Structural study of yeast SNARE proteins on membrane by EPR

by

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# TABLE OF CONTENTS

**ABSTRACT** .................................................................................................................. iv

**CHAPTER 1: GENERAL INTRODUCTION** ...................................................................... 01
  - Introduction .................................................................................................................. 01
  - Dissertation Organization ......................................................................................... 06
  - References .................................................................................................................. 06
  - Figures and Captions ................................................................................................. 12

**CHAPTER 2: A PARTIALLY ZIPPED SNARE COMPLEX STABILIZED BY THE MEMBRANE** .................................................................................................................. 18
  - Abstract ..................................................................................................................... 18
  - Introduction ................................................................................................................ 19
  - Results ......................................................................................................................... 20
  - Discussion .................................................................................................................. 24
  - Materials and Methods ............................................................................................. 25
  - References .................................................................................................................. 29
  - Figures and Captions ................................................................................................. 32

**CHAPTER 3: TRANSMEMBRANE ORGANIZATION OF YEAST SYNTAXIN-ANALOGUE Sso1p** .................................................................................................................. 38
  - Abstract ..................................................................................................................... 38
  - Introduction ................................................................................................................ 39
  - Results ......................................................................................................................... 40
  - Discussion .................................................................................................................. 44
  - Materials and Methods ............................................................................................. 46
  - References .................................................................................................................. 48
  - Figures and Captions ................................................................................................. 53

**CHAPTER 4: STOICHIOMETRY OF Sso1p TMD OLIGOMER AND TMD CONFORMATIONAL CHANGE INVOLVED IN TRANSITION FROM trans-SNARE TO cis-SNARE** .................................................................................................................. 61
Abstract

Introduction

Results

Discussion

Materials and Methods

References

Figures and Captions

CHAPTER 5: GENERAL CONCLUSION

References

ACKNOWLEDGMENTS
ABSTRACT

Membrane fusion is a basic biophysical process involved in cell’s physiological activities. The value of studying the exocytosis of mammalian cells and yeast cells lies in that a highly conserved SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein super family mediates this process and is proposed to be the core protein machinery for the membrane. The SNARE proteins located on plasma membrane and vesicle membrane are assembled together to form a trans-SNARE bridging two separate membranes and initiating membrane.

My research centers on how SNARE assembly involved in yeast cells’ exocytosis promotes membrane fusion between yeast vesicle and plasma membrane. Site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR) are well established techniques in membrane protein structural study. Fluorescent labeled Lipid mixing assay is employed to test the functional activity of SNARE. The data from trans-SNARE assembly on membrane indicates its potential catalyst role in membrane fusion; The transmembrane organization of neural syntaxin-analogue Sso1p is a well defined α-helix across membrane based on accessibility profile of magnetic reagents; Transmembrane domain of Sso1p tends to form oligomers of stoichiometries ranging from 3 to 6 in the membrane, which likely act as a scaffold of formation and clustering of multimeric trans-SNARE supra complex prior to lipid mixing; Sso1p TMD conformational change involved in the transition from trans- to cis-SNARE is also disclosed by EPR spectra, shedding light on the structural and temporal relationship between SNARE assembly intermediates and substeps of membrane fusion.
CHAPTER 1: GENERAL INTRODUCTION

Membrane fusion and SNARE proteins

Material transport in the cell constitutes a fundamental part of life itself. A deepening insight of the material communication between cells and inside individual cells was achieved by studying series of important phenomena in cells, examples of which are neuronal transmitters’ release, virus entry into the infected cells, and cells’ exocytosis and endocytosis (1). All these discoveries involves a basic but a critical biophysical process governing the life metabolism–membrane fusion, in which conserved protein machinery forms a core complex to draw close and bridge two separate membranes, inducing them to fuse into one membrane to release the proteins, hormones and neurotransmitters carried by vesicles to the destination(2).

To better understand the membrane fusion mechanism, some well established physiological models are usually used, including the hottest spot of neuronal transmitters’ release from presynaptic neuronal cells(3), which has a complicated hierarchy of regulation by various regulatory protein candidates such as complexin, nsec1, synaptotagmin(4-8). Another more basic one, and also one of the best characterized model involved in the post-Golgi trafficking in yeast centers on how vesicles originated from trans-Golgi can fuse with plasma membrane constitutively and then release the its cargo outside the cell(2).

The reason to establish such models is based on the highly conserved fusion apparatus utilized by these fusion systems. In eukaryotic pathways, the fusion of a transport vesicle with its target membrane requires the pairing of soluble NSF attachment protein receptor (SNARE) partners, separately anchored to two membranes(2, 9, 10). For fusion to occur, the membrane-proximal “SNARE motif” of the vesicle (v-) SNARE must interact with those of the target membrane (t-) SNARE to form a four–stranded helical bundle(11-16). Based on homology and similar functional activities in intracellular transport, SNARE proteins form a superfamily with 25 members in Saccharomyces cerevisiae, 36 members in humans and 54 members in Arabidopsis thaliana (Figure 1). Although highly conserved in evolution, it is challenging to understand why some systems are regulated so complicatedly while others seem to go in an easy way.
Structure and organization of SNARE complex

It is generally believed that SNARE core complex assembly plays a dominant role in the fusion process: generating the force and the energy necessary to mediate the membrane fusion\((12, 17)\). Almost all researches are investigating the relationship between the SNAREs’ structure and fusion mechanism. The crystal structure and EPR study has clearly showed that SNARE core complex formation is mediated by the SNARE motifs\((11, 12)\). The SNARE motif is 60-80 amino acids in length, usually exhibiting heptad repeat pattern along the peptide sequence\((18)\). In neuron’s case, three SNARE motifs from t-SNARE motifs (one from Syntaxin and another two from N- and C-terminal of SNAP25) assembles with one v-SNARE motif from Vamp2, resulting in an extremely tight parallel four-helix bundle which extending about 12nm (Figure 2a). It should be mentioned here the N-terminal domains of some t-SNARE protein (neural Syntaxin, Yeast Sso1p) usually assume a three-helix-bundle conformation to interact with the SNARE motif (Figure 3b) \((19)\). The resulted t-SNARE protein is in closed conformation inhibiting SNARE assembly \((20)\). Possibly the N-terminal region may act as a platform recruiting other SM (Sec1/Munc18-related) factors and thus is critical step in regulating SNARE complex assembly \((21)\). The assembled coiled coil core structure of SNARE complex is highly stable due to its hydrophobic and hydrophilic interaction between α-helixes. To disassemble it, NSF(ATPase) and SNAP (soluble NSF attachment protein) are required at the recycling stage of fusion\((22)\). The strong binding force comes mostly from extensively arranged hydrophobic interaction along the SNARE motif and displayed a heptad repeat pattern. If with a cross-section view, the center of this bundle contains 16 stacked layers of interacting chains \((16)\) (Figure 2b). They are largely hydrophobic with only exception of a central “0” layer, containing three highly conserved glutamine (Q) and one highly conserved arginine (R) residue \((12)\) (Figure 2c). Accordingly the contributing SNARE motifs are classified into Qa-, Qb-, Qc- and R-SNAREs\((23)\) (Figure 3a). In yeast, functional SNARE complexes mediating exocytosis are also hetero-oligomeric, parallel four helix bundle, composed of Sso1p (Qa-), Sec9 (Qb-/Qc-) and Snc2p (R).

SNARE complex assembly dominates the membrane fusion process

...
SNARE assembly plays an important role during vesicles’ docking and fusion process (Figure 4). It is generally hypothesized that SNARE assembly provides the energy for membrane fusion due to its tight bundle formation and evidences from proteoliposome fusion experiments indicate that SNARE complex constitutes the minimal fusion machinery(24). To initiate the fusion, the coiled coil motifs of t-SNAREs and v-SNAREs are thought to zipper from their membrane-distal N-termini to their membrane-proximal C-termini to pull the membranes together(13, 24-27). At this stage, we call this complex as trans-complex since transmembrane domain of t-SNARE and v-SNARE spans apposite membranes (Figure 5). Although some observation from fusion by neuronal SNAREs support this, it is shown that some partially zippered SNARE was indirectly observed in some critical steps of fusion(28-30), even during the formation of fusion pore, a energy consuming process during fusion(31, 32). Also in yeast case, the zippering model of SNARE formation is challenged by that SNARE complex assembles in a concerted way(33). It is still debated whether the SNARE assembly is truly a source of fusion energy source. Anyway, the trans-SNARE complex plays an important role during the membrane docking and initiation phase of fusion(34).

Following trans-SNARE assembly, it is implied that some mechanical force was exerted on membrane which bends the membrane and disturbs the hydrophobic and hydrophilic boundary of two approaching membrane. Although this force model seems satisfactory in explanation of SNAREs’ role in membrane fusion, it is in conflict with some structural study such as a unstructured linker region between SNARE motif and transmembrane domain(TMD)(35), and a lack of detailed description of fusion intermediates. Based on our lab’s recent discoveries in yeast and neuronal lipid mixing assay(36, 37), a hemifusion state is arrested in the beginning phase of fusion, in which the proximal leaflets of lipid bilayer are mixed, but the distal leaflets retain the original integrity, harboring the negative membrane curvature(38).

Hemifusion then transits to full fusion. Accompanying the transition of these substeps is membrane curvature changes from negative to positive(39-42). On another hand, the SNARE TMD actively participates in the transition of fusion process since GPI-anchored SNAREs lacking the TMDs demonstrates the phenotype of hemifusion(43). It is possible that SNARE TMD conformational change facilitates the membrane curvature change and
transition from hemifusion to full fusion. Alternatively, high density of SNARE on membrane results in full fusion while low density results in hemifusion(44). In vacuoles’ fusion of *Saccharomyces cerevisiae*, SNARE protein and lipid are enriched at a ring-shaped microdomain surrounding apposed boundary membrane disks (45). Also high order SNARE complexes induced by Synaphin are discovered in the squid giant synapse (6). These observations disclose possible cooperation of multimeric SNARE complexes in fusion. While the interactions between SNARE complexes are weak, the formation of multimers of SNARE complexes may depend on transmembrane regions of SNARE protein. In summary, disclosure of the precise stoichiometry of multimeric SNARE complexes, SNARE protein transmembrane organization and relevant conformational change is critical in our defining SNAREs’ role in membrane fusion.

The completion of fusion is usually demonstrated as the formation of *cis*-SNARE complex. At this stage, the cytoplasmic domains of t-SNARE and v-SNARE proteins are tightly bundled with their transmembrane domains residing on the same target plasma membrane. ATPase N-ethylmaleimide-sensitive factor (NSF) is required to disassemble the *cis*-SNARE complex and individual SNAREs are sorted for the next round circle of fusion. A hypothesis concerning *cis*-SNARE is that the formation of heterodimer between transmembrane domains of t- and v-SNAREs helps complete the membrane fusion. Naturally, disclosure of transmembrane domains’ structure of *cis*-SNARE will provide direct clue to answer this question.

**EPR technique employed in the study**

Site directed spin labeling (SDSL) and Electron Paramagnetic Resonance (EPR) spectroscopy are well established methods (46, 47) for studying protein-protein interaction, protein secondary structure, associated protein conformational change and protein dynamics. The attachment of a nitroxide to a specific position (whose amino acid residue has been mutated into a cysteine) in the protein makes it convenient to detect its interaction with surrounding environment in the protein’s natural state (Figure 6a).

EPR spectra analysis is generally performed to study protein-protein interaction. EPR line shape is sensitive to the flexibility (or mobility) of nitroxide and is largely determined by
local structural and viscosity factors. In SNARE assembly, the coiled coil motif of individual SNAREs are mostly unstructured prior to complex formation. When associated with their partners, the polypeptides become α-helix, which significantly reduces the tumbling rates for the peptide backbones as well as for the amino acid side chains. Slower motion gives rise to the larger outer hyperfine splitting (hf) in the EPR spectrum due to incomplete averaging of hyperfine tensors. It also bring about the broadening of the central (M_I=0) line due to the incomplete averaging of the g tensors. Thus, SNARE core formation should accompany large EPR spectrum changes, from a narrow spectrum reflecting the fast motion to a broad spectrum reflecting the slow motion (46-51).

