; Schaub et al., 2006) have shown that SNARE proteins are involved in the hemifusion process. The role of SNARE assembly in bringing about hemifusion has been reported in cell free fusion of yeast vacuoles (Reese et al., 2005). Single cell–cell assays have shown that replacing the transmembrane domain of Sx and Sb leads to the termination of the membrane–membrane interaction in the hemifusion state (Giraudo et al., 2005). Studies which separately monitored the outer leaflet and the inner leaflet mixing, using fluorescently labeled protein free liposomes reconstituted with Sb, with liposomes containing Sx and SNAP25, concluded that mostly sequential fusion occurred on mixing with hemifusion (outer leaflet mixing) dominating at short time scales of 1 min leading to 88% of the liposomes demonstrating complete fusion (inner leaflet mixing) at 150 min (Lu et al., 2005). Using the same assay, it has been shown (Schaub et al., 2006) that an additional 14–20 kDa protein complexin arrests fusion between the above liposomes at the hemifusion state which however can be relieved by synaptotagmin in the presence of calcium. Regarding the last fusion step of pore formation there is limited evidence of the role of SNARE molecules, which were observed to form of ring like structures in ternary SNARE reconstituted bilayers (Cho et al., 2005). Thus the adhesion energies of the SNARE complex might probably be used to overcome only the repulsive interaction of the bilayers and bring the vesicles within a 1 nm of the plasma membrane and lead to hemifusion. The last step of pore formation might require the presence of additional proteins such as complexin and synaptotagmin (Brunger, 2005; Jahn et al., 2003; Rothman, 2002).

Recently a measurement of the interaction energies of the ternary SNARE complex of Sx 1A, Sb 2 and SNAP25 was made using a surface force apparatus (Li et al., 2007). Here the interactions forces between two phospholipids covered mica cylinders, one containing Sb 2 and the other containing complexes of Sx 1A and SNAP25 were measured with a resolution of 1 μN . The forces were modeled using polymer theory to convert them to adhesive energies per unit area of the cylindrical surface. They observed a large range of adhesive energies (forces

corresponding to that between 3 to 20 mN/m) in repetitions of the experiment which was attributed to differences in the densities and therefore differences in the number of the interacting SNARE proteins. Based on the modeling of the number of interacting SNAREs, the average binding energy per complex was calculated to be $35 \pm 7 k_B T$ (Li et al., 2007). In these experiments, the authors report that SNARE complexes were not fully assembled at their membrane proximal C termini and therefore the measured energies are expected to correspond not to the fully assembled SNARE complex but to an intermediate where 20%–40% of the complex is not complete (Li et al., 2007).

Here we report the measurement of the interaction free energies between the heterodimer (binary) complex Sx1A and Sb2 and the heterotrimer (ternary) complex Sx1A-SNAP25B-Sb2 using the Atomic Force Microscopy (AFM) based single molecule techniques (Florin et al., 1994; Marszalek et al., 1999; Merkel, 2001; Oesterhelt et al., 2000; Schwesinger et al., 2000). These SNARE proteins have been subject to previous single molecule investigations using the AFM (Abdulreda et al., 2008; Liu et al., 2006, 2008; Yersin et al., 2003) and Fluorescence Resonance Energy Transfer (FRET) (Bowen et al., 2004, 2005; Weninger et al., 2003). Recently (Liu et al., 2008), we showed that the Jarzynski Equality (Jarzynski, 1997; Liphardt et al., 2002) is a viable method for estimating the binding energy for a pair of interacting single molecules using the case of the binary SNARE system as the standard for comparison. In this report the spontaneous dissociation rates of the binary and ternary complexes are measured using dynamic force spectroscopy where the mechanical properties of the single molecule bonds are studied as a function of the rate of applied force. The temperature dependence of the spontaneous dissociation rate is used to obtain the energy to disassemble the complex from single molecule reaction rate theory. From this the adhesion enthalpy, entropy and free energy are obtained and compared for the binary and ternary interactions of the SNARE complex. The adhesion energy of the two complexes is compared to that required for vesicle membrane fusion. The difference in the adhesion free energy is shown to be consistent with the differences in the spontaneous dissociation rates.

Material and Methods

Protein Synthesis

All plasmids used in this study were kindly provided by Dr. Edwin R. Chapman, University of Wisconsin, Madison, WI. Modified pET vectors were used to generate Sb2 and Sx1A recombinant proteins with their cytoplasmic domains (rat sequence aa1-94 for Sb2 and aa1-266 for Sx1A) tagged with six histidines (H6) at their C-termini (Liu et al., 2006). After purification using nickel-sepharose beads (Qiagen), the proteins were quantified using the Bradford reagent (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as a standard. Recombinant full-length rat SNAP25B was generated using pGEX-2T vector and expressed as a fusion protein having glutathione-S-transferase (GST) at its N-terminus. The resulting fusion protein GST-SNAP25B was purified using glutathione columns (Amersham Biosciences, Piscataway, NJ), and quantified as above. Protein purity was assessed by subjecting recombinant proteins to 15% SDS-PAGE in combination with the silver stain technique. The purified recombinant proteins were found to be 84%–97% of the total protein content as measured through densitometry of silver stained gels, performed using ChemiDoc™ XRS gel documentation system (BioRad Laboratories, Hercules, CA). For SDS-PAGE analysis followed by Western blotting, the proteins were loaded at 1 μ g per lane and then transferred to nitrocellulose membranes. The membranes were then tested using monoclonal antibodies against Sx1 (clone 78.2, Synaptic Systems, cat No. 110 001, 1: 10000 dilution or clone HPC-1, Sigma-Aldrich, cat No. S0664, 1:1000 dilution), Sb2 (clone 69.1, Synaptic Systems, Goettingen, Germany, cat No. 104 201, 1:1000 dilution; note that this product has been recently replaced by the manufacturer with cat No. 104 211) and SNAP25 (clone 71.1, Synaptic Systems, cat No. 111

