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Single-molecule optical tweezers study of regulated SNARE assembly

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Abstract

Intracellular membrane fusion mediates material and information exchange among different cells or cellular compartments with high accuracy and spatiotemporal resolution. Fusion is driven by ordered folding and assembly of soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptors (SNAREs) and regulated by many other proteins. Understanding regulated SNARE assembly is key to dissecting mechanisms and physiologies of various fusion processes and their associated diseases. Yet, it remains challenging to study regulated SNARE assembly using traditional ensemble-based experimental approaches. Here, we describe our new method to measure the energy and kinetics of neuronal SNARE assembly in the presence of α -SNAP, using a single-molecule manipulation approach based on high-resolution optical tweezers. Detailed experimental protocols and methods of data analysis are shown. This approach can be widely applied to elucidate effects of regulatory proteins on SNARE assembly and membrane fusion.

Keywords

Optical tweezers; single-molecule manipulation; SNARE assembly and disassembly; SNAP; NSF; hidden-Markov modeling; energy landscape

1. Introduction

Soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (SNAP) receptor proteins (SNAREs) are a central component of the machinery that mediates intracellular membrane fusion in eukaryotes (1, 2). For example, neuronal SNAREs syntaxin 1 and SNAP-25 on the presynaptic plasma membrane and VAMP2 (also called synaptobrevin) on the synaptic vesicle membrane are responsible for fusion between the two membranes (3), leading to neurotransmitter release and accompanying neurotransmission. All SNARE proteins share characteristic SNARE motifs of ~60 amino acids bearing either glutamine (Q) or arginine (R) in the middle, which categorizes SNAREs into Q- and R-SNAREs (4).

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Individual SNARE motifs are disordered in solution (5), but associate into structurally conserved parallel four-helix bundles containing three Q-SNARE motifs and one R-SNARE motif (3, 6–9) (Fig. 1A). In the complex, different SNARE motifs are held together by 15 layers of hydrophobic amino acids and the middle ionic “0” layer (numbered from –7 to +8). Besides SNARE motifs, most SNAREs contain a C-terminal transmembrane domain (TMD). Many SNAREs also bear other functional domains, such as the N-terminal regulatory domain (NRD) in syntaxin (10, 11). Decades of research have demonstrated that SNARE assembly into the four-helix bundle draws their associated membranes into close proximity, thereby initiating membrane fusion (12, 13). SNARE assembly proceeds stepwise in a zipper-like manner: It starts from slow association of N-terminal SNARE domains (NTDs), pauses in a half-zipped state, and completes with fast zippering of C-terminal SNARE domains (CTDs) and linker domains (LDs) (14–16). In addition, SNAREs frequently mis-assemble into various anti-parallel conformations, which represent dead ends of SNARE assembly and unlikely mediate membrane fusion (17–19). Overall, assembly of SNARE proteins alone is too slow and inaccurate to drive efficient membrane fusion.

SNARE assembly in the cell is exquisitely regulated by many other proteins (1). For synaptic vesicle fusion, the regulatory proteins include Munc18-1, Munc13-1, complexin, synaptotagmin, α -SNAP, and NSF (2, 20). Munc18-1 represents a family of Sec1/Munc18 (SM) proteins that are required for all SNARE-mediated membrane fusion (21, 22). The exact role of SM proteins in SNARE assembly and membrane fusion has not been well understood (23). Evidence suggests that SM proteins enhance or proofread SNARE assembly to promote membrane fusion (2, 24–27). However, the underlying mechanism is not clear. Synaptotagmin senses the calcium influx induced by arrival of an action potential and triggers synchronous synaptic vesicle fusion with the assistance of complexin (1, 20). After membrane fusion, fully assembled SNARE complexes are disassembled by the AAA+ protein NSF and α -SNAP in an ATP-dependent manner (28). Interestingly, NSF does not directly bind to SNARE complexes. Instead, α -SNAP mediates the interactions between NSF and SNARE complexes. Besides its role in SNARE disassembly, it has recently been found that SNAP also modulates SNARE assembly, thereby regulating membrane fusion (29–32). Taken together, regulatory proteins are required to ensure membrane fusion to occur at the right time and location.