In order to study the membrane topology of SNARE protein, EPR saturation method is applied. Accessibility of nitroxide to two magnetic reagents was measured along the whole length of transmembrane domain. The idea of using EPR saturation method to measure accessibility data is based on that collision of the nitroxide with fast relaxing radicals such as oxygen or metal ion complexes causes Heisenberg spin exchange that effectively shortens the spin lattice relaxation time, T1 of the nitroxide. This effect is measured from a saturation curves by power saturation experiment and the result is positively proportional to accessibility profile. Ni-ethylenediaminediacetic acid (NiEDDA) has an increasingly higher gradient distribution from membrane to solution while oxygen which is non-polar accumulates increasingly higher into the membrane. High accessibility profile of oxygen and low accessibility profile of NiEDDA should reflect the membrane spanning properties of transmembrane structure. Also the ratio of $W_{NiEDDA}$ to $W_{O2}$ has been shown to hold a quantitative relationship to the membrane immersion depth (52). By comparing these data with those of standard molecules with known membrane immersion depths, the secondary structure of transmembrane domain of SNARE protein is illustrated (Figure 6b).

Another quantitative method of EPR is the inter-spin distance of two nitrooxide spins whose spatial separation r > 7 Å (Figure 6c). EPR spectrum of the interacting two labels can be treated as a convolution of the non-interacting spectrum and the dipolar broadening function that can be represented as the sum of the dipolar Pake patterns over the distribution of the interspin distances. This function is simply deconvoluted using a Fourier
deconvolution technique. The broadening function also contains information on the distribution of distances due to the flexible nitrooxide side chains (53, 54).

**Functional study by fluorescence lipid mixing study**

The functionality of cysteine and spin-labeled mutants was checked with the proteoliposome fusion assay. This method was proposed by Rothman’s group demonstrating that SNAREs reconstituted into phospholipid vesicles indeed induce membrane fusion (10, 24). Fluorophores NBD (donor) and rhodamines (receptor) are quencher pair. v-SNARE vesicles loaded with NDB and rhodamine, are mixed with t-SNARE vesicles that have no fluorophores. When these two types of vesicles begin to fuse, the fluorophores are diluted and dequenching occurs. Fluorescence change thus observed at the emission wavelength 538nm would be an indicator of actual lipid mixing process. By comparing the lipid mixing efficiency and kinetics we can know the fusion activity of specific SNARE components.

**Dissertation Organization**

Chapter one covers the general background knowledge of the membrane fusion: introduction of SNAREs, intimately coupled SNAREs’ role in membrane fusion and principles of research method. Chapter two discusses the structure of trans-SNARE complex on membrane and has been published in Journal of Biological Chemistry. Chapter three describes the transmembrane organization of yeast t-SNARE protein Sso1p which has been published in Biochemistry. Chapter four identifies the stoichiometry of Sso1p TMD oligomers and discloses the TMD interaction in Sso1p TMD oligomer and cis-SNARE complex on membrane, establishing structural transition between trans-SNARE and cis-SNARE concerning TMDs’ behavior and its implication to membrane fusion. The above research data is summarized in chapter 5.

**References**


Figures and Captions

Figure 1. The assignment of SNAREs to intracellular membrane-trafficking pathways.1

(a). Full complement of QabcR-SNAREs can be assigned to all of the fusion events in the secretory pathway of Saccharomyces cerevisiae. The biosynthetic route begins at the endoplasmic reticulum (ER) and leads, through coatamer protein complex-II (COPII)-coated vesicles, to the Golgi complex. From the trans-Golgi network, transport vesicles are directed to the growth pole (bud) where they fuse with the plasma membrane. A second route leads to the prevacuolar compartment. The retrograde route leads from the plasma membrane, through the endosome/prevacuolar compartment, to the vacuole (this is simplified).

(b). The assignment of SNAREs to trafficking pathways in mammalian cells.

Figure 2. SNARE core complexes.²

(a). A crystal structure of the neuronal SNARE. (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) core complex. This complex contains the SNARE motifs of syntaxin-1 (Qa; red), SNAP-25 (25-kDa synaptosome-associated protein; Qb and Qc; both green), and VAMP (vesicle-associated membrane protein)/synaptobrevin (R; blue).

(b). A skeleton diagram that indicates the position of the central layers of interacting side chains (numbered) in the neuronal SNARE core complex. Cα traces are shown in grey, the helical axes are highlighted by lines that are the same colour as the helices in part a, and the superhelical axis is highlighted by a black line. The '0' layer is coloured red and all other layers are coloured black.

(c). Overlays of individual layers, which are each shown contained in a shaded circle, from the neuronal SNARE core complex (grey) and the endosomal SNARE core complex (coloured).

Figure 3. The general structures of different SNAREs.³

(a). The domain structure of the SNARE subfamilies. Dashed domain borders highlight domains that are missing in some subfamily members. Qa-SNAREs have N-terminal antiparallel three-helix bundles. The various N-terminal domains of Qb-, Qc- and R-SNAREs are represented by a basic oval shape. Qbc-SNAREs represent a small subfamily of SNAREs — the SNAP-25 subfamily — that contain one Qb-SNARE motif and one Qc-SNARE motif. These motifs are connected by a linker that is frequently palmitoylated (zig-zag lines in the figure). Qa-SNARE is shown in red; Qb- SNARE is in light green; Qc-SNARE is in dark green; and R-SNARE is in blue.

(b). The upper panel shows the three-dimensional structure of the isolated N-terminal domain of syntaxin-1. This structure is an N-terminal three-helix bundle that is typical of Qa-SNAREs, as well as of some Qb- and Qc-SNAREs. The lower panel shows the ‘closed’ conformation of syntaxin-1, in which the N-terminal domain of syntaxin-1 (red, as in the upper panel) is associated with part of its own SNARE motif (beige structure; absent in the upper panel). This structure was solved as part of the structure of the Munc18–syntaxin-1 complex.

Figure 4. Model of SNARE-mediated lipid fusion.4

(a). The two membranes are in the vicinity of each other but the SNAREs are not yet in contact.

(b). SNARE complexes start zipping from the amino-terminal end, which draws the two membranes further towards each other.

(c). Zipping proceeds, causing increased curvature and lateral tension of the membranes, exposing the bilayer interior. Spontaneous hemifusion occurs as the separation is sufficiently reduced.

(d). The highly unfavourable void space at the membrane junction in (c) causes the establishment of contacts between the distal membrane leaflets.

(e). The lateral tension in the transbilayer contact area induces membrane breakdown, yielding a fusion pore.

(f). The fusion pore expands and the membrane relaxes. (SNARE, soluble NSF attachment protein receptor, where NSF stands for N-ethyl-maleimide-sensitive fusion protein.)

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Figure 5. Trans-SNARE assembly and zippering model.\textsuperscript{5}

Trans-SNARE is widely thought to be essential intermediates in the fusion pathway. Its assembly is proposed to start at the N termini of the SNARE motifs and then proceeds in a zipper-like fashion towards the C-terminal membrane anchors. At this stage, the transmembrane domains of t- and v- SNARE are anchored in apposite membranes.

Figure 6. Schematic description of site-directed spin labeling (SDSL) and EPR spectroscopy.

(a). Spin labeling reaction;
(b). Spin label immersion depth measurement.
(c). Dipolar interaction of the spin labels.
CHAPTER 2: A PARTIALLY ZIPPED SNARE COMPLEX STABILIZED BY THE MEMBRANE

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Abstract

The SNARE complex acts centrally for intracellular membrane fusion, an essential process for vesicular transport in cells. Association between vesicle-associated (v-) SNARE and target membrane (t-) SNARE results in the coiled coil core that bridges two membranes. Here, the structure of the SNARE complex assembled by recombinant t-SNARE Sso1p/Sec9 and v-SNARE Snc2p, which are involved in post-Golgi trafficking in yeast, was investigated using EPR. In detergent solutions, SNAREs formed a fully assembled core. However, when t-SNAREs were reconstituted into the proteoliposome and mixed with the soluble SNARE motif of Snc2p, a partially zipped core in which the N-terminal region is structured, whereas the C-terminal region is frayed, was detected. The partially zipped and fully assembled complexes coexisted with little free energy difference between them. Thus, the core complex formation of yeast SNAREs might not serve as the energy source for the fusion, which is different from what has been known for neuronal SNAREs. On the other hand, the results from the proteoliposome fusion assay, employing cysteine- and nitroxide-scanning mutants of Sso1p, suggested that the formation of the complete core is required for membrane fusion. This implies that core SNARE assembly plays an essential role in setting up the proper geometry of the lipid-protein complex for the successful fusion.

1 This chapter is published in J. Biol. Chem., 2005, 280(16):15595-15600, Necessary modifications were made to fit the format of this thesis.
Introduction

Membrane fusion is essential for many important life activities such as viral entry to cells, fertilization of eggs, and intracellular material transport (1). Biological membrane fusion is not spontaneous because merging two stable membranes to a single bilayer imposes a high activation energy barrier (2). Thus, specialized fusion proteins are required either to provide the necessary free energy or to lower the fusion energy barrier. Progress in determining three-dimensional structures of these proteins helps understand the mechanism by which the proteins facilitate the fusion of two membranes (3).

In exocytotic pathways, the fusion of a transport vesicle with its target membrane requires the pairing of soluble NSF attachment protein receptor (SNARE) partners, separately anchored to two membranes (4–6). For fusion to occur, the membrane proximal “SNARE motif” of the vesicle (v-) SNARE must interact with those of the target membrane (t-) SNARE to form a four-stranded helical bundle (7–12). The SNARE core complex shares striking structural similarity with viral fusion proteins, including influenza hemagglutinin and human immunodeficiency virus gp41 (3). Thus, it has been postulated that SNARE assembly provides the energy for membrane fusion (8, 13), as is believed, although not proven, for the viral fusion proteins (14).

Association of v- and t-SNAREs might proceed in sequential steps (15–22). The “zipper model” predicts that complex formation starts from the membrane-distal N-terminal region, setting up the stage, and progresses toward the membrane-proximal C-terminal region, closing the gap between the two bilayers. Although the existence of a partially zipped complex has been supported by several biochemical experiments (17, 21, 22), such a complex has not been physically trapped or kinetically identified. Therefore, the structure and the stability of the partially zipped complex, which seem to be important in understanding the exact role of SNARE assembly in membrane fusion, are unknown.

In this work, we investigated the structure of the SNARE core assembled by recombinant t-SNARE Sso1p/Sec9 and v-SNARE Snc2p, a set of SNARE partners involved in post-Golgi trafficking in yeast, using site-directed spin labeling EPR (23, 24). In detergent solutions, SNARE core assembly was spontaneous and complete. However, when t-SNAREs were reconstituted in the proteoliposome and mixed with a soluble recombinant v-SNARE
representing the cytoplasmic domain of Snc2p, an equilibrium was established between the complete SNARE complex and the partially zipped SNARE complex. It appears that the membrane stabilized the partially zipped complex and made it energetically equivalent to the complete SNARE complex. On the other hand, the analysis of the site-directed mutants of Sso1p, using the proteoliposome fusion assay, indicated that complete SNARE core formation is required for membrane fusion. Therefore, the results provide new insights into the structural and energetic roles of SNARE assembly in promoting membrane fusion.

**Results**

*Site-directed Spin Labeling of Yeast t-SNARE Sso1p*—In site-directed spin labeling, native amino acids are site-specifically replaced one by one with cysteines to which the nitroxide side chain is attached. The coiled coil motifs of individual SNAREs are mostly unstructured prior to complex formation. When associated with their partners, however, the polypeptides become $\alpha$-helical (25), which significantly reduces the tumbling rates for the peptide backbone as well as for the amino acid side chains. The EPR line shape is sensitive to the motional rates of the nitroxide (26). Thus, SNARE core formation accompanies large EPR line shape changes, from a narrow spectrum reflecting the fast motion to a broad spectrum reflecting the slow motion (24, 27).

To investigate SNARE core formation, we prepared 26 consecutive cysteine mutants (N215C–L240C) of recombinant Sso1pHT (amino acids 185–283 of Sso1p), which contains the SNARE motif and the transmembrane domain (Fig. 1), and cysteine mutants were labeled with methanethiosulfonate spin label. Spin-labeled mutants were then reconstituted into POPC vesicles containing 15 mol% negatively charged DOPS, a lipid composition commonly used to mimic the native cellular membrane (5, 28, 29). The functionality of the cysteine and spin-labeled mutants was checked with the proteoliposome fusion assay (see below).