001, 1: 10000 dilution). Enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) was used to detect the single immunoreactive bands with appropriate molecular weights for all proteins.

Surface Functionalization

First nickel films (thickness ~150 nm) were thermally evaporated on triangular silicon nitride cantilevers (320 μm long) with pyramidal tips and glass coverslips (Fisher Scientific; cat # 12-545-82-12CIR-1D). After partial film oxidation in air, the cantilever tips were functionalized with recombinant proteins by incubating them in a solution containing proteins for 3 hours at room temperature. The nickel coated glass coverslips were functionalized with recombinant proteins for 1 hour at room temperature. After incubation with the recombinant proteins, the tips and coverslips were rinsed three times with internal solution, and then kept separately submersed in internal solution in a humidified chamber at +4 °C. The internal solution contained (in mM): potassium-gluconate, 140; NaCl, 10, and HEPES, 10 (pH = 7.35). Prior to experiments the glass coverslips were mounted onto AFM sample holders. In experiments on the ternary complex, a solution containing GST-SNAP25B was applied onto the Sx1A functionalized tips for 10 minutes at room temperature, followed by triple wash with internal solution. The cantilever tips and coverslips were used in experiments within 6 hours of functionalization. The Ni^{2+} -H6 sterical coordination followed here for the attachment of the proteins has negligible extensions and is comparatively rigid; the measured mean rupture force of 525 ± 41 pN for the Ni^{2+} -H6 bond exceeds any of the SNARE protein rupture forces studied here (Liu et al., 2006).

Indirect immunocytochemistry was used to confirm the presence of Sx1A/SNAP25B on the functionalized cantilever tips and Sb2 on the functionalized glass coverslips, as described previously (Liu et al., 2006). Mouse monoclonal antibodies were used against Sx1A (clone HPC-1, 1:500) and Sb2 (1:500). In experiments where SNAP25B was complexed onto Sx1A functionalized tips, SNAP25B was probed with a rabbit polyclonal antibody (clone MC-21; 1:200) generously provided by Dr. Pietro DeCamilli (Yale University, New Haven, CT). The cantilevers and coverslips were incubated with the primary antibodies for 1 hour at room temperature, which was then followed by a triple wash with internal solution. After this, the TRIC-conjugated goat anti-mouse or Alexa Fluor® 488-conjugated goat anti-rabbit (Molecular Probes) antibodies were applied and incubated for 1 hour at room temperature, and followed by a triple washout in internal solution. An inverted microscope (Nikon TE 300) equipped with wide-field epifluorescence (Opti-Quip, Highland Mills, NY; 100-W xenon arc lamp), standard rhodamine/TRITC and fluorescein/FITC filter sets (Chroma Technology, Brattleboro, VT), and CoolSNAP-HQ cooled charge-coupled device (CCD) camera (Roper Scientific, Tucson, AZ) driven by V++ imaging software (Digital Optics, Auckland, New Zealand) was used for the visualization.

Dynamic Force Spectroscopy

An Atomic Force Microscope (Nanoscope E, Digital Instruments, Santa Barbara, CA) was used in force spectroscopy mode. The experiments were carried out in a fluid cell that maintained the hydration and osmotic properties of the sample. The spring constants of the cantilevers ranging from 10 to 16 mN/m, were measured using their thermal spectrum (Hutter and Bechhoefer, 1993). The decrease in the cantilever tip to coverslip distance due to the bending of the cantilever was taken into account in the calculation of the extension. The piezoelectric tube extension, including nonlinearities, was calibrated interferometrically for all the different rate of applied force. The experiments were carried out at 24 °C, 14 °C and 4 °C in temperature controlled rooms containing the entire experimental set-up. Specificity of the interaction between Sx1A and Sb2, was confirmed using the light chain of Botulinum

neurotoxin type B, which cleaves Sb2 and thus reduces the probability of interactions between Sx1A and Sb2 (Liu et al., 2006).

