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EPR lineshape analysis to investigate the SNARE folding intermediates

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Abstract

SNARE complex formation, which is believed to drive intracellular membrane fusion, transits through multiple conformational states along the membrane fusion pathway. The SNARE intermediates are biologically important because they serve as targets for fusion regulators and clostridial neurotoxins. Spin labeling EPR has contributed significantly to the understanding of the structures and the dynamics of SNARE intermediates. In particular, the EPR lineshape analysis, which is highly sensitive to protein conformational changes such as the local coil-to-helix transition, has revealed the sequential compacting steps leading to formation of the highly stable four helix bundle.

Keywords

SNAREs; nanodiscs; membrane fusion; EPR; spin labels; exocytosis; neurotransmitter release

1 Introduction

1.1 Layered complexity of SNARE complex formation

It is now widely believed that SNARE proteins, which are highly conserved from yeast to human, drives intracellular membrane fusion (Söllner et al. 1993; Weber et al. 1998). The vesicle (v-) SNARE protein associates with the target membrane (t-) SNARE proteins to form a complex that brings about apposition and subsequently, fusion of two membranes. The SNARE complex is the fusion machine that provides the necessary free energy to overcome the energy barrier for fusion of two separate membranes that are otherwise individually highly stable when undisturbed.

The most critical piece of information to understand the mechanism of SNARE-dependent membrane fusion may be the three dimensional structure of the SNARE complex. The SNARE core complex is a highly stable, all parallel four-stranded coiled coil (Poirier et al. 1998; Stein et al. 2009; Sutton et al. 1998) that forms its parallel structure when it brings two membranes into close proximity. The high stability of the structure ensures the merge of two membranes. This stable formation justifies its structural and energetic role as the core fusion machine.

Equally important is the pathway through which the SNARE complex is assembled. Some SNARE-dependent membrane fusion (for example, synaptic vesicle fusion) is tightly regulated by auxiliary proteins including Ca^{2+} -sensor synaptotagmin 1 and sec1/munc (SM) family proteins (Südhof and Rothman 2009). It is believed that auxiliary proteins target the SNARE folding intermediates (Lou and Shin 2016). Thus, the structural investigations of SNARE folding intermediates appear to be essential towards the understanding of the mechanisms whereby the auxiliary proteins regulate membrane fusion.

At early stages, two t-SNARE proteins, one in the syntaxin family and the other in the SNAP-25 family, assemble into a 1:1 t-SNARE complex, which will serve as the receptor for v-SNARE. In the t-SNARE complex, one SNARE motif (~70 residue-long heptad repeat) from the syntaxin-1A and two N- and C-terminal SNARE motifs from SNAP-25 form a highly dynamic, three-stranded coiled coil, where the C-terminal SNARE motif of SNAP-25 has tendency to uncoil to a great extent (An and Almers 2004; Khounlo et al. 2017). Adding to the complexity, there is evidence that association of v-SNARE with the t-SNARE complex occurs in multiple (at least two) sequential steps: The assembly starts from the membrane-distal N-terminal region and proceeds towards the membrane-proximal C-terminal domain (Sørensen et al. 2006), thereby driving a gradual apposition of two membranes. The folding intermediates are likely to be transient and meta-stable and thus, offer formidable challenges for structural investigations (Gao et al. 2012; Min et al. 2013; Shin et al. 2014a).

1.2 Spin labeling EPR on SNARE complex formation

Over the years, spin labeling EPR has contributed significantly to the understanding of the structure and dynamics of the SNARE core complex and its folding intermediates. In site-directed spin labeling EPR (Hubbell et al. 1998), a specific, selective residue is replaced with a unique cysteine and the cysteine is labeled with an EPR-active nitroxide. EPR of spin labeled mutants offers three powerful experimental avenues to explore the structure and the function of SNARE complexes (McHaourab et al. 2011). The first is the distance measurement between two site-specifically attached nitroxides within the complex (Rabenstein and Shin 1995). The distance measurement method has been used to determine the first four-helix bundle structure of the SNARE core complex (Poirier et al. 1998), the structure of the t-SNARE complex that is consist of syntaxin and SNAP-25 in the 2:1 stoichiometry (Xiao et al. 2001; Zhang et al. 2002), and the conformational change of the transmembrane domain (TMD) of v-SNARE caused by cholesterol (Tong et al. 2009). The detailed methods and the experimental protocols are extensively described in the Method in Molecular Biology article by Oh et al.

The second is the measurements of accessibilities to non-polar O₂ and polar, soluble paramagnetic NiEDDA, the ratio of which is used to measure the membrane immersion depth of the nitroxide attached to the membrane-embedded polypeptide (Altenbach et al. 1994). The method is grossly empirical. Nevertheless, it has proven to yield fairly accurate estimation of the membrane immersion depth. To be an effective fusion machine, the SNARE complex must be able to transfer the force generated by the core region to the transmembrane domains. We believe that the linker region acts as the force transducer. The EPR accessibility measurements reveal that despite highly basic nature of the both v- and t-SNARE linker regions, they are immersed into the membrane with some secondary structures (Chen et al. 2004; Kim et al. 2002; Kweon et al. 2002; Kweon et al. 2003). Thus, they help make a tight connection between the SNARE core and the transmembrane domain and may structurally qualify as the effective force transducer. Furthermore, the determination of the structure of the v-SNARE TMD laid the groundwork for designing the mutant that traps the hemifusion intermediate, leading to the first time discovery of hemifusion in SNARE-dependent membrane fusion (Xu et al. 2005).