It is challenging to observe multiple partially assembled SNARE intermediates and measure the force generated by SNARE assembly using traditional experimental approaches (33, 34). As a result, much of our knowledge on regulated SNARE assembly is only inferred from interactions of regulator proteins with fully assembled SNARE complexes or readouts of membrane fusion (35–40). The single-molecule manipulation approach based on high-resolution optical tweezers is one of a few methods capable of measuring both the structural intermediates of SNARE assembly and their associated forces, energies, and kinetics (15, 16, 33, 41–43). Here, we describe our detailed protocols to characterize SNARE assembly regulated by α -SNAP (42). Our experiments demonstrate that α -SNAP specifically stabilizes the folded four-helix bundle, potentially accounting for the dual roles of α -SNAP in both SNARE assembly and disassembly (28, 30, 31, 44).

2. Materials

2.1 Preparation of SNARE Constructs

2.1.1 Plasmids modifications

1. Dpn I restriction enzyme (10 U/μl) (NEB).
2. XL1-Blue super-competent cells (Agilent Technologies).
3. SOC medium: 20 g/L tryptone, 5 g/L yeast extract, 4.8 g/L MgSO₄, 3.603 g/L dextrose, 0.5g/L NaCl, 0.186 g/L KCl.

2.1.2 Bacterium growth and harvest

1. BL21 Gold (DE3) competent cells (Agilent Technologies).
2. LB medium: Dissolve 25 g LB broth (Miller) into 1 L ddH₂O, autoclave for 30 min.
3. 1 M IPTG (Isopropyl-1-thio-β-D-galactopyranoside) stock solution (1000×, 2.38g/10ml). Filter the solution with 0.22 μm syringe filter.
4. 50 mg/mL kanamycin stock solution (1000×). Filter with 0.22 μm syringe filter.
5. LB-Kanamycin Agar: 25 g LB broth and 15 g Agar per 1 Liter of ddH₂O. Autoclave for 20 min, and then cool to approximate 55 °C. Add 1 mL kanamycin stock solution to a final concentration of 50 μg/mL, and pour into petri dishes.

2.1.3 Protein purification

1. Protease inhibitor cocktail tablet, EDTA free (cOmplete™, Roche).
2. Nickel beads: Ni Sepharose 6 Fast Flow (GE healthcare Lifesciences).
3. Amicon (or Centricon, EMD Millipore).
4. 0.5 M TCEP stock solution: Weight TCEP·HCl (Tris(2-carboxyethyl)phosphine hydrochloride) 1.43 g, add 8 ml ddH₂O, adjust pH to 7.4 using KOH, then make up to 10 ml with water. Store 0.5 mL aliquots at -20°C.
5. Lysis buffer: 25 mM HEPES (pH 7.7), 400 mM KCl, 0.5 mM TCEP, 10% glycerol, 10 mM imidazole. Mix 25 ml HEPES (1 M, pH 7.7), 160 ml KCl (2.5 M), 1 ml TCEP (0.5 M), 100 ml glycerol, 10 ml imidazole stock solution (1 M) and water to final volume of 1 L.
6. Wash buffer I: 25 mM HEPES, pH 7.7, 400 mM KCl, 0.5 mM TCEP, 10% glycerol, 30 mM imidazole. Mix 25 ml HEPES (1 M, pH 7.7), 160 ml KCl (2.5 M), 1 ml TCEP (0.5 M), 100 ml glycerol, 30 ml imidazole stock solution (1 M) and water to final volume of 1 L.
7. Wash buffer II: 25 mM HEPES, pH7.7, 400 mM KCl, 0.5 mM TCEP, 10% glycerol, 50 mM imidazole. Mix 25 ml HEPES (1 M, pH 7.7), 160 ml KCl (2.5 M), 1 ml TCEP (0.5 M), 100 ml glycerol, 50 ml imidazole stock solution (1 M) and water to final volume of 1 L.

8. Elution buffer: 25 mM HEPES, 400 mM KCl, 0.5 mM TCEP, 10% glycerol, 300 mM imidazole. Mix 25 ml HEPES (1 M, pH 7.7), 160 ml KCl (2.5 M), 1 ml TCEP (0.5 M), 100 ml glycerol, 300 ml imidazole stock solution (1 M) and water to final volume of 1 L. NuPAGE[®]Novex 4-12% Bis-Tris precast protein gels (Thermo Fisher).
9. 4 × SDS loading buffer (Thermo Fisher).
10. SDS-PAGE Coomassie staining solution: 0.1-0.2 g Coomassie Brilliant Blue G-250 (Bio-Rad), 225 mL methanol, 225 mL ddH₂O, 50 mL glacial acetic acid.
11. SDS-PAGE Coomassie de-staining solution: 150 mL methanol, 300 mL ddH₂O, 50 mL glacial acetic acid.
12. Running buffer: NuPAGE[®] MOPS SDS running buffer, 20× (Thermo Fisher, #NP0001), dilute to 1X prior to use.