The EPR spectra of the first 11 reconstituted Sso1pHT mutants (N215C–Q225C) had two spectral components. One broad component reflects the slow motion and another sharp component reflects the fast motion of the nitroxide (Fig. 2A). Such composite spectra are characteristic of two coexisting structures, one structured and the other random coil-like. EPR spectra of the next 15 mutants (E226C–L240C) were all fast motional, indicating that
this region is a freely moving random coil. The structure in the N-terminal region might be
due to the self-association of Sso1pHT. Previously, it was shown that Syntaxin, the neuronal
counterpart of Sso1p, self-associates to form dimers (30, 31). To verify whether the N-
terminal structure of Sso1pHT was due to a similar oligomerization, we collected the low
temperature EPR spectra (32) and found that some mutants exhibited apparent spin-spin
interactions (data not shown), supporting the oligomeric state of Sso1pHT.

Partially Zipped SNARE Complex Is Stabilized by the Membrane—As for the core
complex, we first examined SNARE complex formation in detergent solutions as a control.
Spin-labeled Sso1pHTs dissolved in 0.5% Triton X-100 were mixed with a 4-fold excess of
Sec9c (amino acids 401–651 of Sec9) and soluble Snc2pS (amino acids 1–93 of Snc2p)
lacking the transmembrane domain (Fig. 1). The room temperature EPR spectra were
collected after incubating the mixture at 20 °C for 30 min (Fig. 2B). The EPR spectra of all
mutants are broad, reflecting the slow motion of the nitroxide, most likely due to the
formation of the fully assembled coiled coil in the entire region. In fact, the variation of the
EPR spectra along the amino acid sequence appeared to be in qualitative agreement with the
pattern of a coiled coil. Residues 217, 222, 224, 228, 231, 235, and 238 are predicted to be
internal α or ß positions and yielded very broad EPR spectra reflecting nearly completely
frozen motion, indicative of the tight packing of the coiled coil interior. These EPR results
are consistent with the previous NMR study reporting that soluble yeast SNAREs form a well
defined coiled coil (33).

Next, we used reconstituted Sso1pHT into vesicles and mixed it with 4-fold molar
excess of soluble Sec9c and Snc2pS. For the first 14 mutants N215C–V225C, we observed
the broad and slow motional EPR spectra with a small fraction of the sharp spectral
component (Fig. 2C, arrow), indicating that most Sso1p molecules were engaged in complex
formation. In contrast, for the next 12 mutants, the sharp spectral component appears to be
significantly increased, suggesting that a large fraction of Sso1p is uncomplexed and
uncoiled. The addition of an extra 4-fold excess of Sec9c and Snc2pS to the mixture did not
change the EPR spectra, supporting that the sharp component was due to the local
(intramolecular) fraying of the coiled coil and not due to the global (intermolecular)
dissociation of the complex. Also, neither the incubation of the mixture for 1 day at room
temperature nor the incubation at 37 °C for 5 h changed the EPR spectra. Thus, the results support that the protein samples were in equilibrium rather than in a kinetic trap. We, however, note that we were unable to reconstitute the preassembled SNARE complex into the vesicles because of the protein aggregation.

To analyze the data quantitatively, we decomposed the two spectral components into individual ones using spectral subtraction method (34). For each spin-labeled position, the fraction of the unstructured was calculated based on the ratio of the spin concentrations of two species (Fig. 3). For the SNARE samples in the detergent, coiled coil formation was nearly complete across all positions examined. For reconstituted Sso1pHT, however, the N-terminal region showed that ~90% formed the complex, whereas the C-terminal positions showed that only 50% participated in the complex. Therefore, it appeared that 50% of Sso1pHT formed the full SNARE complex, 40% percent formed the partially zipped complex for which the N-terminal region was structured, whereas the C-terminal region was frayed, and the remaining 10% stayed as an uncomplexed species.

The Structure and the Stability of the Partially Zipped Complex—The analysis of the EPR data indicates that the partially zipped core is well structured in the N-terminal region, whereas it might be predominantly a random coil in the C-terminal region. In the meanwhile, we observed a gradual increase of the disordered population of Sso1p from 10 to 50% in the range of D229C–L240C. Such a gradual change in the broad range might suggest that there is a significant degree of heterogeneity in the structure. Further, this region may undergo random fluctuations between the helical conformation and the random coil conformation.

Since the population ratio between the complete complex and the partially zipped complex is known, it is possible to estimate the Gibbs free energy difference ($\Delta G^\circ$) between these two species. Under current conditions, the two species are nearly equally populated, which means that the equilibrium constant ($K$) is close to unity. Since $\Delta G^\circ = -RT \ln K$, where $R$ is the universal gas constant and $T$ is the temperature, the free energy difference comes out to be nearly 0 between these two species. Therefore, we conclude that the transition from the partially zipped SNARE complex to the complete complex may not release the free energy.

Complete Core SNARE Assembly Is Required for Membrane Fusion—The EPR analysis revealed that the partially zipped SNARE complex was stabilized by the membrane and
coexisted with the fully assembled complex. It was also shown that there might be no free energy gain on going from the partially zipped complex to the complete complex. One then might wonder whether the formation of the complete SNARE complex is required for membrane fusion.

To test this possibility, we examined the fusion activity of cysteine- and nitroxide-scanning mutants of Sso1pHT using the proteoliposome fusion assay. The size of the nitroxide side chain is relatively bulky, and it is comparable with that of tryptophane. Therefore, if the formation of the complete coiled coil were required for membrane fusion, the alterations at the internal positions might cause some serious perturbations. With the cysteine- and nitroxide-scanning mutants, we might observe the periodic behavior of the fusion activity along the sequence, consistent with the heptad repeat pattern of the coiled coil (35).

For the lipid mixing fusion assay, we prepared POPC/DOPS vesicles containing mutant t-SNAREs (Sso1pHT mutants/Sec9c). Snc2p was reconstituted into separate vesicles containing fluorescence dyes. When we mixed two vesicle populations, we detected significantly reduced lipid mixing with the mutations at internal a and d positions as compared with that of wild type (Fig. 4), particularly in the C-terminal region below the conserved “0” layer (Q224 of Sso1p). In this region, we observed a periodic behavior of the fusion activity along the sequence that was in phase with the heptad repeat pattern. However, the addition of dithiothreitol that cleaved off the nitroxide side chain from the cysteine residue restored fusion activities significantly at the internal a and d positions (Fig. 4, triangles). Therefore, the results suggested that complete coiled coil formation is required for membrane fusion.

To further examine the necessity of the formation of the complete core, we prepared several Sso1pHT mutants in which the internal d positions in the C-terminal region were replaced with helix-breaking prolines. The proline mutations should block coiled coil formation. When the proline mutants were examined with the lipid mixing fusion assay, we observed significantly reduced lipid mixing as compared with that of wild type, further supporting the assertion that complete core formation is essential for membrane fusion (Fig. 5).
Discussion

In this work, it was shown that membrane-reconstituted SNAREs behaved differently from those in solution. In solution, SNARE motifs of v- and t-SNAREs engaged one another to form the complete coiled coil core. In the membrane, however, the partially zipped complex, in which the C-terminal region of the SNARE core was frayed, became stabilized and coexisted with the complete complex. The EPR analysis suggested that the two complexes were energetically equivalent with little Gibbs free energy difference between the two. Based on these results, it seems difficult to envision that the transition from the partially zipped complex to the complete complex releases the free energy that might be used for membrane fusion.

In contrast, the results from the lipid mixing fusion assay suggested that the formation of the complete complex is still essential for membrane fusion. Moreover, it is highly likely that complete core formation occurs before the lipid mixing (36). How is this possible, given that the SNARE core might not have enough strength to bring two mutually repulsive membranes into contact? One possibility is that membranes might no longer be mutually repulsive at the fusion site where several t- and v- SNAREs are presumably clustered. Both t- and v-SNAREs carry basic membrane-proximal regions that have a strong propensity for the membrane surface (24, 31, 37). The insertion of several membrane-proximal regions into the small patch of the membrane might activate the surfaces to become no longer mutually repulsive. Perhaps complete core formation simply puts, by fluctuation, two activated membranes into a correct geometry necessary for the fusion without the expense of much free energy.

The proposal that SNARE core formation plays a set-up role instead of an energy source for membrane fusion might not be general for all SNARE systems. For example, for neuronal SNAREs involved in neurotransmitter release at synapses, the coiled coil core is extremely stable and is even SDS-resistant (38), whereas the coiled coil of yeast SNAREs is not (39). Therefore, there must be some energy release that could directly assist the apposition of the two membranes. Such an energetic assistance might be necessary for neuronal membrane fusion, in which a fast and controlled release of neurotransmitters is essential.
The possibility that yeast SNAREs might function differently from neuronal SNAREs is not unusual. In fact, there are several other features that yeast SNAREs do not share with their neuronal homologues. First, yeast SNARE assembly is known to be regulated by the N-terminal Habc domain of Sso1p (40), whereas neuronal SNARE assembly is not significantly affected by that of Syntaxin (41). Second, yeast t-SNAREs Sso1p and Sec9 form a 1:1 complex in which the C-terminal region is significantly frayed (33), whereas neuronal Syntaxin and SNAP-25 assemble into a 2:1 complex, the structure of which is virtually identical to that of the ternary SNARE core (42–44). Third, the trans complex formation of yeast SNAREs in vitro proceeds in a single step (36), in contrast to the two-step mechanism proposed for neuronal SNAREs.

In this work, we arbitrarily assumed that the membrane stabilizes the partially zipped complex. However, we do not know whether this is the case or whether the complete SNARE core is destabilized by the membrane. Also, the mechanism by which the membrane influences the relative energy between two forms of SNARE complex is unclear, warranting further investigation.

In summary, we have identified the partially zipped SNARE complex in which the N-terminal regions of individual SNARE motifs engaged one another to form the coiled coil, whereas the C-terminal regions are separate and unstructured. The EPR analysis showed that the partially zipped complex coexisted with the complete SNARE complex with no free energy difference between two forms. Thus, the result suggests that yeast SNAREs play more of a set-up role than serving as the primary energy source for membrane fusion, which is different from what has been proposed for neuronal SNAREs.

Materials and Methods

Plasmids and Site-directed Mutagenesis—DNA sequences encoding Sso1pHT (amino acids 185–290 of Sso1pHT) and Snc2pS (amino acids 1–93 of Snc2p) were inserted into the pGEX-KG vector between EcoRI and HindIII sites as N-terminal glutathione S-transferase (GST) fusion proteins. Sec9c (amino acids 401–651 of Sec9) was inserted into pET-24b(+) between NdeI and XhoI sites as a C-terminal His6-tagged protein. To introduce a unique cysteine residue for the specific nitroxide attachment, native cysteine 266 of Sso1pHT was mutated to alanine. A QuikChange site-directed mutagenesis kit (Stratagene) was used to
generate all mutants; DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.

**Protein Expression, Purification, and Spin Labeling**—Expression of recombinant GST fusion proteins was conducted in *Escherichia coli* Rosetta (DE3) pLysS (Novagene). The cells were grown at 37 °C in LB medium with glucose (2 g/liter), ampicillin (100 μg/ml), and chloramphenicol (25 μg/ml) until the *A*600 reached 0.6–0.8. Isopropyl-D-thiogalactopyranoside was added to a final concentration of 1 mM. Cells were grown for an additional 4 h at 18 °C. Cell pellets were harvested by centrifugation at 6000 rpm for 10 min.

Purification of GST fusion proteins was achieved with affinity chromatography using glutathione-agarose beads (Sigma). Frozen cell pellets were resuspended in PBS buffer (phosphate-buffered saline, pH 7.4, with 0.5% Triton X-100 (v/v)) with 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and 5 mM dithiothreitol. The cells were broken by sonication in an ice bath. As for Sso1pHT, 1% of n-lauroyl sarcosine was added to the solution before sonication. The cell lysate was centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was mixed with glutathione-agarose beads in the resuspension buffer and nutated at 4 °C for 120 min. The protein-bound beads were washed with an excess volume of washing buffer (phosphate-buffered saline, pH 7.4) for at least six rounds. When washing, 0.2% (v/v) Triton X-100 was added to Sso1pHT, whereas no detergent was added to Snc2pS. The beads were then washed with thrombin cleavage buffer (50 mM Tris- HCl, 150 mM NaCl, 2.5 mM CaCl2, pH 8.0), either with 0.2% Triton X-100 for Sso1pHT or without detergent for Snc2pS. Finally, the proteins were cleaved from the resin by thrombin (Sigma) at room temperature for 40 min. AEBSF was added to the protein after the cleavage (2 mM final concentration). The protein was stored at −80 °C with 10% glycerol if needed.