Results

The cytoplasmic tails of Sb2 (amino acids 1-94 of rat Sb2) and Sx1A (amino acids 1-266 of rat Sx1A) with six consecutive histidine molecule (H6) tag at their C-termini and full length rat SNAP25B with glutathione-S-transferase (GST) at its N-terminus (GST-SNAP25B) were used in these single molecule measurements. The ternary interaction between Sx1A-SNAP25B-Sb2 was first studied and the schematic of the experiment is shown in Figure 2(a). The pyramidal tips of the microfabricated AFM cantilevers were nickel coated and exposed to air. Next, the cantilever tips containing Ni^{2+} were functionalized with a recombinant Sx1A-H6 followed by pre-incubation with GST-SNAP25B to form a binary *cis* complex; GST was found to play no significant role in the single molecule interactions of the ternary complex (Liu et al., 2006). Functionalization of the coverslips were done using the same approach, where after coating with nickel and oxidation, they were functionalized with recombinant Sb2-H6.

Next the coverslip and the cantilever were mounted on the AFM (Fig. 2(a)). The Sb2 functionalized coverslips were first attached to the top of the piezoelectric tube. A fluid cell containing internal saline at the room temperature of 24 °C was placed on the coverslip. The Sx1A/SNAP25B functionalized cantilevers were fixed to the top of a fluid cell. The application of voltages to the piezo causes the coverslip to move closer or further away from the cantilever tip. In the AFM, the cantilever bends in response to a force at its tip and this deflection is measured with a laser beam reflected off the cantilever top. The deviation of the reflected laser beam is calibrated to yield the force applied on the proteins attached to the cantilever tip. First the coverslip is moved up using the piezo and the Sx1A/SNAP25B and Sb2 are brought into contact to establish the ternary SNARE complex. The contact time between the proteins and the corresponding contact force are maintained between 0.5–3 s and 0.75–1.2 nN for the range of pulling velocities studied here. Note that for the above forces applied to the proteins on contact, there will be an exponential enhancement in the rate of assembly of the complex by a factor of 10^5 – 10^{11} (Atkins, 1994; Bell, 1978; Evans, 2001; Hummer and Szabo, 2001; Liu et al., 2006; Schumakovitch et al., 2002). Next the coverslip is moved down at a constant velocity of $v = 1.5 \mu\text{m/s}$ (corresponding to a rate of applied force $dF/dt = k * v = 20 \text{ nN/s}$) and the sequence of interactions shown in Figure 2(b) follow, starting with the linear segment *abc* where the tip and coverslip remain in rigid contact as the coverslip is moved down. In the segment *bc*, the tip remains in contact with the coverslip due to non-specific binding; the non-specific nature of this part of measurements was confirmed as we previously described in detail elsewhere (Liu et al., 2006). At point *c* the non-specific bonds are broken and the cantilever immediately jumps to its equilibrium position *d*. The Sx1A-SNAP25B-Sb2 bond remains intact and further downward movement of the coverslip causes the increased application of the force to the molecular system and a corresponding extension as shown by the trace *de* in Figure 2 (b). The increasing molecular extension with the increase in applied force results from the sequential rupture of the bonds. At an extension corresponding to point *e* all the intermolecular bonds are broken and the cantilever snaps back to its equilibrium position *f*. The force corresponding to the length *ef* = 248 pN is the maximum applied force necessary to rupture all the Sx1A-SNAP25B-Sb2 intermolecular bonds. The presence of multiple complexes between the cantilever tip and coverslip can be detected by the recurrence of the trace *def* in the same scan. Here restriction to single molecule events is achieved by controlling the density of the proteins attached to the cantilever and coverslip (Liu et al., 2006). The experiment was repeated and the distribution of rupture forces obtained is shown in Figure 2(c). At this coverslip velocity, the mean value of the rupture force was found to be $256 \pm 11 \text{ pN}$, while the mean value of the extension to rupture all the bonds corresponding to *de* of $12.1 \pm 0.8 \text{ nm}$; both values are consistent with our previous measurements (Liu et al., 2006). Note that the observed

distribution of rupture forces and extensions result due to the thermal energy (Liu et al., 2006) and perhaps partial assembly of the ternary complex in some fraction of the investigated samples. Next the velocity of the downward movement of the coverslip is decreased to $0.16 \mu\text{m/s}$, corresponding to a smaller rate of applied force $dF/dt = 2.4 \text{ nN/s}$ and the experiments are repeated. The corresponding mean value of the rupture force obtained was $186 \pm 9 \text{ pN}$. Similarly, the rupture force distribution for the Sx1A-SNAP25B-Sb2 interaction is measured at other coverslip velocities between $3.8 \mu\text{m/s}$ to $0.04 \mu\text{m/s}$ (rates of applied force ranging from 500 to 60000 pN/s). The mean rupture forces at the various rates of applied force are shown in Figure 2(d) as solid squares. The shift to lower forces at lower rates of applied force can be observed. This feature of dynamic force spectroscopy characterizes the role played by thermal energy and spontaneous dissociation. This decrease in peak of the rupture force distribution can be used to obtain the dissociation rate (Evans, 2001) as is discussed below.

Next the temperature of the complete experimental set-up, including the AFM and the fluid cell, was lowered to 14°C by using a temperature controlled room. As above, the rupture force distribution is again measured at the different rates of applied force. The mean value of the rupture forces at the various rate of applied forces are shown as solid circles in Figure 2(d). Finally, the temperature of the experiment is lowered to 4°C and the measurements are repeated at different rates of applied force ranging from 500 to 60000 pN/s. The corresponding results of the mean rupture force are shown as solid triangles in Figure 2(d).