1.3 EPR lineshape analysis to peel off layers of SNARE complex formation

The third avenue, which is the main focus of this chapter, is the EPR lineshape analysis, taking advantage of the EPR's superb sensitivity to the motional rate of the nitroxide (Columbus and Hubbell 2002). For example, the folding of a polypeptide from a random coil to an α -helix or the binding of the unstructured polypeptide to the membrane gives rise to a dramatic lineshape change from a narrow, fast motional spectrum to a fairly broad, intermediate motional spectrum, which are visually distinguishable from each other. If the nitroxide makes an additional tertiary or steric contact, the lineshape change is even more profound to become very broad which reflects very severely restricted motion. SNARE complex formation involves these types of conformational changes which are accompanied by dramatic EPR lineshape changes for the nitroxide attached to SNARE motifs.

SNARE motifs, when not in the complex, are mostly unstructured and freely moving in solution, resulting in sharp, fast motional EPR spectra for the nitroxides. However, when complexed with other SNARE partners, the motional rate of the nitroxide slows down significantly and the EPR lineshape becomes broad (Chen et al. 2004; Kweon et al. 2003). Very interestingly, however for long SNARE complexes in particular, a conformational change could be localized specifically to a certain part of the protein. For example, SNARE zippering is expected to transition through a partially folded conformation in which the N-terminal coiled coil is intact while the C-terminal region is frayed. The EPR lineshape analysis is uniquely suited to investigate such local conformational changes and has proven powerful in characterizing the structures of SNARE folding intermediates (Khounlo et al. 2017; Zhang et al. 2005).

For the SNARE complex, its working environment is the narrow gap between two closely apposed membranes. However, most structural studies have been carried out by employing isolated proteins, away from such a special situation. Thus, more often than not, the interpretation of the structural outcomes is often ambiguous. Alternatively, the recently advanced nanodisc technology makes it possible to create the membrane platform that

mimics the native-like environment for SNARE complexes. One could place a single SNARE complex within a two nanodisc sandwich by reconstituting t- and v-SNAREs to separate nanodiscs and allowing them to form the *trans* complex between the two nanodiscs. Such an experimental platform has been successfully constructed and the structure of the SNARE complex has been examined using the EPR lineshape analysis (Shin et al. 2014b). The results are exciting and reveal that a half zippered SNARE complex in which the C-terminal half of v-SNARE, which is the downstream of conserved middle 1R3Q layer, is free while the N-terminal half of the SNARE complex is an intact coiled coil has been identified as a likely metastable fusion intermediate.

Although the lineshape analysis is the least explored avenue of spin labeling EPR in structural biology, it has been instrumental in characterizing the structure and the dynamics of SNARE folding intermediates in the native-like environment. Additional contribution with this approach include, but not limited to, the characterization of partially folded t-SNARE core (Khounlo et al. 2017) and structural disruption of the C-terminal region of the SNARE complex by the membrane (Zhang et al. 2005). Overall, EPR has shown to be a powerful technique in observing the structural transitions in SNARE complex formation (Figure 1). In this chapter, we will review the protocols of the sample preparations, EPR experiments, and data analysis for the EPR lineshape analysis on SNARE proteins.

2 Materials

1. Primers: Synthesized by the Iowa State DNA facility.
2. QuikChange Kit: Agilent Technologies QuikChange Site-Directed Mutagenesis Kit.
3. Thermocycler: MJ Mini Thermal Cycler.
4. Restriction enzyme DpnI: FastDigest DpnI.
5. PCR cleanup kit: QIAquick PCR Purification Kit.
6. Nanodrop: Thermo Scientific™ NanoDrop™ 2000 Spectrophotometer.
7. 10 mg/mL tetracycline: 100 mg of tetracycline is added to a final volume of 10 mL of double-deionized water (ddH₂O). Store at −20°C.
8. *E.coli* XL1 Blue and BL21 DE3 competent cells.
9. Luria broth (LB): 25 g/L of premixed 10 g/casein digest peptone, 10 g/L sodium chloride, and 5 g/L yeast extract are dissolved in ddH₂O and autoclaved.
10. 100 mg/mL ampicillin: 1 g of ampicillin is dissolved into a final volume of 10 mL ddH₂O. Store at −20°C.
11. 50 mg/mL kanamycin: 0.5 g of kanamycin is dissolved into a final volume of 10 mL ddH₂O. Store at −20°C.
12. QIAprep Spin Miniprep Kit.