Cysteine mutants of Sso1pHT were spin-labeled before thrombin cleavage. After the cell lysate was incubated with beads and washed with PBS buffer containing 0.2% Triton X-100, dithiothreitol was added to a final concentration of 5 mM. The sample was incubated at 4 °C for 40 min, and the beads were then washed eight times with an excess volume of PBS buffer with 0.2% Triton X-100 to remove dithiothreitol. An ~20-fold excess of 1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl methanethiosulfonate spin label was immediately added to the protein. The reaction mixture was left overnight at 4 °C. Free
methanethiosulfonate spin label was removed by washing with excess PBS buffer with 0.2% Triton X-100. The proteins were cleaved by thrombin in cleavage buffer with 0.2% Triton X-100.

The His6-tagged protein Sec9c was expressed in *E. coli* Rosetta (DE3) pLysS. The cells were grown at 37 °C in LB medium with glucose (2 g/liter), kanamycin (30 μg/ml), and chloramphenicol (25 μg/ml) until the *A*600 reached 0.6–0.8. After isopropyl-β-D-thiogalactopyranoside (1 mM) addition, the cells were incubated further for an additional 4 h at 22 °C. The cell pellets were collected by centrifugation at 6000 rpm for 10 min. For purification, the frozen cell pellet was resuspended in lysis buffer (PBS buffer with 20 mM imidazole, 0.5% Triton X-100, 2 mM AEBSF, pH 8.0). After sonication on ice, the cell lysate was centrifuged at 15,000 × g for 15 min at 4 °C. The supernatant was mixed with nickel-nitrilotriacetic acid-agarose beads (Qiagen) in lysis buffer. The mixture was incubated for binding at 4 °C for 120 min. After binding, the beads were washed with washing buffer (PBS buffer with 50 mM imidazole, pH 8.0). Then the protein was eluted by elution buffer (PBS buffer with 250 mM imidazole, pH 8.0). The protein can be stored at -80 °C with 10% glycerol. All purified proteins were examined with 15% SDS-PAGE.

**Membrane Reconstitution and SNARE Ternary Complex Formation**—Large unilamellar vesicles (~100 nm in diameter) of 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidyicholine (POPC) containing 15% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) were prepared in a detergent-free cleavage buffer using an extruder (24). The total lipid concentration was 100 mM. Proteins were reconstituted into vesicles by the Bio-Beads method (24). Proteins were mixed with vesicles at an ~1:300 protein-to-lipid molar ratio. The detergent was removed by treating the sample with Bio-Beads SM2 (Bio-Rad), which was directly added to the sample in the ratio of 200 mg/1 ml of the mixed solution. After 45 min of incubation, Bio-Beads were removed from the sample by centrifugation at 10,000 × g for 1 min. The same procedure was repeated three times. Reconstitution efficiency was estimated by determining the protein concentration using EPR before and after reconstitution. For all samples, the efficiency was ~70%.

For ternary SNARE complex formation in the membrane, Sso1pHTreconstituted vesicles were mixed with Sec9c in a molar ratio of 1:4 and then incubated at room
temperature for 60 min to help the formation of the binary t-SNARE complex. Next, Snc2ps was added, forming the ternary SNARE complex with the final Sso1p to Snc2ps molar ratio of 1:4. The mixture was incubated at room temperature for 1–5 h. The vesicle solution was concentrated using a 100-kDa cutoff centrifugal filter (Millipore) before taking an EPR spectrum. As for SNARE assembly in detergent, the procedure was the same as described above except that all three proteins were directly mixed in a detergent solution and were concentrated using a 5-kDa cutoff centrifugal filter (Millipore) after a 30-min incubation.

EPR Data Collection—EPR spectra were collected using the Bruker ESP 300 spectrometer equipped with a loop-gap resonator. The modulation amplitude was set at no greater than one-fourth of the line width. Spectra were collected at room temperature.

Proteoliposome Fusion Assay—For the lipid-mixing fusion assay, two different populations of vesicles were separately prepared. Snc2p was reconstituted to vesicles containing POPC, DOPS, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine N-(7-nitro-2,1,3-benzoxadiazol-4-yl) at a molar ratio of 62:35:1.5:1.5. Sso1pHT was reconstituted into the vesicles containing POPC and DOPS at a molar ratio of 65:35. The protein-to-lipid molar ratio was ~1:150. The detergent in the sample was removed by Bio-Beads at three cycles, and then the samples were dialyzed against 2 liters of dialysis buffer at 4 °C overnight. Prior to the fusion assay, Sso1pHT-reconstituted vesicles, Sec9c, and Snc2p-reconstituted vesicles were mixed at a molar ratio 9:9:1. The final solution contained 0.5 mM lipids. Fluorescence was measured at excitation and emission wavelengths of 465 and 530 nm, respectively. Fluorescence changes were recorded with a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 μl with the 2-mm path length. The maximum fluorescence intensity (MFI) was obtained by adding 0.1% reduced Triton X-100. Theoretically, MFI should be obtained at the end of the fusion reaction, in which dyes are distributed homogeneously on every vesicle as a result of the completion of the fusion reaction. As an approximation, we prepared the samples with the homogeneous distribution of dyes. The fluorescence intensities of these samples were measured and compared with those obtained at the onset of the fusion reaction. We found that the MFIs obtained by adding
0.1% reduced Triton X-100 were virtually the same as those obtained with the latter method. All lipid mixing experiments were carried out at 35 °C.

References
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Figures and Captions

**Figure 1. Primary structures of recombinant yeast SNAREs.**

(a). Sso1pHT contains the amino acids 185–290 of Sso1p. This polypeptide includes both the SNARE motif and the transmembrane domain, which are represented by the *cylinder* and the *rectangle*, respectively. The amino acid sequence of the spin-labeled region is shown below the schematic diagram.

(b). Snc2pS is the soluble part of Snc2p (amino acids 1–93) lacking the transmembrane domain (the *dotted rectangle*).

(c). Sec9c represents amino acids 401–651 of Sec9 and contains two SNARE motif regions. The SNARE motif regions are represented by the *cylinders*. 
Figure 2. EPR assay of SNARE complex formation.

(a). EPR spectra for reconstituted Sso1pHT.

(b). EPR spectra for detergent-solubilized Sso1pHT after mixing with the 4-fold molar excess Sec9c and Snc2pS.

(c). EPR spectra for reconstituted Sso1pHT after mixing with the 4-fold molar excess of Sec9c and Snc2pS. The letters (a–g) in the parentheses after the residue number represent the predicted heptad repeats of the coiled coil. The arrows indicate the sharp spectral component, whereas the asterisk points to the broad spectral component. All EPR spectra were taken at 20 °C.
Figure 3. The EPR spectral analysis of local SNARE assembly.

EPR spectra of spin-labeled Sso1pHT are composed of two components. The sharp one represents the unstructured, and the broad one represents the structured. For each spin-labeled position, the composite spectrum can be treated as the sum of the sharp spectrum and the broad spectrum in an appropriate ratio. The standard spectral decomposition analysis (34) provides the fraction of Sso1pHT in the complex ($f_{\text{complex}}$) and in an unstructured form ($f_{\text{free}}$). For each mutant, the fraction complexed was calculated from the equation $[f_{\text{complex}}/(f_{\text{free}} + f_{\text{free}})]$; Sso1pHT in the membrane (open triangles), the ternary SNARE complex in the detergent (open circles), and the ternary SNARE complex in the membrane (filled triangles). The solid lines are arbitrary fits to guide the eyes. For each data point, the error bar represents the standard deviation of the results from three independent decomposition analyses.
Figure 4. Fusion activities of nitroxide- and cysteine-scanning mutants of Sso1pHT.

The fusion activities of the Sso1pHT mutants were determined using proteoliposome lipid mixing fusion assay: spin-labeled mutants (filled circles) and cysteine mutants (filled triangles). For each mutant, the relative fusion efficiency was estimated by comparing the percent of its MFI (see "Experimental Procedures") with that of the wild type. Residue 224 (boxed) is the conserved glutamine in the 0 layer. The letters a and d below the data points in the C-terminal region denote the predicted internal positions of the heptad repeats.
Figure 5. Lipid mixing assay for the proline mutants of Sso1pHT.

The curves represent the lipid mixing when the wild type or proline mutants (A231P, A238P, A245P, and A252P) of Sso1pHT were used in the proteoliposome fusion assay. The data were normalized against MFI obtained by adding 0.1% (v/v) reduced Triton X-100.
CHAPTER 3: TRANSMEMBRANE ORGANIZATION OF YEAST
SYNTAXIN-ANALOGUE Sso1p

Yinghui Zhang and Yeon-Kyun Shin

Abstract

Membrane fusion in secretory pathways is thought to be mediated by SNAREs. It is proposed that membrane fusion transits through hemifusion, a condition in which the outer leaflets of the bilayers are mixed, but the inner leaflets are not. Hemifusion then proceeds to the fusion pore that connects the two internal contents. It is believed that the transmembrane domains (TMDs) of the fusion proteins play an essential role in the transition from hemifusion to the fusion pore. In this work, the structure, dynamics, and membrane topology of the TMD of Sso1p, a target membrane (t-) SNARE involved in the trafficking from Golgi to plasma membrane in yeast, was investigated using site-directed spin labeling and EPR spectroscopy. The EPR analysis of spin-labeled mutants showed that the TMD of Sso1p is a well-defined membrane spanning α-helix. The results also indicate that there is an equilibrium between the monomers and the oligomers. The oligomerization is mainly mediated through the interaction at the N-terminal half of the TMD, whereas the C-terminal half is free of the tertiary interaction. Additionally, the isotropic hyperfine splitting values were examined for nitroxide-scanning mutants, and it was found that the hyperfine splitting values show a V-shaped profile across the bilayer. Thus, hyperfine splitting may be used as an additional parameter to measure bilayer immersion depths of nitroxide.

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1 This chapter is published in Biochemistry (2006) 45, 4173-4181. Necessary modifications were made to fit the format of this thesis.
Introduction

Membrane fusion is a widespread process that eukaryotic cells use to establish material transport between endomembrane organelles as well as the secretion from the cell (1). In secretory pathways, SNAREs are believed to be core constituents of fusion machinery (2-7). Vesicle-associated (v-) SNAREs engage with target membrane (t-) SNAREs to form a helical SNARE complex that bridges two membranes (9-14), facilitating membrane fusion (6, 8). It has been thought that the SNARE complex provides the necessary free energy for membrane fusion (10, 15). However, recent experiments suggest that the SNARE complex acts instead as a catalyst for the fusion of two membranes (16, 17).

SNARE-induced membrane fusion transits through several intermediates (18). There is evidence that it proceeds through an intermediate called hemifusion (19-23), a condition in which the outer leaflets of the two membranes are merged, but the inner leaflets remain intact. In fact, hemifusion has been identified for both class I and class II viral-fusion proteins (24-27), suggesting that hemifusion might be a common intermediate for many biological fusions (28). In the fusion pathway, the hemifusion intermediate subsequently advances to the fusion pore that connects the two aqueous contents (24).

Although soluble parts of fusion proteins play a role in bringing about the apposition of two membranes leading to the formation of hemifusion, the transition from hemifusion to the fusion pore requires the transmembrane domain (TMD). For example, when the TMD of SNAREs are shortened or replaced with lipid anchors (19, 22), the fusion process stops at hemifusion, illustrating the role of the TMD in the transition from hemifusion to complete fusion. Similarly, hemifusion has been the final product for modified influenza hemagglutinin (HA) in which the TMD was replaced with a lipid anchor or shortened below the critical length (29). Therefore, it may be generally true that the TMDs of fusion proteins play a critical role in promoting the formation of the fusion pore.

In an initial attempt toward understanding of the role of the TMDs in SNARE-mediated membrane fusion, we investigated the structure and dynamics of the TMD of Sso1p, a syntaxin-analogue involved in post-Golgi protein trafficking in yeast, using site-directed spin-labeling EPR (30). The results show that the TMD of Sso1p is a transmembrane $\alpha$-helix with some tilt. Interestingly, there exists an equilibrium between the monomers and the
oligomers. Furthermore, the EPR line-shape analysis suggests that the TMD-TMD tertiary contacts are limited in the N-terminal half, whereas the C-terminal half is free of any tertiary contact.