After measuring the single molecule mechanical response of the ternary Sx1A-SNAP25B-Sb2 interaction, we studied the Sx1A-Sb2 interactions without the presence of SNAP25B as we recently reported (Liu et al., 2008). For these studies, the cantilever tips were functionalized with Sx1A-H6 alone, whereas the coverslips were functionalized with Sb2-H6. The cantilever tips and the coverslips were loaded into the fluid cell and the single molecule experiments described above were repeated first at a temperature of 24°C and a rate of applied force of 21 nN/s . While the rupture forces for SX1A-Sb2 ($252 \pm 10 \text{ pN}$; data not shown, but see Liu et al., 2008) are comparable to those of the Sx1A-SNAP25B-Sb2 ternary complex, the extensions were significantly longer at $22.2 \pm 1.0 \text{ nm}$ (data not shown, but see Liu et al., 2008); both measurements are consistent with our previously reported measurements (Liu et al., 2006) using much larger samples. The rupture forces for this binary trans Sx1A-Sb2 complex were measured (44–54 events) at different rates of applied force from 500 pN/s to 60000 pN/s and at three different temperature set points (24°C , 14°C and 4°C) as described above for ternary complex.

The results of the rupture force distribution can be analyzed using a simple analytical model (Evans, 2001) for the behavior of a bound single molecule system under the application of a force which has been adapted from classical chemical reaction rate theory of Kramers and Eyring. In the absence of any force, the bound system diffuses over the barrier due to its thermal energy. On application of a force, the energy barrier is linearly decreased by the force F to “ $\Delta E - Fx_b$ ”, where, x_b is the width of the barrier given by the projection of the dissociation pathway along the direction of the applied force (Bell, 1978; Evans, 2001). This model predicting a linear decrease of the energy barrier for disassembly with force has been verified in a variety of single molecule ligand-receptor experiments and in the unbinding of proteins (Florin et al., 1994; Marszalek et al., 1999; Merkel, 2001; Oesterhelt et al., 2000; Schumakovitch et al., 2002; Schwesinger et al., 2000). The linear decrease of the barrier for disassembly due to the applied force is valid in the experiments here due to the small value of the cantilever spring constant which is much less than the effective curvature of the barrier for dissociation (Hummer and Szabo, 2001). This decrease in the dissociation barrier due to the applied force F leads to an increase in the dissociation rate K_{off}^F given by (Evans, 2001):

$$K_{\text{off}}^F = K_D * \exp[-(\Delta E - Fx_b)/k_B T] \quad (1)$$

where K_D is the thermal attempt frequency. In the standard analysis, $\Delta E \approx \Delta H$, which is the change in enthalpy to the transition state (48). Then, $K_D = K_a e^{\Delta S/k}$, where $K_a = k_B T/h = 6.2 * 10^{12} \text{ s}^{-1}$ is used for the Eyring model while $K_a = 3.3 * 10^9 \text{ s}^{-1}$ is used for applying the Kramers model of chemical reactions for the interaction of leucine zipper type homodimeric α -coils and the folding of small peptides in solution (Evans, 2001); ΔS is the change in entropy. The most probable rupture forces F_p resulting from Eq. (1) can be written as (Evans, 2001):

$$F_p = \frac{k_B T}{x_b} \left\{ \ln \frac{dF}{dt} + \ln \left(\frac{x_b}{K_{\text{off}}^0 k_B T} \right) \right\} \quad (2)$$

where K_{off}^0 is the spontaneous dissociation rate of the intermolecular bonds when the applied force $F = 0$. For a series of bonds sequentially broken for the “zipper” type system as in the SNARE interaction reported here (Liu et al., 2006), the lifetime K_{off} represents the lifetime for the dissociation of all the bonds.

We can apply the experimental results to Eqs. (1) and (2) of single molecule reaction rate theory to obtain the change in enthalpy ΔH . First, the mean values of the rupture force as a function of the rate of applied force shown in Figure 2(d) can be used with Eq. (2) to obtain the barrier height x_b and the spontaneous dissociation rate K_{off}^0 from the slope and the intercept, respectively. For the Sx1A-Sb2 complex the values of K_{off}^0 are 5.6, 2.5, and 0.51 s^{-1} at the 3 different temperatures 24 °C, 14 °C and 4 °C, respectively. The corresponding values of K_{off}^0 for the ternary SNARE complex made of Sx1A-SNAP25B-Sb2 are 0.48, 0.15, and 0.021 s^{-1} at the 3 different temperatures 24 °C, 14 °C and 4 °C, respectively. Knowing the K_{off}^0 at the three different temperatures, one can use Eq. (1) (setting $F = 0$) to solve for the ΔE . This is done graphically by plotting $\ln(K_{\text{off}}^0)$ versus $(1/T)$ as shown in Figure 3. The K_{off}^0 for the binary Sx1A-Sb2 and the ternary Sx1A-SNAP25B-Sb2 SNARE system are shown as solid circles and squares, respectively. A linear fit is made to the two sets of K_{off}^0 (Marszalek et al., 1999; Schwesinger et al., 2000) and from the slope $\Delta E \approx \Delta H$ is found to be equal to $19.8 \pm 3.5 \text{ kcal/mol} = 33 \pm 6 k_B T$ and $25.7 \pm 3.0 \text{ kcal/mol} = 43 \pm 5 k_B T$ for the binary and ternary complex, respectively. Here, the unit $k_B T$ is defined at 300 K. From the intercepts, the entropy change (ΔS) for the two systems is found and listed in Table 1. In the case of the binary Sx1A-Sb2 interaction, the net $\Delta G = \Delta H - T\Delta S$ can next be calculated and is found to be $16.6 \pm 4.9 \text{ kcal/mol} = 28 \pm 8 k_B T$ when using the Eyring model and $12.4 \pm 4.9 \text{ kcal/mol} = 21 \pm 8 k_B T$ when using the Kramer's model for the entropy. For the case of the ternary Sx1A-SNAP25B-Sb2 interaction, the corresponding values are $18.0 \pm 4.5 \text{ kcal/mol} = 30 \pm 8 k_B T$ and $13.8 \pm 4.5 \text{ kcal/mol} = 23 \pm 8 k_B T$ when using the Eyring and Kramers model, respectively.