13. 1 M isopropyl- β -D-thiogalactopyranoside (IPTG): 2.38 g of IPTG is dissolved in a final volume of 10 mL ddH₂O. Store at -20°C .
14. Centrifuge and rotors: A Beckman Coulter Avanti J-25 centrifuge is used in conjunction with a JA-14 rotor for cell pelleting and a JA-25.5 rotor for spinning down cell lysate.
15. 10X Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, and 1.8 mM KH₂PO₄ with a pH of 7.4. The pH is not adjusted. Store at 4°C .
16. Phosphate-buffered saline with triton (PBST): Generated from 10X PBS. Final concentration of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 1.8 mM KH₂PO₄ and 0.4% Triton X-100 with a pH of 7.4. Store at 4°C .
17. 1 M DL-dithiothreitol (DTT): 1.54 g of DTT is dissolved in a final volume of 10 mL ddH₂O. Store at -20°C .
18. AEBSF: 0.25 g of AEBSF is dissolved in a final volume of 5 mL ddH₂O. Store at -20°C .
19. 20% N-lauroylsarcosine: 2 g of N-lauroylsarcosine is dissolved in a final volume of 10 mL ddH₂O. Store at 4°C .
20. GSH beads: Glutathione Agarose Beads.
21. Phosphate-buffered saline with N-octyl-B-D-glucopyranoside (PBS-OG): Generated from 10X PBS. Final concentration of 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂PO₄, 1.8 mM KH₂PO₄ and 0.8% OG with a pH of 7.4. Store at 4°C .
22. Thrombin.
23. Glycerol.
24. Bio-rad RC DC Protein Assay Kit II.
25. Spin concentrators: Amicon® Ultra – 0.5mL Centrifugal Filters Ultracel® - 3K.
26. Desalting column: PD MiniTrap™ G-25 (GE Healthcare).
27. 100 mM MTSSL: 50 mg methanethiosulfonate (MTSSL) (Fisher Scientific) is added to 1.89 mL of acetonitrile. Store at -20°C wrapped in aluminum foil.
28. 100 mM TEMPOL: Add 172 mg of 4-Hydroxy-TEMPO (Sigma Aldrich) to a final volume of 100 mL ddH₂O. Store at 4°C .
29. Bruker Elexsys E500 X-band EPR spectrometer equipped with the loop-gap resonator (Medical Advances) and a low-noise microwave amplifier (Militech).
30. XEPR: Bruker Xepr software suite version 2.6b.54 is used in Linux (OpenSuse 11.3).
31. Lipids: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) are removed from their vials

and transferred to amber, glass bottles. All bottles are capped, sealed with parafilm, and vacuum sealed for storage. Store at -20°C in the dark.

32. 100 mg/mL cholesterol: 100 mg of cholesterol powder is dissolved to a final volume of 1 mL using chloroform in an amber, glass bottle. All bottles are capped, sealed with parafilm, and vacuum sealed for storage. Store at -20°C in the dark.
33. Vacuum desiccator: Bel-Art™ SP Scienceware™ Space Saver Vacuum Desiccators.
34. Kimtech wipes: Kimberly-Clark™ Professional Kimtech Science™ Kimwipes™ Delicate Task Wipers, 1-Ply.
35. Phosphate-buffered saline (10% OG): 100 mg of OG is added to 100 μL of 10X PBS. The final volume is adjusted to 1 ml with ddH₂O. Store at room temperature.
36. 500 μM sodium cholate: 3 α ,7 α ,12 α -Trihydroxy-5 β -Cholan-24-Oic Acid (Anatrace). 215 mg of sodium cholate is dissolved in a final volume of 1 mL using T150. It is very important that this comes from a company that synthesizes it, not one that purifies it from a biological source. The enzyme contaminants in the biologically purified sodium cholate will degrade lipids and membrane proteins.
37. Apo-A1 is recombinantly expressed and purified (*see* Note 10).
38. T150 buffer: 10 mM tris base and 150 mM sodium chloride. This pH is adjusted to 7.4. Store at 4°C .
39. Bio-Beads: Bio-Beads SM-2 Resin (Bio-Rad). A 1:1 solution of bio-beads in T150 is made by treating the bio-beads with methanol to remove air and equilibrating to desired buffer. Add 10 mL of bio-beads to a 25 mL batch column. The bio-beads are washed with 10 column volumes (CV) of methanol, ensuring that the bio-beads are constantly submerged. At the end of the last wash, immediately wash with another 10 CV of T150 (or buffer of choice). Near the end of the last wash, cap the tip of the column, fill the column 20 mL with

¹⁰His-Apo-A1 is in a pET28b vector and GST-Apo-A1 is in a pGEX-KG vector. Both are recombinantly expressed in *E. Coli* BL21 DE3 cells grown in 500 mL of LB medium with either kanamycin (50 $\mu\text{g}/\text{ml}$) for His-Apo-A1 or ampicillin (100 $\mu\text{g}/\text{ml}$) for GST-Apo-A1 at 37°C and 200 rpm to an OD₆₀₀ of 0.6–0.8. Once at the optimal OD₆₀₀, the cells are induced with 150 μL of IPTG (1 M) and grown for an additional 16–18 hours at 16°C . The cells are pelleted at $3,800 \times g$ and 4°C for 10 minutes in a JA-14 rotor and then, resuspended in ~15 ml of lysis solution. The lysis solution should consist of PBS pH 7.4 along with 2.5 mM DTT, 60 μM AEBSF, and 0.15% N-lauroylsarcosine (with 20 mM imidazole for his-Apo-A1). The cells are lysed with 3 passes through a cell homogenizer. The lysate is spun down at $27,200 \times g$ and 4°C for 30 minutes in a JA-25.5 rotor. ~1 ml of the affinity beads are equilibrated in a 25 mL batch column with their respective lysis buffers while the cells are centrifuging, Ni-NTA Agarose (his-Apo-A1) or GSH beads (GST-Apo-A1). The supernatant is then added to the beads and nutated at 4°C for 2 hours. The supernatant is drained from the columns and the beads are washed with 5 CV PBS pH 7.4 (with 20 mM imidazole for his-Apo-A1). His-Apo-A1 is eluted with PBS pH 7.4 with 200 mM imidazole 1 mL at a time. Significant amounts of protein are found in fractions 2–4. The GST-Apo-A1 is cleaved off of the GSH beads by incubating with 30U of thrombin in 1 mL of PBS for 2 hours at room temperature and eluting 1 mL at a time in the same buffer. 177 μL of glycerol is added to each 1 mL eluted fractions of both types of Apo-A1 to have a final concentration of 15% glycerol. After the concentration has been checked, they are divided in stock concentrations and stored at -80°C . These remain active for ~1 year.