One of the important parameters of the EPR spectrum is the hyperfine splitting resulting from electron-nucleus dipolar coupling. The hyperfine splitting of a nitroxide is sensitive to the polarity of the environment (31) and has been used, for example, as a qualitative indicator of the location of nitroxide in the membrane environment (33-35). Because the TMD of Sso1p is now shown to be a well-defined membrane-spanning α-helix, its nitroxide-scanning mutants may serve as an ideal model system to examine the variation of hyperfine splitting across the bilayer. The plot of isotropic hyperfine splitting versus the residue number shows a symmetric V-shaped curve, which may be used as a calibration curve to estimate the immersion depth of nitroxide in the membrane.

Results

Site-Directed Spin-Labeling EPR for the TMD of Yeast t-SNARE Sso1p. To investigate the transmembrane structure of yeast t-SNARE Sso1p using EPR, native residues were replaced one by one with cysteines that were modified with a nitroxide spin label. The Sso1pHT used in this study consisted of amino acids 185-290 of Sso1p without the N-terminal regulatory Habc domain. We made 35 single-cysteine mutants of Sso1pHT ranging from a.a. 256 through 290, which include the putative linker region (a.a. 256-265) and the putative TMD region (a.a. 266-280) (Figure 1). The spin labeling efficiency was greater than 80% for all mutants. Each spin-labeled mutant was reconstituted into the vesicles made of 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) (molar ratio of 85:15), lipids commonly used for in vitro studies of SNARE proteins. The functionality of the spin-labeled Sso1pHT mutants was tested using an in vitro fluorescence-fusion assay (36). All mutants showed at least 57% of the fusion activity of wild-type Sso1pHT (Figure 2).

After the reconstitution of Sso1pHT mutants into phospholipid vesicles, the EPR spectra were collected for spin-labeled mutants at room temperature (Figure 3). For A256CA259C, the EPR spectra are composed of two components, one sharp (arrow) and the other relatively
broad (asterisk). Because these positions are located near the water-membrane interface, we expect that the region might be in equilibrium between the free and membrane-bound states. Thus, it is likely that the sharp component represents the subpopulation that is free in solution, whereas the broad component represents the subpopulation that interacts with the membrane surface and tumbles slowly in the viscous headgroup region.

EPR spectra for R260C to A266C are all broad, indicating that the region is fully inserted into the membrane. The extent of line broadening in this region is prototypical for the nitroxide embedded in the headgroup region of the bilayer (37, 38) except for K261C, where we observed an extensive line broadening perhaps due to the tertiary interaction with the neighboring molecules. The EPR spectra for W267C and L268C are slightly narrower than those of the preceding positions, suggesting that residues following position 267 enter into the less viscous acyl chain region. Among those, positions 269, 270, 272, 273, and 276 manifest broad spectral components, indicative of tertiary contacts most likely with the neighboring TMDs. Importantly, the presence of two spectral components suggests that there may be equilibrium between the monomeric TMD and the oligomeric TMDs. We note that the five interacting positions reside within the N-terminal half of the TMD, revealing that the TMD-TMD interaction is mostly confined within the N-terminal half of the TMD.

The inverse of the central line width is often used as a semiquantitative measure of the tumbling rates of nitroxide (39). The line-shape parameter is plotted as a function of the residue number in Figure 4. In this Figure, it is clearly demonstrated that residue 259 is located right at the water-headgroup boundary. The line-shape parameter for residues from 260 to 276 remained near 0.24, although there are some variations along the sequence. However, we observe a significant and gradual increase in the lower half of the TMD, indicative of the increased dynamics at the C-terminal half of the SsO1pHT TMD.

Transmembrane Structure of SsO1pHT TMD Determined by Accessibility Measurements. We then investigated the nitroxide-scanning mutants of SsO1pHT using the EPR saturation method to determine the topology of the SsO1pHT TMD in the membrane (36, 40). With this EPR method, we measured the accessibility of nitroxide to a water-soluble paramagnetic reagent, nickel-ethylenediaminediacetic acid (NiEDDA) ($W_{\text{NiEDDA}}$), to estimate the extent of the seclusion of the spin-labeled site from the aqueous phase. We also measured
the accessibility of the nitroxide to a nonpolar paramagnetic reagent, molecular oxygen ($W_{O_2}$), to probe the immersion into the nonpolar membrane interior.

In Figure 5a, the $W_{NiEDDA}$ and $W_{O_2}$ values for the spinlabeled mutants of Sso1pHT are plotted as functions of the residue number. The $W_{NiEDDA}$ values show the representative U-shaped curve expected for a polypeptide passing through the entire bilayer. The collision with NiEDDA starts with very high values, indicative of the solvent exposure of the first few residues. The valley of low-collision frequency in the middle reflects the passage of the peptide chain through the bilayer. The collision frequency increases again to high values, showing the re-emergence of the peptide chain to the solution phase. In contrast, the collision with oxygen is inversely correlated with the behavior of the collision with NiEDDA; when the $W_{NiEDDA}$ values are low, the $W_{O_2}$ values are high and vice versa. Such an inverse correlation of $W_{NiEDDA}$ and $W_{O_2}$ has been considered as strong evidence of the membrane-spanning peptide. Over the entire range, the $W_{NiEDDA}$ profile and the $W_{O_2}$ profile crisscross each other twice, once near positions 263-265 and again near position 284.

For I269C, I273C, and V276C, the $W_{O_2}$ values are noticeably lower than those of other membrane-spanning residues. It is important to note that these three positions belong to the group of residues that show a broad spectral component stemming from tertiary contacts. In the helical wheel diagram (Figure 5c), these positions (color coded in red) are lined on one side of the helical surface, revealing the interacting surface of Sso1pHT TMD.

Quantitatively, it has been shown that the immersion depth of nitroxide is proportional to the logarithm of the ratio of $W_{NiEDDA}$ to $W_{O_2}$. For individual positions, the immersion depths were calculated by comparing the ratios of $W_{NiEDDA}$ to $W_{O_2}$ to a standard curve (40, 41) and the results were plotted with respect to residue numbers in Figure 5b. As expected, we obtained an overall V-shaped curve with the lowest point at position 275, which is located approximately 19 Å from the phosphate group near the center of the bilayer. It was also evident that residues 262-264 reside near the boundary between the headgroup region and the acyl chain region, which is located approximately 6 Å below the phosphate group (0 Å). On the other side of the membrane, residue 284 (proline) sits at the headgroup-acyl chain boundary. Therefore, from the immersion depth data, it appears that approximately 21 residues (from residue 264 to residue 284) span the 27-30 Å thick acyl chain region.
Considering the geometry of a standard α-helix (1.5 Å/residue), this result supports the idea that the TMD is a membrane-spanning α-helix with some tilt with respect to the membrane normal. The tilt angle was estimated to be less than 30°, although the exact value was difficult to determine. Furthermore, it is interesting to find that the data appears more scattered in the N-terminal half of the TMD than it does in the C-terminal half. This is likely due to the TMD-TMD interactions in the N-terminal half because the tertiary interaction at several residues makes accessibility measurements uncertain and leads to larger errors.

*Low-Temperature EPR to Measure Interspin Distances.* The EPR line-shape analysis indicated that residues 269, 270, 272, 273, and 276 might have partial tertiary contacts with neighboring TMDs; the EPR spectra are composed of two spectral components, indicating that there might be equilibrium between the monomeric TMD and multimeric TMDs. The Fourier deconvolution method (42) has proven to be powerful in determining the interspin distance between two interacting nitroxides and in estimating the percentage of the interacting spins when there is equilibrium between the interacting population and the noninteracting population (43). The low-temperature EPR spectra taken at 130 K (Figure 6, left panel) showed the line broadening due to the spin-spin interaction for all positions except position 270. The Fourier spectral analysis revealed that the monomer fraction consists of 60, 66, 56, and 68% for positions 269, 272, 273, and 276, respectively; Figure 6, right panel). The interspin distances in the oligomeric state were estimated by analyzing the remaining interacting factions, and they were 12-13 Å for all four positions. Thus, the Fourier deconvolution method strongly supports the idea that residues 269, 272, 273, and 276 participate in the intermolecular interaction between the TMDs. Position 270 is likely to be a peripheral residue for which the distance between residues is larger than 25 Å.

To verify the equilibrium between the monomeric TMD and the multimeric TMDs, we collected room-temperature EPR spectra for residue 273 at three different concentrations (Figure 7). We found that the broad spectral component (arrows in Figure 7), reflecting the oligomeric TMDs, increased noticeably as the protein concentration was increased, strongly supporting the idea that the monomers and multimers are in equilibrium.

*Isotropic Hyperfine Splitting Reflects the Membrane Immersion Depths.* One of the important parameters of the EPR spectrum is the hyperfine splitting resulting from electron-
nucleus dipolar coupling. The hyperfine splitting of a nitroxide is sensitive to the polarity of the environment and has been used as a means to measure the partitioning of a small nitroxide between water and the membrane, which has been used to detect the bilayer phase transition (32). Furthermore, this parameter has also been used as a qualitative indicator of the location of the nitroxide in the membrane environment (33, 34, 35, 44). Because the detailed structural features of the Sso1pHT TMD are now determined, the isotropic hyperfine values of the Sso1pHT mutants can serve as a model to explore the relationship between the hyperfine splitting profile across the bilayer and the membrane immersion depth. We attempted to plot the isotropic hyperfine splitting values of individual positions along the sequence (Figure 8). The plot shows an overall V-shaped curve; higher hyperfine splitting value as the positions move away from the center of the bilayer. Interestingly, the curve is symmetric and linear in the range of a.a. 266-284 which form the core helix spanning the acyl chain region of the bilayer. Near the headgroup-acyl chain interface, the curve shows discontinuities: one on the N-terminal side (a.a. 263-265) and another near residue 284 on the C-terminal side. The discontinuity at the N-terminal side might indicate that this region might not exist as an α-helix, consistent with previous results for syntaxin (38). However, we note that residue 284 is proline, which is a known helix breaker. Therefore, the break in the profile near position 284 may be attributed to the discontinuity in the α-helix structure near the headgroup-acyl chain interface. The results suggest that hyperfine splitting can be used as a complimentary measure of the membrane immersion depth of nitroxide for membrane-embedded helices.

**Discussion**

The EPR analysis of spin-labeled mutants suggests that the TMDs of Sso1pHT have a tendency to interact with one another, most likely to form an oligomeric structure. The results also show that there may be an equilibrium between the monomeric form and the oligomeric forms in a molar ratio of approximately 6:4 at the lipid-to-protein ratio of 300:1. Interestingly, the EPR line shapes showed that the tertiary contacts are limited within the N-terminal half, whereas the C-terminal half is free of such interactions. This result is consistent with the significant increase in the motional dynamics in the C-terminal region of the TMD.
(Figure 4). Potentially, the interaction between the TMDs would help the oligomerization of the SNARE complex at the fusion site. The oligomerization of the neuronal counterpart syntaxin has also been reported (45-47).

Combining the EPR line-shape analysis, accessibility measurements, and the Fourier-deconvolution analysis, it seems that residues 269, 273, and 276 are the positions involved in the tertiary interactions when the TMDs of Sso1pHT gather together. These residues are located on one side of the helix and form a well-defined interacting surface (see the helical wheel diagram in Figure 5c). Interestingly, the sequence alignment between neuronal syntaxin and yeast Sso1pHT (Figure 5d) reveals that residues 269, 273, and 276 match one-to-one to the residues in syntaxin that are proposed to line the transmembrane pore (48).

Oligomerization appears to be generally required for a variety of fusion proteins. For SNAREs, it has been shown that the coordination of at least three SNARE complexes is necessary for successful fusion (49). For influenza hemagglutinin (HA), it appears that 4-6 HA molecules work together at the fusion site (50). On the basis of electrical measurements, it has been proposed that 5-8 syntaxin TMDs form a cluster (48). In contrast, it has been previously shown that syntaxin forms dimers in the membrane (38). In the present EPR, it was not possible to estimate the exact stoichiometry of the oligomeric Sso1pHT TMDs, although partial clustering is clearly demonstrated.

Over the last several years, the EPR saturation method has proven powerful in determining the structure and topology of membrane-bound peptides and proteins. Although isotropic hyperfine splitting has often been used qualitatively to verify membrane insertion of polypeptides, its entire profile across the membrane was not extensively investigated. The TMD of Sso1pHT is a well-defined transmembrane helix and has served as an excellent system to determine the full profile of hyperfine splitting. The clean profile in Figure 8 demonstrates that a simple measurement of isotropic hyperfine splitting may be useful in assessing the immersion depths of nitrooxides, in addition to the results from a well-established EPR saturation method.