2.1.4 Protein biotinylation and SUMO cleavage

1. BirA biotin ligase, purified by ourselves.
2. 10× 100 mM ATP: Weight 0.605 g ATP (Adenosine 5-triphosphate disodium salt trihydrate Disodium salt), dissolve in 10 ml ddH₂O, adjust pH to 7.4 using NaOH. Store 1 mL aliquots at −80°C.
3. 10× 100 mM MgOAc: Weight 0.214g (CH₃COO)₂Mg·4H₂O, and dissolve in 10 ml 25 mM PH 7.7 HEPES buffer. Store at room temperature.
4. 10× 500 μM d-biotin: Weight 1.22 mg d-biotin, and dissolve in 10 ml ddH₂O. Store 2 mL aliquots at −20°C.
5. Streptavidin (1 mg/mL): Add 1 mL ddH₂O to 1 mg streptavidin powder (Promega).
6. Biotinylation buffer: 25 mM HEPES, pH 7.7, 200 mM L-Glutamic acid potassium, 0.5 mM TCEP, 300 mM imidazole. Mix 2.5ml 1 M HEPES, 100 μl 0.5 M TCEP and 30 ml 1 M imidazole stock solution, 4.06 g Glutamic acid potassium salt monohydrate and ddH₂O to final volume of 100 ml. (Note 1)
7. SUMO protease, purified by ourselves.

2.2 Prepare of DNA Handles

1. Taq DNA polymerase with ThermoPol buffer (NEB).
2. dNTP mix (10 mM).
3. DNA primer 1: Dual-Digoxigen modified oligo (Dual-Dig-ATCATCCAAGGCTGAGCCTGCAGGTCT), DNA primer 2: Thiol-modified oligo (Thiol-AAAT C GAC GCTCAAGT C AGAGGTG)

¹Some commonly used chemicals reduce the activity of BirA enzyme, including NaCl and glycerol, whose concentrations should be minimized in the biotinylation reaction.

4. Template plasmid: pUC19 plasmid with a modified multi-cloning site (45, 46). The sequences of the original and modified pUC19 multi-cloning sites are compared as follows:

Modified multi-cloning site:

AATTCACCGTCTAAGATCTGATTCGAGCCCGGTACTCGGGGATCCT
CTAGAGTAGACCTGCAGGCTCAGCCTTGGATGATGCA

Original pUC19 multi-cloning site:

AATT-----
CGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGG
C-----ATGCA

Here the inserted or mutated bases in the first sequence are underlined and the dashed lines in the second sequence indicate the positions of sequence insertion.

2.3 Crosslinking

1. 0.2 M sodium phosphate buffer, pH 5.8: Weight 2.40 g Sodium phosphate monobasic, dissolve in 90 ml ddH₂O, adjust pH to 5.8 using NaOH, and add ddH₂O up to 100 ml.
2. 0.2 M sodium phosphate buffer, pH 8.5: Weight 2.40 g Sodium phosphate monobasic, dissolve in 90 ml ddH₂O, adjust pH to 8.5 using NaOH, and add ddH₂O up to 100 ml.
3. 5 M NaCl solution.
4. Buffer A: 0.1 M sodium phosphate buffer, pH 5.8, 400 mM NaCl: Mix 50 ml 0.2 M sodium phosphate buffer, pH 5.8, 8 ml 5 M NaCl, and add ddH₂O up to 100 ml.
5. Buffer C: 0.1 M sodium phosphate buffer, pH 8.5, 400 mM NaCl: mix 50 ml 0.2 M sodium phosphate buffer, pH 8.5, 8 ml 5 M NaCl, and add ddH₂O up to 100 ml.
6. Elute buffer C: 0.1 M sodium phosphate buffer, pH 8.5, 400 mM NaCl, 300 mM imidazole: take 5 ml 0.2 M sodium phosphate buffer, pH 8.5, 3 ml 1 M imidazole, and make up to 10 ml with ddH₂O.
7. 10 mM 2,2'-dithiodipyridine disulfide (DTDP) stock solution: Dissolve 2.2 mg DTDP powder into 0.2 mL acetonitrile and then add 0.8 mL ddH₂O to dilute to final concentration of 10 mM.
8. Bio-spin columns (Micro Bio-spin 6 columns, Bio-RAD)