T150 (or buffer of choice), and transfer the bio-beads to a 50 mL tube for storage at 4°C.

40. FPLC: BioLogic DuoFlow 10 System Fraction collector (Bio-Rad).
41. Size exclusion column (SEC): GE Healthcare Life Science Superdex 200 10/300 GL.
42. Ni-NTA Agarose Resin: Thermo Scientific™ HisPur™ Ni-NTA Resin.
43. 5 M imidazole: 17.02 g of imidazole is dissolved in a final volume of to 50 mL ddH₂O. The pH is corrected to 7.4. Store at 4°C wrapped in aluminum foil.
44. EPR sample loading tips: Fisherbrand™ Gel-Loading Tips, 0.5–10 µL.
45. Capillary tubes: Borosilicate capillary tubes with an internal diameter (i.d.) of 0.6 mm and an outer diameter (o.d.) of 0.84 mm (VetroCom) are sealed at one end with a Bunsen burner.
46. Ethanol.

3 Methods

3.1 Generation of cysteine mutant plasmid

1. Design primers (*see* Note 1).
2. Site-directed mutagenesis. The thermocycler is set at following protocol: Heat lid to 105°C, 98°C for 2 minutes, 98°C for 15 seconds, 55°C for 1 minutes, 68°C for 5.5 minutes, repeat steps 2–4 for 16 cycles (17 cycles in total), 68°C for 11 minutes, 4°C until stopped.
3. Transformation into *E. coli* XL1 Blue competent cells (*see* Note 2). 100 ng of purified mutant plasmid is transformed into ~50 µL XL1 Blue competent cells. Transformed cells are incubated with 1 ml Luria Broth (LB) at 37°C for 1–2 hours shaking at 200 rotations per minute (rpm). After incubation, the cells are plated onto ampicillin plates and incubated upside down at 37°C for 16–18 hours.

¹When using site-directed spin labeling to study a change in structure or conformation, an important criterion is that the label will minimally interfere with the native structure or binding sites. Specifically for SNARE proteins, sites are chosen to introduce cysteines that face the outside of the four-helical bundle. Primers are designed according to the Agilent QuikChange II Site-Directed Mutagenesis Kit protocol (QuikChange II Site-Directed Mutagenesis Kit: Instruction Manual). The most effective primers are ~33 nucleic acid-long (15 before the site of desired mutagenesis, then the cysteine codons (TGT or TGC), and 15 after the site of mutagenesis). When necessary, the length of the primer can be extended with native nucleotides, so that the primers begin and end with multiple G or C's to allow for tighter annealing (e.g. GC, CC, GG, and CG). The Northwestern Oligonucleotide Properties Calculator (Kibbe 2007) is used to measure GC%, melting temperature, and test for self-complementarity for generated primers. Normally, the GC% and melting temperature are satisfactory according to the Agilent QuikChange protocol (QuikChange II Site-Directed Mutagenesis Kit: Instruction Manual) (>40% and >78°C, respectively). However, the most common problem is with self-complementarity. To address this issue, the codon either right before or after the introduced cysteine is changed to an alternative codon for the same amino acid in *E. coli*. Once the issue is resolved, the reverse complementary primer sequence is obtained from the same web page. The primers for our studies are synthesized by the Iowa State DNA facility.

²When using a new plasmid, controls are necessary to ensure a proper transformation. The method is adapted from the Addgene heat-shock transformation protocol (Addgene: Bacterial Transformation 2017). Transformations are grown on LB-agarose antibiotic plates. A positive control is performed by transforming and plating a plasmid with a known antibiotic resistance to test for competency of cells. A negative control is performed by plating the competent cells directly on an ampicillin agar LB plate to verify absence of native resistance. If the transformed SNARE colonies have not formed within 24 hours, repeat the transformation. If colonies still do not form, repeat the PCR reaction. If colonies still do not form, design new primers.

4. Screen for the desired mutation. Pick off 3–5 isolated colonies with different sterile pipette tips and add each colony to separate 50 mL sterile tubes containing 10 mL LB-ampicillin (100 µg/mL). Cap the tubes, but do not screw on the cap tightly. If necessary, tape the cap on to prevent it from falling off. Incubate the colonies for 16–18 hours at 37°C while shaking at 200 rpm.
5. Purifying amplified plasmids. The QIAprep Spin Miniprep Kit and protocol are used (QIAprep® Miniprep Handbook 2012). The DNA concentration is measured using a Nanodrop in the dsDNA nucleic acid mode.
6. Plasmids are sequenced by the Iowa State University DNA facility using Sanger Sequencing and sequences are aligned with the wild-type sequence to verify that only the desired mutation is made.