Materials and Methods
Plasmid Construction and Site-directed Mutagenesis. The DNA sequence encoding Sso1pHT (amino acids 185-290 of Sso1p) was inserted into the pGEX-KG vector between EcoRI and HindIII sites as N-terminal glutathione S-transferase (GST) fusion proteins (16). To introduce a unique cysteine residue for the specific nitroxide attachment, native cysteine 266 of Sso1pHT was mutated to alanine. A QuickChange site-directed mutagenesis kit (Stratagene) was used to generate all mutants; DNA sequences were confirmed by the Iowa State University DNA sequencing facility.

Protein Expression, Purification, and Spin Labeling. The expression of recombinant GST fusion proteins was conducted in E. coli Rosetta (DE3) pLysS (Novagene). The cells were grown at 37 °C in an LB medium with glucose (2 g/L), ampicillin (100 μg/mL), and chloramphenicol (25 μg/mL) until the A600 value reached 0.6-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. The cells were grown for four more hours at 18 °C. The cell pellets were harvested by centrifugation at 6000 rpm for 10 min.

Purification of GST fusion proteins was achieved with affinity chromatography using glutathione-agarose beads (Sigma). Frozen cell pellets were resuspended in a PBS buffer (phosphate-buffered saline at pH 7.4 with 0.5% Triton X-100 (v/v), PBST) with 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and 5 mM dithiothreitol (DTT). The cells were broken by sonication in an ice bath. As for Sso1pHT, 1% of n-lauroyl sarcosine was added to the solution before sonication. The cell lysate was centrifuged at 15 000g for 20 min at 4 °C. The supernatant was mixed with glutathione-agarose beads in the resuspension buffer and mutatned at 4 °C for 120 min. The protein-bound beads were washed with an excess volume of washing buffer (phosphate-buffered saline at pH 7.4) for at least six rounds. When washing, 0.2% (v/v) Triton X-100 was added to Sso1pHT. The beads were then washed with a thrombin-cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, and 2.5 mM CaCl2 at pH 8.0) and 0.2% Triton X-100. Finally, the proteins were cleaved from the resin by thrombin (Sigma) at room temperature for 40 min. AEBSF was added to the protein after cleavage (2 mM final concentration) to inhibit continuous cleavage by thrombin. The protein was stored at -80 °C with 10% glycerol.
Cysteine mutants of Sso1pHT were spin labeled before thrombin cleavage. After the cell lysate was incubated with beads and washed with the PBS buffer containing 0.2% Triton X-100, DTT was added to a final concentration of 5 mM. The sample was incubated at 4 °C for 40 min, and the beads were then washed six times with an excess volume of the PBS buffer with 0.2% Triton X-100 to remove DTT. About 20-fold excess of (1-oxyl-2,2,5,5-tetramethylpyrrolinyl-3-methyl) methanethiosulfonate spin label (MTSSL) was immediately added to the protein. The reaction mixture was left overnight at 4 °C. Free MTSSL was removed by washing with excess PBS buffer with 0.2% Triton X-100. The proteins were cleaved by thrombin in a cleavage buffer with 0.2% Triton X-100. Thrombin was not removed from the protein samples. It was expected that the small amount of thrombin (3 μM) would not influence the experimental data.

Membrane Reconstitution. Large unilamellar vesicles (~100 nm in diameter) of 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine (POPC) containing 15% 1,2-dioleoylsn- glycero-3-phosphatidyserine (DOPS) were prepared in a detergent-free cleavage buffer using an extruder (36). The total lipid concentration was 100 mM. The proteins were reconstituted into vesicles by the Bio-Beads method (36): The proteins were mixed with vesicles at a ~1:300 protein-to-lipid molar ratio. The detergent was removed by treating the sample with Bio-beads SM2 (Bio-rad), which was directly added to the sample in the ratio of 200 mg/1 mL of the mixed solution. After 45 min of nutation, the Bio-beads were removed from the sample by centrifugation at 10,000g for 1 min. The same procedure was repeated three times. Reconstitution efficiency was estimated by determining the protein concentration using EPR before and after reconstitution. For all samples, the efficiency was approximately 70%. To determine the orientation of reconstituted Sso1pHT on the vesicle, we treated the reconstituted protein with 2 mM trypsin for 1 h. An SDS-PAGE analysis indicated that the Sso1pHT protein was almost completely digested to small fragments (data not shown), suggesting that a majority of Sso1pHT analogues were oriented toward the outside of the vesicle.

EPR Experiments and Analysis. EPR spectra were obtained with a Bruker ESP 300 EPR spectrometer equipped with a loop-gap resonator. The modulation amplitude was set at no greater than one-fourth of the line width. Spectra were collected at either room temperature
or 130 K in the first-derivative mode. At room temperature, the microwave power was kept at 1 mW, but it was set at 8 μW at 130 K to avoid the saturation of EPR lines. All protein samples contained 10-15% glycerol as a cryoprotectant. For low-temperature EPR, the capillary sample tubes were plunged quickly into liquid nitrogen for fast freezing.

For saturation EPR, the gas exchange to the protein sample was achieved with a TPX tube for the loop-gap resonator. For individual mutants, power saturation curves were obtained from the peak-to-peak amplitude of the central line \((M_1 = 0)\) of the first-derivative EPR spectrum as a function of incident microwave power in the range of 0.1-40 mW. Three power saturation curves were obtained for each mutant after equilibration with (1) N\(_2\), (2) air (O\(_2\)), and (3) N\(_2\) in the presence of 200 mM NiEDDA (nickel ethylenediaminediacetic acid). From the saturation curves, the microwave power \(P_{1/2}\) (mW) where the first-derivative amplitude is reduced to one-half of its unsaturated value was calculated. The quantity \(\Delta P_{1/2}\) is the difference in \(P_{1/2}\) values in the presence and absence of a paramagnetic reagent, which is proportional to the diffusion coefficient times the frequency of the collision of nitroxide with freely diffusing reagents such as oxygen and NiEDDA. Thus, \(\Delta P_{1/2}\) is considered to be equivalent to the accessibility \(W\). The immersion depth is calculated on the basis of the reference curves determined from a set of lipid molecules spin-labeled at different acyl chain positions (40).

The isotropic hyperfine splitting for each spin-labeled mutant was determined empirically by measuring the peak-to-peak separation between the first and center lines of the EPR spectrum. This empirical value should be considered as an approximate measure of isotropic hyperfine splitting. It is expected that isotropic hyperfine splitting is independent of motional broadening or inhomogeneous line broadening as long as the molecules are not macroscopically oriented.

**References**

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Figure 1. Primary structure of yeast Sso1pHT.
Sso1pHT contains Sso1p amino acids 185-290. This polypeptide includes both the SNARE motif and the transmembrane domain, which are represented by a cylinder and a rectangle, respectively. Between them is the linker region that contains amino acids 256-265. The spin-labeled positions are shown below the schematic diagram.
**Figure 2. Fusion activity of spin-labeled Sso1pHT mutants.**

The fusion activity of each spin-labeled Sso1pHT mutant was measured using an *in vitro* fusion assay. The percent fusion activity of the mutant was calculated in reference to the fusion activity of the wild type.
Figure 3. Room-temperature EPR spectra of spin-labeled Sso1pHT transmembrane mutants.

The EPR spectra for A256C-A259C are composed of two spectral components: one sharp (arrows) and the other, relatively broad (asterisks).
Figure 4. Dynamic profile across the Sso1pHT TMD.

The inverse line width $(\Delta H)^{-1}$, which reflects the motion of the nitroxide, is plotted as a function of the residue number with arbitrary units. The inverse line width was calculated from the peak-to-peak width of the central line. The high $(\Delta H)^{-1}$ value reflects the fast motion and the low $(\Delta H)^{-1}$ value reflects the slow motion. The error bar represents the standard deviation of the results from three independent measurements of line width.
Figure 5. Structural determination of the Sso1pHT transmembrane domain with EPR accessibility measurements.

(a). Accessibility parameters $W_{O2}$ (open triangle) and $W_{NEDDA}$ (filled triangle) are plotted as functions of the residue number.

(b). Membrane immersion depths vs the residue number.

(c). A helical wheel diagram for the Sso1pHT TMD. Positions 269, 270, 272, 273, and 276, which show the tertiary contacts in the EPR spectra, are marked with asterisks, whereas positions 269, 273, and 276, which show the reduced $W_{O2}$ values, are color coded in red.

(d). Sequence alignment between the TMDs of neuronal syntaxin and Sso1p. Positions 269, 273, and 276, which show the tertiary interaction in this work, match one-to-one to the proposed pore-lining positions in syntaxin.
Figure 6. Low-temperature EPR spectra and Fourier deconvolution analysis for spin labeled Sso1pHT.

Low-temperature absorbance (integrated) spectra for spin-labeled mutants (-) were compared with a noninteracting reference spectrum (....) in the left panel. The dipolar broadening functions (in Fourier space) are shown in the right panel. The data was fitted with a sum of two Gaussians (-). In the Fourier space, the fraction of noninteracting monomers appears as a constant y-axis offset (42, 43). The y-axis offsets were around 60%.
Figure 7. Room-temperature EPR spectra of spin-labeled Sso1pHT mutant I273C. The molar lipid-to-protein ratios are given in parentheses. The arrows indicate the broad spectral component reflecting the multimers.
Figure 8. Profile of isotropic hyperfine splitting across the bilayer for the Sso1pHT TMD.

Isotropic hyperfine splitting for each spin-labeled mutant was determined by measuring the peak-to-peak separation between the first and center lines of the EPR spectrum. The error bars represent the standard deviation of results from three independent isotropic hyperfine-splitting analyses.
CHAPTER 4: STOICHIOMETRY OF Sso1p TMD Oligomer and TMD Conformational Change Involved in Transition From trans-SNARE to cis-SNARE

Abstract

Membrane fusion in eukaryotic cells is mediated by SNARE (soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptor) proteins. It is proposed that trans-SNARE complex assembly renders the docking of vesicles to the target membrane and initiates membrane fusion. Multi SNARE complexes may cooperate to promote this process. Trans-SNARE complex is converted to cis-SNARE complex as membrane fusion proceeds from hemifusion to full fusion. In this work, the stoichiometry of Sso1p transmembrane domain (TMD) oligomer determined from EPR spectral subtraction method ranges from 3 to 6. Combined with FRET data, it appears Sso1p TMD oligomer may act as a scaffold to recruit the Snc2p into the trans-SNARE formation prior to membrane fusion. Comparison of the structure of full Sso1p TMD oligomer at high surface SNARE density to that of partial TMD oligomer at low surface SNARE density provides possible structural explanation of transition from hemifusion to full fusion. Additionally, EPR spectra show upon the formation of cis-SNARE complex, the Sso1p TMD oligomer is broken apart, while the TMD heterodimerization between v-SNARE and t-SNARE in cis-SNARE on membrane does not show full interaction. Structural information concerning TMD conformational change implies its role in the transition from hemifusion to fusion pore formation. These data outlines a catalyst role of SNARE assembly throughout membrane fusion by displaying the synergy of cytoplasmic domain and transmembrane domain of SNARE protein in this process.
Introduction

Membrane fusion is a general process involved in material transport between endomembrane organelles as well as the exocytosis of in eukaryotic cells (1). In secretory pathways, SNAREs are well established to be the core fusion machinery (2-7). Generally vesicle-attached(v-) SNAREs engage with target membrane (t-) SNAREs to form a tightly bundled coiled coil SNARE complex that bridges two apposite membranes (8-13), facilitating membrane fusion (6, 14). Structural study of yeast trans-SNARE assembly on membrane implies a catalyst role of SNARE complex for membrane fusion (15).

SNARE-mediated membrane fusion transits through several intermediates (16). Trans-SNARE assembly may play an important role in the docking of vesicles onto the plasma membrane (17). The membrane fusion reaches a hemifusion intermediate at the beginning of lipid mixing, in which outer leaflets of the two apposite membranes are merged while inner leaflets remain intact (17-20). The hemifusion has been proposed to be a common intermediate for many biological fusions, which subsequently advances to the fusion pore leading to release of cargo loaded in vesicles outside of the target membrane (21, 22).