2.4. Single-molecule SNARE assembly assay

2.4.1 Home-built dual-trap high-resolution optical tweezers—Our dual-trap optical tweezer setup is home-built and calibrated as previously described (47–49). Briefly, the tweezers are mainly composed of five parts: the laser and power control part, the beam

expanding part, the beam steering stage, the trap and sample stage, and the detection stage. The tweezer setup is built in an isolated room in a basement to minimize environment noises and vibrations. The room temperature is controlled at 23 °C. A 1064 nm laser (CW, Nd:YVO4 laser with a maximum power of 5 W, Spectra-Physics) beam is expanded by two telescopes. Between the telescopes, the laser beam is split into two orthogonally polarized beams. One polarized beam is steered by a mirror attached to a nano-positioning stage (Mad City Labs, WI), which is used to move the optical trap in the sample plane. The two beams pass through a 60× water immersion front objective (NA = 1.2, Olympus, PA) and form two optical traps inside the central channel of the microfluidic flow cell (Fig. 1B,C). The outgoing laser beams are collected by a second identical back objective, split again by polarization, and projected onto two position-sensitive detectors (Pacific Silicon Sensor, CA) to detect bead positions using back-focal-plane interferometry (50, 51).

2.4.2 Chamber preparation

1. Laser engraver. Epilog Zing laser engraving and cutting system. Used to cut holes in coverslips and Nescofilms into patterns to form the microfluidic channels (Fig. 1B).
2. Nescofilm (Karlson research)
3. Microscope cover glass (60 × 24 × 0.17 mm) (Fisherbrand).
4. Plastic tubing: Polyethylene tubing PE10 and PE50 (Becton Dickinson).
5. Customized glass tubing (King Precision Glass, Inc., CA): Bead dispenser tubes with 100 µm outer diameter (OD) and 25 µm inner diameter (ID); protein injection tubes with 80 µm OD and 40 µm ID.
6. Heat block.

2.5.2 Optical trapping assay

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4.
2. Catalase from bovine liver: aqueous suspension, 10,000 to 40,000 units/mL (Sigma-Aldrich).
3. Glucose oxidase from *Aspergillus niger*: 50 mg/mL, 100 units/mg (Sigma-Aldrich).
4. 400 mg/mL Glucose stock solution: Weight 20 g Glucose, add to the 50 ml ddH₂O slowly to make sure that glucose dissolve completely. Store 1 mL aliquots at −20°C.
5. PBS buffer with oxygen scavenging system: Add 75 µL glucose, 6 µL glucose oxidase (final concentration 10 units/mL), and 4.7 µL catalase (final concentration 15.7-62.7 units/mL) to 3 ml PBS.

6. Polystyrene beads: 1% w/v anti-digoxigenin antibody coated polystyrene particles (2.1 μm , called DIG beads), streptavidin-coated polystyrene particles (1.8 μm), both from Spherotech, IL.
7. Sodium azide solvent: 0.02% sodium azide solution and filter with 0.22 μm syringe filter.

3. Methods

SNARE construct

The cytoplasmic domains of neuronal SNAREs syntaxin 1A, SNAP-25B, and VAMP2 are used in our assay shown in this work. We pulled a single cytoplasmic SNARE complex from the C-termini of syntaxin and VAMP2 via a 2,260 bp DNA handle (52), with their N-termini crosslinked at around -6 layer through a disulfide bridge (Fig. 1A). To facilitate the N-terminal crosslinking, we introduced cysteine mutations in VAMP2 (Q36C) and Syntaxin (L205C). A flexible spacer sequence GGSGNGGSGS followed by an AviTag sequence (GLNDIFEAQ**KIEWHE**) was added to C-terminus of syntaxin. The biotin-ligase BirA recognizes the AviTag sequence and catalyzes addition of a biotin moiety to the lysine residue in the sequence (shown in bold), which allows attachment of syntaxin to a streptavidin-coated polystyrene bead. Similarly, a flexible space sequence GGSGNGSGGLC was added to the C-terminus of VAMP2. The underlined C-terminal cysteine residue was crosslinking to the thiol group on the DNA handle at one end. The other end of the DNA handle is labeled with two digoxigenin moieties, which bind to anti-digoxigenin antibodies immobilized on the polystyrene bead. The spacer sequences and the DNA handle facilitate attachment of a single SNARE complex to two beads and minimize nonspecific interactions between SNARE protein and bead surfaces. Finally, the natural cysteine residues in the loop of the SNAP-25 and syntaxin proteins were mutated to serine.