3.2 Purification of recombinant proteins

1. Transform verified cysteine mutant plasmid into *E. coli* BL21 (DE3) competent cells.
2. Grow the starter culture. Follow the same protocol as 3.1 step 4.
3. Grow a large culture. Inoculate 500 mL of LB-amp with 5 mL of the starter culture and incubate at 37°C while shaking at 200 rpm until the optical density at 600 nm (OD₆₀₀) reaches 0.6–0.8. Chill for at least 30 minutes at 4°C, induce with 150 µL of IPTG (1 M), and incubate at 16°C and 200 rpm for 16–18 hours.
4. Lyse the cells. After induction, pour out the large culture into 250 mL centrifuge tubes. Pellet the cells by centrifuging at 4°C for 10 minutes at 3,800 × g in a JA-14 rotor. Once cells are pelleted, pour out the supernatant and resuspend the cells in 20 mL of lysis buffer (see Note 3). Add 75 µL DTT (1 M), 30 µL AEBSF (50 mg/mL), and 150 µL N-lauroylsarcosine (20% w/v) to the resuspended cells. Lyse cells using a homogenizer. Lysate should change from a viscous opaque color to a fluid clear color indicating successful lysis.
5. Bind protein to affinity beads. Add the lysate to 50 mL centrifuge tubes and centrifuge with at 4°C for 30 minutes at 27,200 × g in a JA-25.5 rotor. While the lysate is centrifuging, add ~1 mL of GSH beads to a 25 mL batch column and wash with 3 column volumes (CV) of ddH₂O, then 1 CV of lysis buffer to equilibrate the column ensuring the beads stay continuously hydrated. Cap the tip of the column and add the supernatant from the centrifuged lysate to equilibrated beads. Cap the top of the column and nutate the mixture at 4°C for ~2 hours.

³Phosphate-buffered saline (PBS) at pH 6.85 is the lysis buffer for soluble proteins (SNAP-25). Phosphate-buffered saline with triton X-100 (PBST) at pH 6.85 is the lysis buffer for membrane proteins (syntaxin-1A (stx), VAMP2 or synaptobrevin 2 (VpF)). Phosphate buffers are preferred over tris-base buffers due to the unfavorably low MTSSL-cysteine reactivity in the presence of tris. The pH of PBS is set to 6.85 to reduce nonspecific labeling (e.g. to amines) at more basic pH levels. The elution buffer for soluble proteins is the same as the lysis buffer. The elution buffer for membrane proteins is phosphate-buffered saline 0.8%-octyl-beta-Glucoside (PBS-OG) at pH 6.85.

6. Purify the protein of interest. After ~2 hours, drain the supernatant from the column by first removing the cap from the top of the column and then the cap from the tip of the column. Wash the beads with 5 CV of lysis buffer. After washing, the beads are buffer-exchanged into their elution buffers (*see Note 3*). Buffer exchange by adding three 1 mL aliquots to the washed beads. Let each aliquot fully flow through before adding the next. Cap the tip of the column and add 1 mL of elution buffer and 30 μ L of thrombin (1U/ μ L) to the buffer-exchanged beads. Cap the top once all the contents are added. Cleave off the purified protein by either incubating the column at room temperature for 1.5 hours or 4°C for 16 hours. Ensure homogenous distribution of the thrombin by cleaving on a nutator.
7. Elute purified protein. Remove the cap off the top of the column first and then remove the cap off tip of the column over an Eppendorf tube to collect the first fraction. Elute the remaining protein by adding 1 mL aliquots of the elution buffer to the beads and collecting. Significant amounts of protein should appear in the first three fractions. Add 177 μ L of glycerol to each 1 mL fraction making the final solution 15% glycerol (v/v). Glycerol serves as a cryo-protectant for storage at -80°C.
8. Check the protein purity. Hand cast 12% SDS-PAGE gels according to the Bio-Rad protocol (Bio-Rad: Handcasting Polyacrylamide Gels). Aliquot out 10 μ L of the eluted protein (*see Note 4*). Add 5X SDS-PAGE loading dye to purified protein so that volume ratio of dye to protein is 1:4. Load the gel into the electrophoresis system and load the entire protein-dye sample along with a protein ladder into the wells. Run at 40 mA for 35 minutes for a single gel or 60 minutes for two.
9. Estimate the protein concentration. Use the Bio-Rad RC DC kit (Bio-Rad: RC DC Protein Assay) and protocol to estimate the protein concentration. Concentrate and store at -80°C (*see Note 5*).

4. When a new protein is purified for the first time, it is advised that every step in the purification process be checked. This has proven to be especially true when purifying SNARE proteins in the manner described. A SDS-PAGE gel can easily help track the progress in the purification of the protein of interest by saving an aliquot after each step. 10 μ L is saved from the resuspended cells before lysis, the lysate, the supernatant and pellet after centrifugation, the flow through as the supernatant leaves the column in step, the washed beads before cleavage and after cleavage, and the eluted protein. Dilute the aliquots taken from resuspended cells, lysis, supernatant, pellet, and flow through to 50 μ L with the lysis buffer. Take 10 μ L of each of these, add 2.5 μ L of 5X SDS-PAGE loading dye, and boil for 10 minutes. During the boiling process, do not let the caps of the Eppendorf tubes pop open. Perform a quick spindown to gather all the liquid back to the bottom of the tube. Add 2.5 μ L of 5X SDS-loading dye to the other undiluted saved aliquots. Load and run the entire sample for each step using a 15% SDS-PAGE gel along with a ladder.