The study of in vitro yeast SNARE-mediated fusion assay displays hemifusion can convert into full fusion when surface density of SNARE protein increases (18, 19). This observation stimulates hypothesis of more than one SNARE complex may be involved in this process. Many researches have reported that oligomerization of SNAREs may be important for membrane fusion. In squid giant synapse, Synaphin helps SNARE complex be oligomerized into a high order structure which is required for neurotransmitter release (23). It suggests that oligomerization of SNARE complexes occurs prior to membrane fusion and arranged in a ring-like structure (24). There is still controversy about the stoichiometry of this multi-SNARE complex oligomer. In the fusion study of large dense-core granules of PC12 cells the inhibitory effect of increasing amounts of soluble SNARE domain of Vamp on the kinetics of calcium-induced granule release was monitored. The fitted data suggested three SNARE complexes cooperate in the fusion (25). Electrophysiological experiment estimated 5 or 8 Syntaxin lined fusion pore (26). Disclosing the exact stoichiometry of multimeric SNARE complex remains a challenging problem as to how SNARE promotes membrane fusion.
TMDs of yeast t-SNARE Sso1p protein tends to self associate into TMD oligomer in the membrane. Our previous data shows that Sso1p TMD oligomer exists in equilibrium with Sso1p TMD monomer (27). In an attempt to infer the stoichiometry of Sso1p TMD oligomer, EPR spectral subtraction method and FRET data suggests Sso1p TMD oligomers preformed in the membrane prior to membrane fusion and acted as a scaffold to recruit Snc2p in forming multimeric trans-SNARE complex. Another interesting discovery is that at low surface density of SNAREs, the TMD oligomer is only partially oligomerized in the upper half of TMD, while at high surface density, the Sso1p TMD monomers interact with other monomers strongly in the oligomer all along TMD domain.

Since trans-SNARE transits to cis-SNARE as fusion continues to full fusion, TMD conformational change concerning transition from Sso1p TMD oligomer to cis-SNARE complex is disclosed. EPR spectra of Sso1p TMD in cis-SNARE appear overall relatively sharp compared to that of Sso1p TMD full oligomer, indicating lack of full interaction between TMD domains of t-SNARE and v-SNARE proteins. Intriguingly, Sso1p TMD oligomer is likely broken apart upon the formation of cis-SNARE on membrane. Kinetics data disclosed N-terminal of trans-SNARE assembly occurs prior to membrane fusion while C-terminal TMD interaction of cis-SNARE occurs simultaneously with completion of full fusion. The structural information confirms the synergy of cytoplasmic domain and transmembrane domain of SNAREs play a catalyst role throughout the membrane fusion process.

Results

TMDs of t-SNARE Form Oligomers of Different Stoichiometries. In this study we studied the SNARE proteins involved in yeast exocytosis. Yeast SNARE shared a great functional and structural homology with neural SNAREs mediating neural transmitters’ release (30). The yeast SNARE protein Sso1p is a neural syntaxin analogue, anchored by its transmembrane domain in plasma membrane and thus is classified as t-SNARE (target-membrane SNAREs) (Figure 1a). Sec9 is also t-SNARE protein which contains two different SNARE motifs binding Sso1p to form a binary SNARE complex on plasma membrane (Figure 1c). Its neural analogue is SNAP-25. Snc2p is vesicle-attached SNARE protein as
neural Vamp2 (Figure 1b), which is required for the ternary SNARE assembly inducing fusion between vesicle and plasma membrane.

Our previous data have verified the equilibrium between monomeric TMDs and multimeric TMDs in the membrane. The room-temperature EPR spectra for residue 273 at different concentrations demonstrate that the broad spectral component reflecting tertiary contact between oligomeric TMDs increased significantly as the protein concentration was increased (27). To quantitatively analyze the equilibrium, site-directed spin labeling EPR (31) and EPR spectral subtraction analysis are employed (32).

In site-directed spin labeling, native amino acid residues are site-specifically replaced one by one with cysteines to which nitroxide side chain is attached. For Sso1p TMD oligomers in the membrane, there are potential tertiary contact positions between Sso1p TMD monomers. Upon formation of Sso1p TMD oligomers, the dynamics of TMD peptides would be reduced. Accordingly, the tumbling rates of amino acid side chains are significantly reduced. The EPR line shape is sensitive to the motional rates of the nitroxide (33). Thus, Sso1p TMD oligomer formation will be accompanied with large EPR line shape changes, from a narrow spectrum reflecting the fast motion to a broad spectrum reflecting the slow motion (27).

As each EPR spectrum can be convolution of a sharp spectrum and a broad one (Figure 2b), here we made a simple assumption for each spectrum: sharp component represents as a TMD monomer due to unrestricted motion of spin label attached to TMD monomer and broad component as a TMD oligomer. At each protein concentration, the fraction of TMDs forming oligomers in equilibrium with TMD monomers is equivalent to fraction of broad component calculated from EPR spectral subtraction method. Here we define it as x, C is the surface density of the protein, $K$ is the equilibrium constant and n is stoichiometric number.

$$K$$

$nM \leftrightarrow Mn$

4 different positions (269, 272, 273, 284) showing possible tertiary contact along the TMD sequence were selected (labeled by asterisk in Figure 1a). Four different surface densities (1:400, 1:200, 1:100, 1:50) of each spin labeled mutant were reconstituted onto the POPC vesicle containing 15 mol% negatively charged DOPS, a lipid composition commonly
used to mimic the native cellular membranes (6, 14, 34). Room temperature EPR spectra were collected for every sample (Figure 2A). After spectral subtraction analysis of each EPR spectrum, 4 “x” values corresponding to 4 surface protein densities respectively were obtained for each mutant position. The specific concentrations were plotted with respect to the fraction of Sso1p TMD oligomer (Figure 3A,B). The data then was fitted with formula derived from the formula calculating equilibrium constant. 4 stoichiometric “n” values were appropriately estimated: 269 produced 4, 272 produced 3, 273 produced 6 and 284 produced 6. Fluctuation of n values for different positions may reflect various spatial arrangements of the transmembrane domain in different oligomers. Thus, in the membrane there seems to be different Sso1p TMD oligomers with stoichiometries ranging from 3 to 6. Their internal tertiary arrangement may also differ.

*Sso1p TMD Forms Full Oligomers at High Surface Density of Protein.* We also discovered that at high protein concentration (1:50) on membrane, the EPR spectra for position 284 become very broad, indicating a strong interaction in this position. 284 is located in the C terminal of Sso1p TMD. It looks like a full Sso1p TMD oligomer forms at this concentration.

In order to test this, we made 30 single-cysteine mutants of Sso1p ranging from a.a. 261 through a.a. 290, which includes part of putative linker region (a.a.256-265) and the TMD region(a.a. 266-280)(Figure1A). The spin labeling efficiency was greater than 80% for all mutants. Each spin-labeled mutant was reconstituted into POPC vesicle containing 15 mol% negatively charged DOPS. All spin labeled mutants showed at least 57% of the fusion activity (data not shown here).

To show the formation of full Sso1p TMD oligomer in the membrane, each mutant was reconstituted onto the vesicle at protein to lipid ratio of 1:50. All the EPR spectra were collected at room temperature (column A of Figure 4). EPR spectra for the putative linker region from 261 to 265 become broad indicating nitroxides attached to these regions of Sso1p molecules were embedded into the negatively charged headgroup region. At the same time extensive line broadening at positions 261 and 262 showed possible tertiary interactions between neighboring molecules. Pattern of EPR spectra change from 285 to 290 is similar to above data, consistent with this segment of peptide’s similar location in the headgroup region.
on the other side of lipid bilayer. Following the linker region is the transmembrane domain ranging from 266 to 284 which is confirmed by the previous EPR power saturation data (27). The EPR spectra are demonstrating an overall line shape broadening all along the TMD. For positions 268, 269, 270, 273, 276, 282, 283, 284, the EPR spectra become very broad indicating almost all mutants in these regions have been fully assembled into the oilgomers (column A of Figure 4). Consistently, the above EPR spectral subtraction method also showed about 90% of Sso1p TMD had been oligomerized at 269, 273, 284 (Figure 3). In addition to that, the line shapes of these EPR spectra suggest potential spin-spin interaction between adjacent Sso1p TMD monomers. Dipolar EPR experiment is performed at these positions, the spin-spin distance is 12-13Å, confirming the closely associated Sso1p TMD monomers in the membrane (data not shown). In summing up above data, the transmembrane domains of Sso1p self-associate into a full Sso1p TMD oligomer in the membrane at high protein concentration.

Interestingly, when we compare the EPR spectra for the Sso1p oligomer at low protein concentration of 1:300 and high protein concentration 1:50, there appears to present a conformational change between these two kinds of oligomers. Sso1p TMD oligomer appears to be partial at low protein concentration with only upper half of TMD oligomerized (27). Upon increase of protein concentration the oligomer formation extends to the full length of transmembrane domain. As this conformational change is somewhat like a scissoring motion, we hypothesize these two conformation may favor two kind of membrane curvature: partial Sso1p TMD oligomer favors the negative membrane curvature while full TMD oligomer favors positive curvature. This provides a possible structural explanation of hemifusion arrested at low protein concentration and full fusion resulted from high protein concentration (18, 19). Whether this dynamic conformational change occurs in actual membrane fusion remains to be studied.

TMD Heterodimerization in cis-SNARE and TMD Conformational Change Involved in Transition from t-SNARE TMD Oligomer to cis-SNARE Complex. Trans-SNARE initiates membrane fusion and forms cis-SNARE as fusion completes (35). Since the cytoplasmic SNARE motifs of SNARE complex is required to be fully assembled for the fusion, it is also hypothesized coiled coil assembly may extend into the membrane forming a heterodimer
between TMDs of t-and v-SNAREs in cis-SNARE. The TMD heterodimerization may help complete the fusion.

Cis-SNARE formation is scanned with same 30 Sso1p TMD mutants covering part of putative linker region and transmembrane domain. At first, Sso1p, Sec9 and Snc2p are mixed at equal ratio to form the cis-SNARE complex in detergent before reconstitution into the lipid vesicle at protein to lipid ratio of 1:50. Choosing this concentration is aimed at comparing the Sso1p TMD’s behavior in transition from trans-SNARE to cis-SNARE complex. Here Sso1p TMD oligomer is considered as part of trans-SNARE structure in the membrane. The successful reconstitution of cis-SNARE on the membrane was checked by the EPR spectra of spin labels attached to two positions (66, 70) on Snc2p SNARE motif, which showed obvious broadening (data not shown).

EPR spectra for the linker region from 261 to 265 showed apparent line shape broadening, indicating potential tertiary contact in these regions (column B of Figure 4). Considering the tightly assembled core SNARE complex in the cytoplasmic domain, this interaction is likely resulted from the juxtaposition of two linker regions belonging to t-SNARE Sso1p and v-SNARE Snc2p. For the following region entering the membrane, most of the EPR spectra become less broad along the TMD, which showed in cis-SNARE complex, the interaction between Sso1p TMD and Snc2p TMD is not that strong as hypothesized. Still we can observe some apparent interaction at positions of 272, 274, 280, 282, 283 in Sso1p TMD (column B of Figure 4). We attribute the interaction to the TMD heterodimerization between Sso1p and Snc2p in the cis-SNARE complex because the fact of the spectra remaining the same even as cis-SNARE complex reconstituted at low protein concentration precludes the possible interaction between Sso1p TMD monomers (data not shown).

Above TMD stoichiometric estimation implies multimeric trans-SNARE complexes may form at the beginning of fusion as Sso1p oligomer potentially acts like a scaffold to recruit other t- and v- SNAREs. As the cis-SNARE complex is the final state of SNARE complex, we want to disclose the structural clues of the transition process from trans-SNARE to cis-SNARE concerning behavior of Sso1p TMDs. The EPR spectra corresponding to the positions of 268, 269, 270, 273, 276, 282, 284 in the Sso1p TMD
oligomer demonstrate some spin-spin interaction between Sso1p TMD monomers, while spectra corresponding to positions in cis-SNARE shows no such interactions and are relatively sharp. It seems that Sso1p TMD no longer has close interaction between each other in the membrane once cis-SNARE complex is formed. Logically, in the middle of membrane fusion process Sso1p TMD oligomer may be broken apart upon the cis-SNARE formation on membrane.

Comparison of Kinetics of SNARE Assembly. SNARE Proteins’ Oligomerization, and Lipid Mixing Process Implies SNARE Assembly Covers the Whole Membrane Fusion Process\(^1\). As we have identified the Sso1p TMD oligomer and disclosed the possible implication of transition from Sso1p TMD oligomer to cis-SNARE complex, the next step is to establish a structural and temporal relationship between the SNARE assembly across the membrane and lipid mixing process.