Discussion

Comparison to Coiled-Coil Formation Energies

The adhesion energies found above can be compared to those of coiled-coils which have been studied using NMR, Differential Scanning Calorimetry, and Circular Dichroism (Bosshard et al., 2001; Ibarra-Molero et al., 2001). One of the best studied is GCN4-p1, a 33 residue dimeric leucine zipper domain of the yeast transcriptional activator GCN4, where $\Delta G = 12.4 \pm 5.0 \text{ kcal/mol}$ of dimer for the Kramers model and $\Delta G = 18.0 \pm 5.0 \text{ kcal/mol}$ of the dimer for the

Eyring model was found (Ibarra-Molero et al., 2001). These values are consistent with those found for the SNARE complex here. Given the 2 hydrophobic bonds and the heptad repeat pattern in α -coils, this leads to 20 hydrophobic residues involved and thus about 0.6–0.9 kcal per hydrophobic residue of the GCN4-p1 coiled-coil. Note that the polar residues contribute only a negligible energy to the interaction (Mittl et al., 2000; Nozaki and Tanford, 1971; Spek et al., 1998). This value of the interaction energy per residue can be compared to the case of the Sx1A-Sb2 interaction, where based on the longest ~ 23 nm (22.2 ± 1.0 nm in the present study) mean extension to rupture all the bonds and its modification when Sx1A cognate peptides were used (Liu et al., 2006) we can conclude that the entire SNARE domains of the two proteins are involved in a leucine zipper type interaction of two α -helices wound around each other in coiled-coil or superhelix. Such a parallel coiled-coil interaction for the Sx1A-Sb2 interaction was also observed in FRET experiments (Bowen et al., 2004). Note that anti-parallel Sx1A-Sb2 interactions will not be observed in the experiments here as they would result in rupture extensions smaller than 1 nm (as the C-termini of the proteins are fixed to the surfaces) and thus would be disregarded as part of the non-specific bonds (segment *abcd* in Fig. 2b). From Figure 1, this implies that 15 hydrophobic bonds and one electrostatic bond at the “0” layer need to be unwound for rupture of the binary complex. If we assume that the bond energies are shared equally by the 15 hydrophobic bonds, this would lead to an average free energy of $16.6/30 = 0.55$ kcal/mol per residue for the Eyring model and 0.4 kcal/mol per residue for the Kramers model. Note that even though the X-ray structure of the SNARE complex resembles that of the leucine zipper, the distribution of the leucine, isoleucine and valine residues and the geometry of the structure are very different (Sollner et al., 1993) and therefore corresponding deviations in the interaction energies are expected. Another well studied coiled-coil Jun-Iz of 32 residues (d'Avignon et al., 2006) has interaction energies per residue which are less by a factor of 3. The interaction energies between the hydrophobic residues listed in Table 1 for the binary Sx1A-Sb2 and the 4 coil Sx1A-SNAP25-Sb2 ternary complex can be calculated using the tabulated values based on the energy change for the exposure of the non polar residues from water to ethanol (Nozaki and Tanford, 1971). This method yields hydrophobic interaction energies between 1–3 kcal per residue, which are somewhat larger than the ones measured here. The discrepancy is probably due to the fact that this energy evaluation method assumes complete burial of the non-polar residues inside the coiled-coil and no contact with water.

In the case of the Sx1A-SNAP25B-Sb2 ternary interaction, a direct comparison of the 18.0 (Eyring model) – 13.8 (Kramers model) kcal/mol interaction energy cannot be made, due to the lack of energy measurements in similar tetrameric coiled-coils. However, based on the X-ray structure and the measured extension at rupture of 12.1 ± 0.8 nm [note that we previously reported the mean of 12.5 nm in Liu et al., 2006], the 7 layers of hydrophobic interactions preceding the “0” layer have to be ruptured to unbind the complex. Thus a total of $7 * 4 = 28$ hydrophobic bonds are broken, leading to an average hydrophobic energy of $18.0/28 = 0.6$ kcal/mol per residue (Eyring Model) and $13.8/28 = 0.5$ kcal/mol per residue (Kramers Model), consistent with the case of the GCN4-p1 leucine zippers.