3.1 Plasmids preparation.

Genes of VAMP2 and syntaxin are inserted into the pet-SUMO vector containing a 6 $\times$ His tag and a SUMO fusion protein at the N-terminus. SNAP-25B was cloned into the pET-15b vector (Novagen) that introduces a 6 $\times$ His tag to the N-terminus of the protein. These plasmids were modified from those previously described (16) by the QuikChange site-directed mutagenesis kit (Agilent Technologies) as shown below:

1. Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequences.
2. Prepare the PCR reaction: Mix 5 μl of 10 $\times$ reaction buffer, 5–50 ng plasmid, 125 ng oligonucleotide primer #1, 125 ng oligonucleotide primer #2, 1 μl of dNTP, and ddH₂O to a final volume of 50 μl . Then add 1 μl of PfuUltra HF DNA polymerase (2.5 U/ μl).
3. Perform PCR amplification using the cycling parameters outlined below: initial denaturation at 95 $^{\circ}\text{C}$ for 3 min, 18 cycles of denaturation at 95 $^{\circ}\text{C}$ for 30 s and annealing at 55 $^{\circ}\text{C}$ for 1min, and extension at 68 $^{\circ}\text{C}$ for 10 min, followed by a final extension for 5 min at 72 $^{\circ}\text{C}$, cooling to 4 $^{\circ}\text{C}$.

4. Add 1 μ L of the Dpn I restriction enzyme (10 U/ μ L) directly to the PCR reaction mixture. Gently and thoroughly mix the reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixture in a micro-centrifuge for 1 minute and immediately incubate the reaction at 37°C for 1 hour to digest the parent supercoiled dsDNA.
5. Gently thaw 50 μ L XL1-Blue super-competent cells on ice.
6. Transfer 1 μ L of the Dpn I-treated DNA to the super-competent cells, gently swirl the transformation reaction, and incubate the reaction on ice for 30 minutes.
7. Heat shock the transformation reaction for 45 seconds at 42°C and then place the reaction on ice for 2 minutes.
8. Add 0.5 ml of S.O.C broth pre-heated to 42°C and incubate the transformation reaction at 37°C for 1 hour while shaking at 225–250 rpm.
9. Plate the appropriate volume of the transformation reaction on agar plates containing the appropriate antibiotic for the plasmid vector.
10. Pick 5 colonies from a freshly streaked selective plate and inoculate each colony in a culture of 5 ml LB medium containing the appropriate selective antibiotic. Incubate the cells for 12–16 h at 37°C while shaking at 225–250 rpm.
11. Harvest the bacterial cells by centrifugation at > 8000 rpm (6800 $\times$ g) in a cooled micro-centrifuge for 5 min.
12. Extract plasmids.
13. Sequence and confirm the protein genes in the extracted plasmids.

3.2 Protein expression and purification

1. Transform 5–10 ng protein-encoding plasmids in a 1 to 5 μ L volume to BL21 Gold (DE3) competent cells and mix by stirring gently with the pipette tip.
2. Incubate on ice for 30 min.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice and wait for 2 minutes.
5. Add 200 μ L of room temperature S.O.C. medium and shake at 37 °C with 225–250 rpm for one hour.
6. Spread 100 μ L of the competent cells evenly on a selective LB plate containing 50 μ g/ml kanamycin pre-warmed to 37 °C.
7. Incubate at 37 °C overnight.
8. Pick a single colony from the plate and re-suspend it in 100–200 ml LB with 50 μ g/ml kanamycin. Grow overnight at 37 °C while shaking at 225–250 rpm. This is the preculture sample.

9. Inoculate 1 L of LB containing 50 µg/ml kanamycin with pre-cultured bacterial sample grow the culture for about 2 hours until OD₆₀₀ is 0.6 to 0.8 (mid-logarithmic phase).
10. Induce expression of proteins by adding IPTG to a final concentration of 1 mM.
11. Grow at 37 °C with shaking the culture for 3 to 5 hours. Harvest the cells by centrifuging the culture at 4500 g for 15 min at 4 °C.
12. Proceed to purification or store the cells at –20 °C for future use.
13. Thaw the cell pellet for 15 min on ice. Re-suspend cells in 30 ml lysis buffer containing protease inhibitor cocktail.
14. Disrupt cells using a French press method.
15. Centrifuge the cell suspension at 200,000 g for 1 hour at 4 °C and keep the clarified supernatant.
16. Wash Ni-NTA beads using lysis buffer three times and centrifuge at 500 g for 3 min.
17. Add the Ni-NTA beads (500 µl bead/1 L E. coli culture) to the supernatant from step 15 and rotate the bead solution at 4 °C for two hours.
18. Centrifuge the Ni-NTA beads at 500 g for 3 min at 4 °C. Collect the protein-bound Ni-NTA beads at the bottom.
19. Wash the beads with 10 ml wash buffer I and I three times, respectively to remove nonspecifically bound proteins. After add buffer, rotate the bead solution at 4 °C for 10-15 min. Centrifuge at 500 g for 3 min at 4 °C to collect the Ni-NTA beads.
20. For SNAP-25, BirA ligase, SUMO protease, and VAMP2, directly elute the protein from the Ni-NTA bead by adding elute buffer to beads and rotate at 4 °C for 1 hour. Centrifuge the Ni-NTA beads at 500 g for 3 min and collect the supernatant (Note 2, 3).
21. For VAMP2, use Amicon to change buffer to the lysis buffer. Add SUMO protease to cleave the N-terminal 6×His-tag and SUMO fusion protein and incubate at 4 °C overnight. Then add Ni-NTA beads into the solution to bind the cleaved His-tag-SUMO proteins. Centrifuge to precipitate the beads and keep the supernatant containing purified VAMP2 proteins.
22. Run SDS PAGE to check the protein quality and concentration.