The SDS-PAGE gel is also useful for determining the efficiency of the SNARE protein purification. If the resuspended cells or lysate do not contain large amounts of SNARE protein, try performing a fresh transformation. It is advised to make a fresh transformation for every expression. If the majority of the SNARE protein is in the pellet, this usually means that the cells are not sufficiently lysed. It can also mean that the expression temperature was too high, which can produce inclusion bodies. If neither of these solutions resolve the issue of low yield, then the detergent concentration for the PBST lysis buffer can be increased. This will weaken the lipid membrane of the cells allowing for a more efficient lysis. If a significant amount of SNARE protein is in the flow through, there is most likely an issue with the amount of effectiveness of the affinity beads. If the protein appears impure on beads prior to cleavage, additional washes are needed. If the majority of SNARE protein remain on the beads after cleavage, then try increasing the salt concentration in the elution buffer. The salt concentration can be increased up to a maximum of 500 mM for effective elution. If the eluted fractions contain higher molecular weight impurities, this is most likely due to the residual amounts of thrombin in solution. 1 μ L of AEBSF (200 mM) is added to each 1 mL elution to deactivate the residual thrombin.

5. The ideal labeled SNARE protein concentration for EPR is 50 μ M. Using our method of spin labeling, consecutive steps of spin concentrations and PD-10 desalting columns are necessary. Each time one of these steps is performed, a small amount of protein is lost, decreasing the labeled protein concentration. In EPR experiments, the labeled proteins are mixed with unlabeled SNARE partners to form the SNARE complex. The mixing of the two or three proteins also decreases the labeled protein concentration. To address

3.3 Spin Labeling Recombinant Proteins

1. Reduce protein for efficient spin labeling. An aliquot of purified protein is thawed on ice. The protein is then diluted to a final volume of 500 μ L in a solution of PBS + DTT (5 mM). This mixture simultaneously reduces the cysteines and prepares the protein for the PD-10 desalting column. The mixture is incubated for 30 minutes at 4°C. While the protein is being reduced, the desalting columns are prepared by pouring off the storage buffer and equilibrating with 3 CV of elution buffer. Calculate the volume of MTSSL in order to have the spin label : protein ratio at 10 : 1 (*see* Note 6). After incubation, the mix is added to the equilibrated desalting column. Once the mixture has fully loaded into the column, 1 mL of elution buffer is used to elute the reduced protein off the column. The flow through from this step contains the reduced protein and should be collected.
2. Spin label reduced protein. Add the calculated volume of MTSSL as soon as reduced protein elutes from the desalting column. The protein is spin labeled overnight by nutating for 16–18 hours at 4°C.
3. Remove excess spin label. Concentrate the labeled protein to 500 μ L using a 3K spin concentrator. During the centrifugation, prepare another desalting column as in 3.3 step 1. A concentration cycle should be about $14,000 \times g$ at 4°C for 8 minutes with resuspension between cycles to prevent aggregation. Load the concentrated spin labeled protein onto the equilibrated PD-10 desalting column. This desalting column removes a majority of the excess MTSSL from the labeled protein. Elute the spin labeled protein and collect.
4. Remove residual excess spin label. Spin wash the eluted labeled protein by concentrating it to ~250 μ L in a new 3K spin concentrator and then filling the remaining portion of the spin concentrator full of elution buffer. Use the same concentration cycle procedure as in 3.3 step 3. Three spin wash cycles should remove the remaining of excess MTSSL. Re-estimate the protein concentration using the Bio-Rad RCDC kit (Bio-Rad: RC DC Protein Assay).
5. Measuring labeling efficiency (*see* Note 7). EPR spectra of TEMPOL standard solutions of known concentrations are collected. The EPR spectra are processed by correcting for the baseline and double integrating. These double integration values are plotted against the spin concentrations to generate a standard curve. The EPR spectrum of the spin labeled protein of a known protein concentration

these issues, the SNARE proteins are aliquoted into stock concentrations well above 50 μ M. It is preferable to store 150 μ L aliquots at a concentration greater than 100 μ M protein.

⁶The nitroxide spin label (MTSSL) is dissolved in an acetonitrile solution. Acetonitrile may cause proteins to aggregate and fall out of solution, so it is ideal to limit the amount that is added when spin labeling. The 20 mM MTSSL stock is recommended when using volumes equal to or less than 20 μ L. If a larger volume is required, use the 100 mM stock. It is also important to add MTSSL to the protein instead of vice-versa. This way the protein is able to interact with the most dilute amount of the acetonitrile preventing aggregation.

⁷A 200 mM stock solution of TEMPOL is made by dissolving 344 mg of 4-Hydroxy-TEMPOL (Sigma Aldrich) in 100 mL of ddH₂O. This is quantitatively made in a volumetric flask. This stock solution is diluted to generate the TEMPOL standard solutions at 10, 25, 50, 75, and 100 μ M. These concentrations cover the concentration range of the raw SNARE proteins when purified. The standards must generate a linear curve with an R^2 value of 0.95 or higher for an accurate measurement.

is measured and processed under the same conditions as the TEMPOL standards. The spin label concentration of the protein sample is determined by comparing its double-integration value with the TEMPOL standard curve. The spin label concentration of the protein sample is then divided by the protein concentration determined by the Bio-Rad RCDC kit (Bio-Rad: RC DC Protein Assay) to obtain the spin-labeling efficiency. Spin labeling efficiencies using our method are usually over 90%.