Comparison of Binary and Ternary Free Energies to the Dissociation Rates

On comparing the binary Sx1A-Sb2 complex and the ternary Sx1A-SNAP25B-Sb2 complex, we find that regardless of the model (Eyring or Kramers) used the latter complex has 1.4 kcal/mol ($2.3 k_B T$) higher interaction free energy. Thus the Sx1A-SNAP25B-Sb2 complex is much more stable than the Sx1A-Sb2 complex. This energy difference can be compared to the ratio of their spontaneous dissociation rates. For example, the binary Sx1A-Sb2 has a spontaneous dissociation rate K_{off}^0 , binary of 5.6 s^{-1} as compared to K_{off}^0 , ternary of 0.48 s^{-1} for the Sx1A-SNAP25B-Sb2 ternary system at 24 °C. In this case, translating the free energy difference to a ratio of spontaneous dissociation rates leads to

$$K_{\text{off, ternary}}^0 / K_{\text{off, binary}}^0 \approx \exp[(\Delta G_{\text{ternary}} - \Delta G_{\text{Binary}}) / k_B T] = \exp(2.3) = 10 \text{ in good agreement with}$$

the $K_{\text{off, ternary}}^0/K_{\text{off, binary}}^0 = 5.6/0.48 = 11.6$ also measured here. The same ratio of the K_{off}^0 is 17 at 14 °C and 24 at 4 °C. These correspond to free energy differences between the ternary and the binary of less than $3.2 k_B T$. These values are consistent with our measured values of ΔG for the binary and ternary complexes. The spontaneous dissociation rate of 0.48 s^{-1} for the ternary complex is much lower than that obtained in experiments using GdnHCl denaturants (Fasshauer et al., 2002) or estimated in experiments using the surface force apparatus (Li et al., 2007). The former assumes that the rate determining step does not change when the GdnHCl is set to zero. In the latter estimation, K_D in Eq. (1) is *a priori* assumed to be 10^9 s^{-1} . However, protein systems exhibit a wide range of K_D between 10^{13} – 10^{17} s^{-1} (Schwesinger et al., 2000).

Comparison of the Adhesion Energies to Energetics of Vesicle Tethering/Docking and Fusion

The fusion of lipid bilayers requires the application of external force. The energy released on the formation of the SNARE complex is considered necessary to overcome the inter-membrane repulsive interactions and bring the vesicles in close proximity to the plasma membrane and partially overcome the barrier for intracellular vesicle-plasma membrane fusion. The adhesion enthalpies ΔH measured here for the formation of both the Sx1A-Sb2 binary $19.8 \pm 3.5 \text{ kcal/mol} = 33 \pm 6 k_B T$ and the Sx1A-SNAP25B-Sb2 ternary $25.7 \pm 3.0 \text{ kcal/mol} = 43 \pm 5 k_B T$ complexes are consistent with this model. The first step of tethering/docking the vesicle within 1 nm of the plasma membrane requires energies of 5 – $10 k_B T$. In membrane fusion, the first two stages of stalk formation and hemifusion are expected to require only $13 k_B T$, thus both the binary and ternary complexes might have sufficient energy to drive the process (Markin and Albanesi, 2002). The final stage of pore formation requires average energies estimated to be $46 k_B T$ (Markin and Albanesi, 2002). In the fusion of secretory granules of mouse mast cells the activation energy for pore formation was reported to be $23 \text{ kcal/mol} = 39 k_B T$ based on membrane capacitance measurements (Oberhauser et al., 1992). The fusion of viral particles to membranes such as that from pH-induced fusion of vesicular stomatitis virus to erythrocytes ghosts yielded activation energies of $42 \text{ kcal/mol} = 71 k_B T$ (Clague et al., 1990). When protein free fusion between vesicles triggered by the introduction of polyethylene glycol was studied through proton transfer, three steps with activation free energies of $37 \text{ kcal/mol} = 62.5 k_B T$, $27 \text{ kcal/mol} = 45.6 k_B T$ and $22 \text{ kcal/mol} = 45.6 k_B T$ were observed (Lee and Lentz, 1998). It should be noted that the fusion process of viral particles while sharing pathways with those mediated by SNAREs, have many differences as they contain the entire fusion machinery only on one surface, fuse to an incompetent surface, and takes minutes compared to a few hundred microseconds (Chernomordik and Kozlov, 2005). Thus based on the theoretical modeling and the available experimental evidence, one can estimate that lipid bilayer fusion and pore formation requires energies around or greater than $57 k_B T$ (Chernomordik and Kozlov, 2005; Cohen and Melikyan, 2004; Markin and Albanesi, 2002).