3.3 Syntaxin biotinylation

Syntaxin biotinylation was performed following step 19 in the preceding protein purification protocol:

².The volume of elution buffer should be greater than the volume of Ni-NTA beads.

³.One elution is often not enough to elute all proteins from the Ni-NTA beads. The elution step can be repeated for 3~4 times for maximum yield protein elution.

1. Add the biotinylation buffer containing 300 mM imidazole to the syntaxin-bound Ni-NTA bead solution (Note 4). Add ATP, MgOAc and d-biotin to a final 1X concentration
2. Incubate at 4 °C overnight.
3. Centrifuge and keep the supernatant containing biotinylated syntaxin.
4. Test the biotinylation level of syntaxin using SDS gel as previously described (53).
5. Carry out step 21 in 3.3 to remove the SUMO fusion tag in syntaxin.

3.4 SNARE complex formation and DNA handle crosslinking

1. Run PCR to generate the DNA handles labeled with a thiol group at one end and two digoxigenin moieties at the other end (Fig. 1A).
2. Purify DNA handles using PCR Purification Kit.
3. Reduce DNA handles with 2 mM TCEP first and then remove free TCEP. Activate the thiol groups in the DNA handle with 1 mM DTDP and remove free DTDP.
4. Measure the DNA concentration.
5. Mix the purified syntaxin, SNAP-25 and VAMP2 proteins in a molar ratio of 0.8:1:1.2 lysis buffer and incubate the mixture at 4 °C overnight to form ternary SNARE complexes.
6. Purify the SNARE complexes: Add above reaction mixture to appropriate amount of washed Ni-NTA beads, incubate while rotating the bead solution at 4 °C for 2 hours to allow SNAP-25 bind to Ni-NTA beads through its N-terminal His tag. Both free SNAP-25 and ternary SNARE complexes bound to Ni-NTA beads, but only ternary complexes could bind to beads and be pulled.
7. Drain and then wash the beads with 500 µL Buffer C three times to remove TCEP and free VAMP2 and syntaxin molecules.
8. Elute the SNARE complex using elute Buffer C.
9. Measure the concentration of the eluted SNARE complexes.
10. Immediately crosslink the SNARE complex and the DNA handle (Fig. 1A): Mix the DTDP-treated DNA handles with the purified SNARE complexes at a molar ratio of 1:100.

3.6 Preparation of the microfluidic flow cell

1. Use the laser engraver to cut the Nesco-film into the pattern with three channels (Fig. 1B).
2. Use the laser engraver to drill six holes in coverslips.

⁴For biotinylation, the protein concentration should be as high as possible.

3. Clean the coverslips and Nesco-films with ethanol and then allow them to dry in air.
4. Place a Nesco-film on each coverslip with holes and channels aligned by gently pressing the film against the coverslip, which allows the film to stick to the coverslip.
5. Place two bead dispenser tubes onto the Nesco-film, one between the top and central channel, and the other between the central and bottom channel. Place a protein injection tube with one end pointing the central channel. All tubes should be arranged as shown in Fig. 1B.
6. Place a film-coverslip assembly prepared in Step 4 on top of the above tube-aligned coverslip (prepared in Step 5) so that the two films touch each other and all channels and holes are aligned.
7. Sandwich the two coverslips together by heating the above two-coverslip assembly on a heating block at 95 °C until the films melt and seal the flow channels.
8. Install the flow cell on a holder, as is shown in Fig. 1.2 in ref. (45).
9. Test the flow cell, including the channel connectivity, by injecting water to three channels using a syringe.
10. Install the flow cell on a motorized stage that positions the flow cell between two objectives.
11. Inject beads to the top and bottom channels and the PBS buffer or α -SNAP solution to the central channel (Fig. 1C).