3.4 Reconstitution of spin labeled protein into nanodiscs

1. Prepare the stock lipid mixture. Lipids are carefully mixed in a glass tube so that when resuspended in 100 μ L of T150 buffer, the total lipid concentration is 50 mM. The final lipid mixture of PC : PS : cholesterol is at a molar ratio of 65 : 15 : 20. The chloroform in the mixture is evaporated under an air stream to dry the lipids. The dried lipid film is placed in a vacuum desiccator at room temperature overnight (16–18 hours) (*see* Note 8). Resuspend with 100 μ L of T150 buffer by incubating in a 42°C water bath for 1 minute and vortexing for 1 minute. Repeat until the lipid film has been resuspended. The lipid stock can be stored at –80°C for ~2 weeks.
2. Preparing nanodisc mixture. Section 3.4 steps 2–3 are summarized in Figure 2. The goal is for the end product of lipids : labeled protein : Apo-A1 to be at a molar ratio of 400 : 1 : 4. This will be referred to as the nanodisc mixture. 5 μ L of the lipid stock solution is added to sodium cholate so that the final concentration of sodium cholate in the nanodisc mixture is 50 mM. This mixture is incubated on ice for 5 minutes.
3. Adding SNAREs to the nanodisc mixture (*see* Note 9). The v- and t-SNARE proteins are added to the separate sodium cholate lipid mixtures. The proteins are added in a lipid : protein ratio of 400 : 1. When incorporating the t-SNARE proteins, Stx is used to determine the 400 : 1 ratio. This mixture is incubated on ice for 5 minutes. Apo-A1 (*see* Note 10) is added to the mixture in a lipid : Apo-A1 ratio of 100 : 1 and it is incubated on ice for 5 minutes. His-tagged Apo-A1 is used when incorporating t-SNAREs and untagged Apo-A1 is used for v-SNARE when forming the *trans*-SNARE complex between two nanodiscs with the intention to purify the complex with the Ni-NTA column.
4. Reconstitution of labeled SNAREs into nanodiscs. Bio-beads are added to the mixture at a 1 : 2 volume ratio. This is incubated on ice for 5 minutes and shortly

⁸When drying the lipids in the glass tube, start with soft air pressure while constantly rotating the glass tube. This generates a thin lipid film that forms around the inner edge, ideally on the bottom centimeter of the tube. Once the film has mostly dried, increase the air pressure to ensure the lipids are completely dried. It is important to apply soft pressure initially to avoid clumping the lipids at the bottom of the tube. Clumped lipids are found to generate inconsistent vesicles. After the lipids are fully dried, a Kimtech wipe is rubber banded across the opening of the tube to prevent particulates from entering. This is then stored in the dark inside a vacuum desiccator at room temperature overnight (16–18 hours).

⁹Prior to reconstitution, the t-SNARE proteins, Stx and SN25, are premixed and incubated for 30 minutes at room temperature. Since SN25 is not a membrane protein, PBS (10% OG) is added to maintain the detergent concentration in the solution above critical micelle concentration (0.08%). The mixture of Stx : SN25 : PBS (10% OG) is added at a ratio of 1 : 1.5 : 0.12. An excess amount of SN25 is used in comparison to Stx in order to prevent formation of an off-pathway 2 : 1 complex. After incubation, the t-SNARE complex is stored on ice until needed.

spun to pellet the bio-beads. Repeat the same process on the supernatant using the same amount of fresh bio-beads. It is easier to collect the supernatant from the bio-beads if a small cavity is made in the pelleted bio-beads (*see Note 11*). The supernatant volume is either concentrated to 120 μ L using a spin concentrator or diluted to 120 μ L with T150 buffer. Filter supernatant with a Spin-X centrifuge filter and store on ice.

5. Purification of SNARE-reconstituted nanodiscs using size exclusion chromatography. All samples used on the size exclusion column (SEC) must be filtered and buffers must be both filtered and degassed. Filter and degas 500 mL of T150. Wash SEC with 2 CV of filtered and degassed T150 at 0.5 mL / minute. Inject the sample into the sample loop and load with 2 mL of T150 at the 0.5 mL / minute flow rate. Elute with 1.5 CV of T150 at the flow rate of 0.5 mL / minute. Nanodiscs usually elute between the 12–14 mL fractions.
6. Form *trans*-SNARE complex within two nanodiscs. **This step can be skipped if only a single species of the nanodisc is desired.** ~1 mL of Ni-NTA beads is added to a 25 mL batch column, washed with 2 CV of ddH₂O, and equilibrated with 1 CV of T150. The tip of the column is then capped. Both v-SNARE nanodiscs and t-SNARE nanodiscs purified from FPLC are added to the Ni-NTA beads, the top of the column capped, and the mixture is nutated overnight at 4°C. The top of the column is first opened and then the tip removed from the bottom allowing unbound nanodiscs to run off the column. The remainder of unbound nanodiscs are washed off with 2 CV of T150 buffer. The nanodiscs that have formed the *trans*-SNARE complex are eluted with 300 mM imidazole in T150 buffer.

3.5 Electron paramagnetic resonance (EPR)

1. Prepare samples for EPR. This process is summarized in Figure 3. Pipette up 10 μ L of sample into EPR tube loading tips. Take the loaded EPR loading tip and put it in the open end of a sealed EPR capillary tube. Place the EPR capillary tube and tip into a 15 mL tube without the cap. The 15 mL tube is used as an adaptor to centrifuge the sample in the loading tip into the EPR tube. Place 15 mL tube in a clinical centrifuge at the maximum speed for 30 seconds. The protein solution should have moved from the EPR loading tip and be settled at the bottom part of the EPR capillary tube. Clean the EPR capillary tube by dipping it in ethanol and drying with a Kimtech wipe. Place the EPR capillary tube in the loop-gap resonator and collect the spectrum. Measure EPR spectra (*see Note 12*).