In the case of the binary Sx1A-Sb2 complex, based on the activation energy and formation enthalpy measured here of $33 \pm 6 k_B T$, we can conclude that it has only a very low probability of driving the complete process of membrane fusion and pore formation. However the attachment energy far exceeds the sum of the vesicle thermal fluctuation energy and the inter-membrane repulsive energy, and thus just one Sx-Sb interaction would be sufficient to tether/dock a vesicle to the plasma membrane. This value of the binary Sx-Sb interaction energy is also consistent with earlier observation (Bowen et al., 2004) that energy deficit for fusion in the case of the Sx-Sb interaction can be overcome by thermally activating the process. On the other hand, the ternary Sx1A-SNAP25B-Sb2 complex formation which would lead to an energy release of $43 \pm 5 k_B T$ is closer to the $\approx 57 k_B T$ estimated as required for the complete spontaneous or chemically evoked fusion. It should also be pointed out that for ternary complex, the energy values suggest that as few as one set of SNARE molecules is sufficient

for driving fusion, consistent with earlier estimates using single molecule techniques based on the AFM (Liu et al., 2006) and fluorescence microscopy (Bowen et al., 2004). The higher adhesion energies for the ternary complex again suggest that the energetics of membrane fusion require the presence of SNAP25 for increasing the probability of fusion. Note that other proteins such as complexin and synaptotagmin interact with the SNARE complex, along with lipid molecules and play important roles in membrane fusion (Bai and Chapman, 2004; Brose et al., 1992; Brunger, 2005; Chapman et al., 1995; Deak et al., 2006; Giraudo et al., 2005; Hay, 2007; Hu et al., 2003; Jahn et al., 2003; Koh and Bellen, 2003; Lu et al., 2005; Martens et al., 2007; Reese et al., 2005; Rothman, 2002; Sudhof and Rizo, 1996; Weber et al., 1998). For example, it was found that complexin binding to the SNARE proteins arrests membrane fusion at the hemifusion state (Schaub et al., 2006) and that Ca^{2+} binding to synaptotagmin 1 leads to the formation of membrane tubules with high positive curvature due to its interaction with negatively charged phospholipids such as phosphatidylserine and phosphatidylinositol-4,5-bisphosphate thereby lowering the barrier for fusion (Martens et al., 2007). The formation energy of the ternary SNARE complex measured here is slightly less than that required for complete fusion. This is consistent with the hypothesis that the SNARE assembly brings the vesicle and plasma membranes to the hemifusion stage, and additional interactions provided by complexin and synaptotagmin triggered by a Ca^{2+} influx may provide a supplementary release of energy to fully complete the pore formation process. This is also consistent with the observation that Ca^{2+} evoked fusion was abolished in SNAP25 knockout mice (Washbourne et al., 2002).

The formation of the two different complexes Sx1-Sb2 and Sx1A-SNAP25-Sb2 suggests that vesicles can be tethered/docked to the plasma membrane in both ways. However the larger binding energy involved in the case of Sx1A-SNAP25-Sb2 complex points to the unique role of SNAP25 in exocytosis, in providing a much stronger and more stable docking of the vesicles. This higher interaction energy for the ternary complex is in spite of the fact that only a 12.1 nm mean extension and 7 hydrophobic layers is required for rupture of the complex compared to the 22.2 nm extension and 15 hydrophobic bonds for rupture of the Sx1A-Sb2 binary complex. The shorter extension required to rupture the ternary complex, perhaps means that the role of SNAP25 is to tether the position of the vesicles in close proximity to the plasma membrane. The longer ~22 nm extension required to completely break the binary Sx1A-Sb2 interaction suggests the ability of the interaction to tether/dock vesicles floating at distances as large as ~22 nm away from the plasma membrane. The multiple methods of SNARE interactions responsible for vesicle tethering/docking are consistent with the low probability of Ca^{2+} evoked fusion events. As merely 10%–20% of the available vesicles are observed to fuse on arrival of the Ca^{2+} signal (Rosenmund and Stevens, 1996), only a small fraction of the tethered/docked vesicles attached with the ternary SNARE complex are energetically favored for complete fusion.

Conclusion

In conclusion, we have provided a single molecule based experimental measurement of activation free energies for the interaction of the SNARE proteins Sx1A, SNAP25B and Sb2. The exocytotic proteins Sx 1A and Sb2 were attached with pointlike relatively rigid Ni^{2+} -H6 linkers on an AFM cantilever tip and coverslip, respectively. Coiled-coils formed between the binary heterodimer Sx1A-Sb2 and the ternary heterotrimer Sx1A- SNAP25B-Sb2 where studied. The dissociation rates were obtained from the rupture force distribution as a function of the rate of applied force. The temperature dependence of the dissociation rates was used to obtain the interaction energy to the transition state and found to be $19.8 \pm 3.5 \text{ kcal/mol} = 33 \pm 6 k_B T$ and $25.7 \pm 3.0 \text{ kcal/mol} = 43 \pm 5 k_B T$ for the binary Sx1A-Sb2 and ternary Sx1A-SNAP25-Sb2 complex, respectively. These activation energies are consistent with those measured previously for the ternary system. They are also of the order expected for the tethering/docking