3.7 Single protein folding studies using optical tweezers

Single SNARE complexes were pulled in the absence and presence of α -SNAP in the solution. Two kinds of signals are obtained: force-extension curves (Fig. 2A) and time-dependent extension trajectories at constant trap separations or mean forces (Fig. 3A). From these measurements, information was derived on SNARE folding and unfolding transitions and α -SNAP binding and unbinding (Figs. 1B and 2B).

3.7.1 Measure the force-extension curves in the absence and presence of α -SNAP.

1. Wash all three microfluidic channels with PBS.
2. Mix the crosslinked SNARE-DNA sample (10-30 ng DNA) with 20 μ L DIG beads and incubate at room temperature for 15 min. Dilute the beads into 1 mL PBS buffer and transfer the bead solution to a 1 mL syringe.
3. Add 2 μ L streptavidin-coated beads in the stock solution into 1 mL PBS. Transfer the bead solution to a 1 mL syringe.
4. Connect the syringes to the top and bottom channels of the microfluidic cell and a 0.5 mL centrifuge tube to the protein injection tube with PE10 tubing (see Fig.

1.2 in ref. (45)). The centrifuge tube is also connected to a nitrogen source whose pressure can be controlled by the computer.

5. Add 50-100 μL 5 μM α -SNAP in PBS to the 0.5 mL tube.
6. Transfer 3 mL PBS containing the oxygen scavenging system to a 10 mL syringe and connect the syringe to the central channel. The pressure in the 10 mL syringe can also be computer-controlled.
7. Inject the beads to top and bottom channel manually. Catch a single SNARE-DNA-bound DIG bead and a single streptavidin-coated bead in each optical trap.
8. Calibrate the optical traps to determine their stiffness. The stiffness of each optical trap is determined by measuring the power-spectrum density distribution of the Brownian motion of the trapped bead at 80 kHz and fitting the distribution with the Lorentz distribution (45).
9. Bring the two beads close to form a tether (53).
10. Pull the tether at a trap separation speed of 10 nm/s to completely unfold the SNARE complex and then relax the protein at the same speed (Fig. 2A, FEC #1).
11. Inject α -SNAP to the central channel, wait for 60 second, and finally repeat the pulling and relaxation cycle as described in Step 10 (Fig. 2A, #2-3).
12. Flush away the α -SNAP solution from the central channel.
13. Test more single SNARE complexes by repeating Steps 8-11.

Representative FECs obtained from a single SNARE complex is shown in Fig. 2A. In this experiment, all measurements were acquired at 20 kHz, filtered online to 10 kHz, and stored on a hard-disk for further data analysis.

3.7.2 Measure time-dependent extension trajectories at constant trap separations—To better determine the effect of α -SNAP on SNARE folding and assembly, time-dependent extension trajectories were detected at a range of constant trap separations with a long time for each, revealing detailed kinetics of SNARE transitions and α -SNAP binding.

1. Pull a single SNARE complex in the absence of α -SNAP to a force to observe rare SNARE unfolding, hold the complex at the trap separation, and record the time-dependent extension and force for typically 30-120 seconds.
2. Increase the trap separation in a stepwise manner and repeat the extension and force recordings until the full force range of reversible SNARE transitions is covered.
3. Repeat Steps 1 and 2 in the presence of α -SNAP in the solution.
4. Pull the SNARE complex to higher force range to completely unfold the SNARE complex and record the corresponding FEC.

3.8. Data analysis

The purpose of data analysis is to extract protein folding information from the two types of signals from optical tweezers: Force-extension curves and extension-time trajectories at constant mean forces or trap separations. Force-extension curves are generally obtained by pulling or relaxing a single protein when the movable trap moves away or towards the other trap at a constant speed, typically 10 nm/s (Fig. 2A). These curves give the phase-diagram of protein transitions in a large range of forces, including the number of states involved in the protein folding and unfolding transitions, their transition pathways, and structural information of different states (Fig. 2B). However, since these transitions occur during constant pulling, some slow transitions may not reach thermodynamic equilibrium, leading to hysteresis in the force extension curves (16, 33).