¹¹The solution of bio-beads needs to be thoroughly resuspended before measuring out bio-beads because they quickly fall out of solution. Pipetting bio-beads is made much easier if the last third of a 200 μ L tip is cut off. It is easiest to collect the supernatant from the bio-beads if a small cavity is made in the pelleted bio-beads. This is done by decanting a small amount of the supernatant with a pipette tip, submerging the tip below the bio-beads layer, injecting the supernatant from the tip to create a cavity, moving the pipette tip to the bottom of the tube, and then quantitatively decanting all the supernatant from the cavity. This method prevents bio-beads from clogging the pipette tip while decanting the supernatant.

¹²Spectra are collected at 1 mW incident microwave power using a field modulation of 2 Gauss at 100 kHz. The scans are performed at room temperature with a scan width of 120 Gauss and 1024 data points per scan. The time constant is set to 40.96 ms and conversion time of 40.96 ms. 20 scans was sufficient to obtain a clear EPR spectra.

2. Spectral subtraction. The process is summarized in Figure 4. All data analysis is performed in Bruker's EPR suite Xepr vs. 2.6b.54. Collect the EPR spectrum of uncomplexed (or unbound) SNARE and that of the SNARE complex. The former has a narrow lineshape, reflective of freely moving random coil while the latter has a composite (narrow + broad) lineshape, reflective of the equilibrium coexistence of a random coil species and the structured SNARE complex (Figure 4A). Process the spectra using the baseline correction and the normalization functions of the Xepr software suite. After processing, both spectra are brought back to the derivative spectral mode by double derivatization for direct comparison (Figure 4B). Baseline correction is usually performed using a 1st order polynomial linear fit to the 20 outermost data points on either end of the spectra. Center and overlay two spectra on top of each other (Figure 4C). Adjust the gain of the unbound spectrum so that the height of the 3rd peak in the unbound spectrum roughly match the height of the shape component of the 3rd peak in the composite spectra. Subtract the unbound from the composite to obtain the bound fraction spectrum (Figure 4E). Adjust the gain carefully to yield a smooth, broad spectrum, reflecting the bound species. The adjusted gain is equivalent to the percentage of unbound population in the composite spectrum.

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Figure 1. Exploring the pathway of SNARE complex formation with SDSL EPR

A) Diagram of a disulfide-linked nitroxide side chain (MTSSL). B) The t-SNARE is in a state where the C-helix (SC) of SNAP-25 is unstructured and highly dynamic. The dynamic structure of the SC domain was investigated by attaching a nitroxide spin label to a site-specific cysteine. C) The t-SNARE is in a state where the SC is in a structured α -helix. The nitroxide spin label is sensitive to the local environment and produces a broader EPR lineshape than the dynamic SC does. The EPR lineshape analysis revealed that the SC is in a dynamic equilibrium, alternating between a bound and an unbound state. D) Dynamic *trans*-SNARE complex in which the N-terminal of VAMP2 is locally structured, but the C-terminus is locally dynamic. The structured t-SNARE complex is the precursor for VAMP2 binding and SNARE complex formation. When VAMP2 binds, SNARE zippering occurs from the N- to C-terminus. This pre-fusion state of the SNARE complex was studied using a SNAREpin formed in the chasm of two nanodiscs. E) Structured *trans*-SNARE complex. The nanodiscs allow the SNARE proteins to form the stable four helix bundle, but stop the full progression by arresting it at a half-zipped state. F) *Cis*-SNARE complex. A stable four helix bundle as a post-fusion complex. The post-fusion state of the SNARE complex was studied within a single nanodisc.

Figure 2. Reconstitution of SNARE proteins into a lipid nanodisc

A step by step flow chart for the reconstitution of full-length SNAREs into lipid nanodiscs.

Figure 3. Preparation of the sample tube for EPR

Diagram of EPR tube assembly. The spin labeled sample is pipetted up and kept in the loading tip. This loading tip is inserted into the top, open end of the EPR tube with the opposite side sealed off using a Bunsen burner. The EPR tube with loading tip is then placed into a 15 mL tube which is inserted into a centrifuge adaptor, where it is briefly centrifuged. This will make the sample to evacuate the loading tip and fill the EPR tube from the bottom to the top. After centrifugation, the tube is cleaned with ethanol and dried with a Kimtech wipe. The sample is now ready for EPR.

Figure 4. Spectral subtraction analysis

A) Raw unbound EPR spectrum (red) and raw composite (mixture of labeled unbound species with interacting species) EPR spectrum (purple) are obtained directly from EPR. B) Both spectra are baseline-corrected and normalized. C) The processed unbound and composite spectra are centered and overlaid on top of each other. The point of comparison between the two spectra is the 3rd peak indicated by the arrow. The unbound spectrum has a higher intensity representing the spectrum when 100% are unbound. D) The gain of the unbound spectrum is coarsely adjusted, so the height of the 3rd peak matches the composite spectra. E) The gain is finely adjusted, so the spectral subtraction results in a bound spectrum (blue) that is smooth and broad. The total adjusted gain is equivalent to the percentage of unbound population within the composite spectra. This can be used to calculate the bound population in the composite spectra as well.