of the vesicle and lipid bilayer hemifusion as expected from theoretical models and available experiments. From the activation energy barrier the ΔH , ΔS and ΔG were calculated using Eyring's and Kramer's reaction rate theory applied to peptides in solution. The ΔG was found to be $12.4\text{--}16.6\text{ kcal/mol} = 21\text{--}28k_B T$ and $13.8\text{--}18.0\text{ kcal/mol} = 23\text{--}30k_B T$ for the binary and ternary complex, respectively. The values obtained were found to be consistent with energy measurements in other coiled-coil systems such as the well studied yeast transcriptional activator GCN4. The differences in measured free energies of the binary and ternary system are also consistent with the difference in the spontaneous dissociation rates. The values of the adhesion energies suggest that only one set of proteins is sufficient for tethering/docking of individual vesicles. In addition, the adhesion energy of the Sx-Sb2 heterodimer system indicates that it is sufficient for tethering/docking but insufficient to bring about complete vesicle-membrane fusion. The higher adhesion energies measured for the ternary system along with the shorter extensions required for rupture of the complex points to the unique and important role of SNAP25B in profoundly increasing the probability for membrane fusion. These quantitative results will allow a better understanding of the various interactions of the SNARE proteins and clarify their roles in exocytosis. The availability of the interaction free energy along with the structural information should lead to robust molecular modeling of the exocytotic process.

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	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7	+8																																					
Sx1A	I	I	K	L	E	N	R	L	R	E	L	H	D	M	F	M	D	M	A	M	L	V	E	S	Q	G	E	M	I	D	R	I	E	Y	N	V	E	H	A	V	D	Y	V	E	R	A	V	S	D	T	K	K	A
Sb2	L	Q	Q	T	Q	A	Q	V	D	E	V	V	D	I	M	R	V	N	V	D	K	V	L	E	R	D	Q	K	L	S	E	L	D	D	R	A	D	A	L	Q	A	G	A	S	Q	F	E	T	S	A	A	K	L
SN1	T	R	R	M	L	Q	L	V	E	E	S	K	D	A	G	I	R	T	L	V	M	L	D	E	Q	G	E	Q	L	E	R	I	E	E	G	D	Q	H	I	N	K	D	M	K	E	A	E	K	N	L	T	D	L
SN2	L	E	Q	V	S	G	I	I	G	N	L	R	H	M	A	L	D	M	G	N	E	I	D	T	Q	N	R	Q	I	D	R	I	M	E	K	A	D	S	N	K	T	R	I	D	E	A	N	Q	R	A	T	K	M

Fig. 1.

The amino acid sequence of the partial SNARE domains of proteins contributing to the ternary SNARE complex. The 15 hydrophobic layers (−7 to −1 and +1 to +8) are labeled in bold and '0' ionic layer is italicized. Protein sequences: syntaxin 1A (Sx1A), GeneBank accession number AF217191; synaptobrevin 2 (Sb2), GeneBank accession number BC074003; and SNAP25B (SN1 and SN2 domains), GeneBank accession number AB003992.

Fig. 2.

(a) Schematic of experimental setup. Syntaxin 1A (green) is co-complexed with SNAP25B (blue) on the cantilever tip. This functionalized tip is used to probe synaptobrevin 2 (red) attached to the coverslip. These proteins are brought to proximity (approach; arrow pointing down) by means of the piezoelectric element and then taken apart (retract, arrow pointing up). At contact they form ternary SNARE complex. The drawing is not to scale. (b) A typical force curve obtained where the segment 'ef' represents the bond rupture force and 'de' represents the extension at the point of rupture of the intermolecular bond. In the segments 'abc' (see results for details), the coverslip and the cantilever tip are still in contact. (c) Distribution of the forces at rupture for Sx1A-SNAP25B-Sb2 single intermolecular bonds. Arrowhead indicates the mean value. (d) The mean rupture force for the ternary Sx1A-SNAP25B-Sb2 complex as a function of the rate of applied force for three different temperatures of 24 °C, 14 °C and 4 °C are shown as squares, circles and triangles, respectively. Each point represents the mean value of 40–65 events. The corresponding best fit straight lines are indicated as solid, dashed and dotted lines.

Fig. 3.

The natural logarithm of the spontaneous dissociation rate (K_{off}^0) for the Sx1A-Sb2 binary complex [circle; adapted from Figure 5 of Liu et al., 2008] and Sx1A-SNAP25-Sb2 ternary complex (square) plotted as a function of the inverse temperature. The slopes lead to the change in enthalpy from the bound state to the transition state ($\Delta E \approx \Delta H$) and the intercepts to the corresponding change in entropy. The values are listed in Table 1.

The change in enthalpy (ΔH), entropy (ΔS) and free energy (ΔG) from the bound state to the transition state for SNARE complexes. The ternary complex is more stable by 1.4 Kcal mol⁻¹ using either model, which is consistent with our independent measurement of a factor of 12 difference in their respective spontaneous dissociation rates.

Table 1

	ΔH	ΔS		ΔG	
		cal mol ⁻¹ K ⁻¹		kcal mol ⁻¹	
		Eyring	Kramers	Eyring	Kramers
Sx1A-Sb2	19.8 ± 3.5	10.8 ± 11.5	24.9 ± 11.5	16.6 ± 4.9	12.4 ± 4.9
Sx1A-SNAP25B-Sb2	25.7 ± 3.0	25.9 ± 11.1	40.0 ± 11.1	18.0 ± 4.5	13.8 ± 4.5

Note: To convert from kcal mol⁻¹ to $k_B T$ multiply with 1.67.