Time-dependent extension trajectories at constant trap separations can reveal detailed dynamics of a specific protein transition under thermodynamic equilibrium, including number of states and their populations, lifetimes, energies, and associated transition kinetics (Fig. 3A). The major steps to analyze these trajectories are listed below:

1. Estimate the state number, positions, and populations using the histogram analysis (53). Filter a time-dependent extension trajectory with proper time window, calculate the probability density distribution of the extension, and fit the distribution with a sum of multiple Gaussian functions to determine the number of distinct states involved in the protein transition and their associated average extensions and populations (Fig. 3B).
2. Determine average state positions, populations, extensions, lifetimes, transition rates, and state transitions using hidden-Markov modeling (HMM) (41) (Fig. 4A,B). HMM requires the state number and approximate state positions from the histogram analysis shown in Step 1 as inputs. Calculate the average state forces from the corresponding time-dependent force trajectory using the derived state transitions. The mean of these state forces is chosen as the force plotted for the force-dependent state populations and transition rates (Fig. 4C).
3. Derive the state energies and transition rates at zero force by fitting the force-dependent state populations, forces, rates, and relative state positions using a theoretical model (Fig. 4C). The model accounts for the effect of force and DNA handle on protein folding in optical traps, as is described in detail elsewhere (43).

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Fig. 1.

Experimental setup to study regulated SNARE folding and assembly using dual-trap high-resolution optical tweezers and microfluidics. **(A)** Schematic diagram of optical tweezers and crystal structures of the neuronal SNARE four-helix bundle, the N-terminal regulatory domain (NRD) of syntaxin 1 (red), and the yeast α -SNAP homolog Sec17 (gray). A single SNARE complex is attached to two polystyrene beads via a 2,260 bp DNA handle and pulled in the absence or presence of α -SNAP in the solution. **(B)** Microfluidic flow cell used in optical trapping experiments. The flow cell contains three approximately parallel channels, with the top and bottom channels connected to the central channel by glass tubing. The flow cell is formed by sandwiching two coverslips by Nesco-films with a total thickness of $\sim 180\ \mu\text{m}$. **(C)** Diagram illustrating flows and connectivity of the channels. Single SNARE complexes are pulled in the central channel that may contain the α -SNAP solution. The figure is reproduced from our earlier work with permission (45).

Fig. 2.

Force-extension curves (FECs) showing different SNARE folding and α -SNAP binding states. **(A)** FECs obtained by pulling and then relaxing a single SNARE complex for three rounds (designated #1-3) in the absence of α -SNAP (#1) and presence of 5 μ M α -SNAP (#2-3) in the solution. Different pulling and relaxation phases are shown in different colors. Reversible transitions of the linker domain (LD) and the four-helix bundle domain (4HB) are marked by red rectangles. Irreversible unfolding of the t-SNARE complex and accompanying SNAP-25 dissociation is indicated by black arrows. SNARE folding states associated with different FEC regions are shown by the corresponding state numbers and illustrated in B. Solid red lines are best fits by the worm-like chain model (54, 55). **(B)** Schematic diagrams of different SNARE folding and α -SNAP binding states and their transitions. SNAP-25 contains an N-terminal SNARE motif (designated “SN1”) and a C-terminal SNARE motifs (“SN2”) connected by a disordered polypeptide. The figure is reproduced from our earlier work with permission (42).

Fig. 3. α -SNAP specifically stabilizes the SNARE four-helix bundle. (A) Extension-time trajectories at a constant mean force of 16.5 pN showing reversible SNARE four-helix bundle transitions and α -SNAP binding and dissociation. The average extensions of different folding and binding states are marked by green dashed lines and indicated by the corresponding state numbers. The α -SNAP-bound state (2 α) is colored red. All data here were obtained on a single SNARE complex under the same force in the absence (– α -SNAP) or presence (+ α -SNAP) of α -SNAP in the solution. Close-up views of the boxed regions in

the top two traces are shown at the bottom. **(B)** Probability-density distributions of the extensions shown in A (symbols) and their best fits by a sum of three Gaussian functions (curves). A comparison of the distributions in the absence (black) and presence (red) of α -SNAP indicates that α -SNAP specifically stabilizes the four-helix bundle state. The figure is reproduced from our earlier work with permission (42).

Fig. 4. Hidden-Markov modeling (HMM) reveals kinetics of reversible SNARE folding and unfolding and α -SNAP binding and unbinding. **(A)** Extension-time trajectories of a single SNARE complex at different constant mean forces (gray traces) and their idealized extension trajectories derived by HMM (colored symbols). **(B)** Close-up view of the boxed region in A. **(C)** State probabilities and transition rates as a function of force. Experimental

data (symbols) are fit by a model (curves) to reveal the energies and transition rates of different states at zero force. The figure is reproduced from our earlier work (42).