First, we want to know whether Sso1p and Snc2p were oligomerized during fusion. To achieve this, fusion assay was adopted in which one of SNARE protein was labeled with cy3 (Sso1p) or cy5 (Snc2p) at N-terminus or C-terminus with other proteins being wild type. During fusion process, if the same SNARE protein monomers self associated with each other, their mutual distance would be shorter, energy transfer should happen between adjacent fluorophores, resulting in decrease of the fluorescence intensity called self quench. On the other hand, if the labeled proteins are oligomerized at the beginning and then get away from each other, we would observe the increase of the fluorescence intensity. This is called self dequench.

For four cy3 or cy5 labeled mutants (Snc2p P13C, Snc2p S115C, Sso1p E185C, Sso1p R290C), only Snc2p C-terminal labeled protein (Snc2p S115C) showed the decrease of fluorescence intensity at the first 10 min indicating Snc2p c-terminal forming oligomers. We can further deduce that Snc2p proteins become oligomerized with its TMDs interacting with each other as fusion initiated. As self quench is not observed either on N-terminal or C-terminal of Sso1p during whole fusion process, it can be explained that Sso1p oligomer preformed prior to membrane fusion (Figure 5).

\(^1\) This part of work was performed by Xiaobing Lu. It is quoted here with permission as an important part to complement this work.
Next we want to compare the SNARE assembly kinetics with that of lipid mixing. Fluorescence lipid mixing assay showed in the lipid mixing process, outer leaflet mixing occurred faster than inner leaflet mixing (Figure 6), evidence of hemifusion happening at first. FRET was observed both in N-terminal and C-terminal assembly of SNARE complex across the membrane. According to the kinetics curve, N-terminal assembly was faster than C-terminal (Figure 6). When normalized kinetics of outer and inner leaflet mixing are compared with that of SNARE assembly, it is discovered N-terminal SNARE complex assembled faster than the outer leaflet mixing while C-terminal SNARE assembly occurred almost simultaneously with inner lipid leaflet mixing. Since outer leaflet mixing and inner leaflet mixing represents the start and completion of membrane fusion respectively, the data established that in the membrane fusion process, trans-SNARE complex was first assembled in N-terminal of cytoplasmic domain prior to lipid mixing and C-terminal of TMD forms heterodimer in cis-SNARE complex at last, concurrent with full fusion.

Notably, overlapping of kinetics curve of SNARE N-terminal assembly and Snc2p C-terminal oligomerization demonstrated the two processes happened at the same time, foretelling trans-SNARE assembly tightly coupled with self-association of Snc2p monomers in the membranes. This confirms our hypothesis that Sso1p oligomer performs as a scaffold recruiting Snc2p and Sec9 in the formation of multimeric trans-SNARE complexes prior to lipid mixing.

Discussion

In this work sotichiometry of t-SNARE protein Sso1p TMD oligomer was estimated, which indicates Sso1p forms oligomers of different stoichiometries anchored to membrane by self-associating TMDs. Prior to membrane fusion it acts as a scaffold for initial multimeric trans-SNARE complex formation. Upon high protein surface density the whole length of TMD is fully oligomerized. As conversion from trans-SNARE to cis-SNARE drives hemifusion to full fusion, full SNARE assembly is extended to C-terminal of TMD region. Structurally, the Sso1p TMD oligomer may be broken apart after the cis-SNARE complex forms in the membrane.
Oligomerization of fusion protein appears to be generally required for various kinds of membrane fusion activities. In the yeast homotypic vacuole fusion, SNAREs and other chaperones are drawn together into a vertex ring which is ring-shaped microdomain surrounding boundary membrane—a spatial arrangement for possible formation of high order SNARE complex (36). On the basis of electrical measurements it has been estimated that 5 or 8 syntaxin TMDs form a cluster (26). Alternatively, three SNARE complexes were obtained by fitting a fusion kinetics assay to a theoretical function (25). The existence of multi SNARE complexes in the cells was not only confirmed through extract of high order SNARE complexes from squid synaptosomes or brain (23), it is also observed on artificial membrane by atomic force microscopy (24).

Based on the equilibrium between Sso1p TMD monomers and TMD oligomer, the quantitative EPR spectral subtraction method helps us estimate the likely stoichiometry of TMD oligomer in the membrane. It seems, in the membrane there exist different TMD oligomers with stoichiometries ranging from 3 to 6. Different “n” values for selected positions may reflect specific tertiary arrangements within oligomers of specific stoichiometry. This result is quite consistent with other stoichiometric estimation of multimeric SNARE complexes involved in exocytosis. Still, we don’t know how Sso1p TMD oligomers of different stoichiometries are kinetically assembled. Is there any dynamic conversion between these oligomers? These questions remain to be further studied.

Hypotheses have been made to explain how these multimeric SNARE complexes are clustered. One of explanation is the domain swapping of SNAP 25 interconnects adjacent SNARE four helix bundles (37). This explanation may not be appropriate in the yeast multimeric SNARE complexes’ formation since structural data indicates prior to fusion, the yeast trans-SNARE is partially zipped with parts of the SNARE motif unstructured and flexible (from preliminary data of our lab). Other studies established transmembrane segments’ potential for the high order complex formation (38-41). Our structural and kinetic data illustrates the t-SNARE Sso1p TMDs’ tendency to form oligomer in the membrane. To form initial trans-SNARE complex, it induced the oligomerization of Snc2p in the vesicle. All these indicate Sso1p oligomers act as a scaffold to recruit Sec9 and Snc2p to form a supra trans-SNARE complexes prior to membrane fusion. Another deduction of this discovery is
TMD oligomers of v-SNARE and t-SNARE may form a gap-junction like structure as the initial fusion machinery in the fusion area before the lipid mixing. It is recently reported that TMDs of syntaxin 7 and endobrevin (Qa and R protein respectively) are responsible for the multimerization of endosomal SNARE complexes (42). Our discovery makes a big jump from that by offering clues on how the TMDs of t-and v-SNAREs might be organized in this process before membrane fusion initiates.

Hemifusion is an intermediate in the membrane fusion. The transition from hemifusion to full fusion is accompanied with the change of membrane curvature. The negative membrane curvature is supposed to favor the hemifusion since negative-curvature-promoting PE enhances hemifusion while positive-curvature-promoting lysolipid inhibits this process (21, 43). It is believed that transmembrane domain of SNARE protein play a role in the transition process. One of the proposed mechanisms is self-interaction of TMDs of t-SNARE induces the hemifusion-to-full fusion transition (44). Another relevant study showed hemifusion can be trapped by low surface density while full fusion results from high protein concentration (18, 19). In this study, the hemifusion-to-full fusion transition may be structurally established by a possible transition of partial Sso1p TMD oligomer to full TMD oligomer caused by surface density change of SNARE protein. Although this process wasn’t recapitulated by FRET from the fluorophore attached to position 290, this may be due to position’s locating outside interacting transmembrane domains and bulky group of fluorophore may not be sensitive to detect the delicate scissor-like conformational change in the partial-to-full TMD oligomer transition.

Hemifusion tends to form fusion pore which will further expands to lead to full fusion process (41, 45, 46). Formation of the fusion pore requires the participation of TMD of v-SNARE: Structurally, half truncated v-SNARE TMD mutant trapped the fusion at hemifusion implying that fusion pore could not open (18); Temporally, C-terminal TMD interaction in cis-SNARE occurred simultaneously with inner leaflet mixing. It stimulates us to propose TMD interaction of v- and t-SNARE complex induces the fusion pore formation. Based on the partial and relatively weak interaction between TMDs in the cis-SNARE, a structural scenario of fusion pore formation is suggested: initially the TMDs of v- and t-SNARE may interact with each other tentatively to open a small membrane pore. If this
interaction can not proceed all the way along the whole length of TMD and possibly the two TMDs are separated again, the pore may close prematurely before inner leaflet lipid mixing. Once TMDs of v-SNARE and t-SNARE begin to interact with each other fully, a mature fusion pore forms and proceeds to full fusion irreversibly. Because this process is dynamic and involves some uncertainties, the fusion pore formation may be the most time-consuming process in membrane fusion, in good agreement with data from observation of single vesicles’ fusion (47). Also, the structural comparison indicates the formation of cis-SNARE complex may break apart the fully assembled Sso1p TMD oligomers, it is logical to infer that this structural transition may eliminate protein barriers around fusion area to facilitate the fusion pore enlargement.

Based on these data and previous work, it is reasonable to propose a model on how SNARE assembly promotes membrane fusion. Initially, a preformed t-SNARE Sso1p oligomer was anchored in the membrane establishing a scaffold to recruit v-SNARE Snc2p from vesicles with the help of Sec9. This made it possible of 3 to 6 trans-SNAREs assembled and clustered simultaneously with self-association of Snc2p monomers in the membrane. As trans-SNARE formation proceeds from a partially zipped SNARE to a full SNARE complex, two separate membranes begin to fuse and first reach a hemifusion state. The scissor-like conformational change from a partial Sso1p TMD oligomer to a full Sso1p TMD oligomer may set up a proper membrane curvature change from negative to positive which favors fusion pore formation. As trans-SNARE assembly may further extends into transmembrane domain, the tentative contact of TMDs from t- and v-SNAREs opens a pore from the hemifusion diaphragm while only final fully interacting TMDs may render the mature fusion pore formation leading to irreversible full fusion. Simultaneously, the disassembly of Sso1p TMD oligomers induced by the cis-SNARE formation further facilitates the ongoing membrane fusion by eliminating protein barrier to fusion pore enlargement and catalyzing full fusion.

In summing up above structural and kinetic data in this study, we can see that membrane fusion involves both multi-steps of lipid mixing and several intermediates of SNARE assembly corresponding to cytoplasmic domains and transmembrane domains. This established a structural and temporal relationship between specific SNARE protein’s
conformational change and relevant lipid mixing state. The work here presents a novel structural perspective in our understanding of membrane fusion mechanism: synergy of transmembrane domains and cytoplasmic domains of SNARE demonstrates an essential catalyst role throughout membrane fusion.

Materials and Methods

**Plasmids and Site-directed Mutagenesis.** DNA sequences encoding Sso1pHT (amino acids 185–290 of Sso1pHT) and Snc2p (containing amino acids 1–115) were inserted into the pGEX-KG vector between *Eco*RI and *Hind*III sites as N-terminal glutathione S-transferase (GST) fusion proteins. Sec9c (amino acids 401–651 of Sec9) was inserted into pET-24b(+) between *Nde*I and *Xho*I sites as a C-terminal His6-tagged protein. To introduce a unique cysteine residue for the specific nitroxide attachment, native cysteine 266 of Sso1pHT was mutated to alanine. A QuickChange site-directed mutagenesis kit (Stratagene) was used to generate all mutants; DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.

**Protein Expression, Purification, and Labeling.** Expression of recombinant GST fusion proteins was conducted in *Escherichia coli* Rosetta (DE3) pLysS (Novagene). The cells were grown at 37°C in LB medium with glucose (2 g/liter), ampicillin (100 μg/ml), and chloramphenicol (25 μg/ml) until the A<sub>600</sub> reached 0.6–0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells were grown for an additional 4 hours at 16°C. Cell pellets were harvested by centrifugation at 6000 r.p.m. for 10 min.

Purification of GST fusion proteins was achieved with affinity chromatography using glutathione-agarose beads (Sigma). Frozen cell pellets were resuspended in PBS buffer (phosphate-buffered saline, pH 7.4, with 0.5% Triton X-100 (v/v), PBST) with 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and 5 mM dithiothreitol (DTT). The cells were broken by sonication in an ice bath. As for Sso1pHT, 1% of *n*-lauroyl sarcosine was added to the solution before sonication. The cell lysate was centrifuged at 15 000g for 20 min at 4°C. The supernatant was mixed with glutathione-agarose beads in the resuspension buffer and nutated at 4°C for 120 min. The protein-bound beads were washed with an excess
volume of washing buffer (phosphate-buffered saline, pH 7.4) for at least 6 rounds. When washing, 0.2% (v/v) Triton X-100 was added to Sso1pHT. The beads were then washed with thrombin cleavage buffer (50 mM Tris–HCl, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0), and 0.2% Triton X-100 or 1% OG depending on latter reconstitution method. Finally, the proteins were cleaved from the resin by thrombin (Sigma) at room temperature for 40 min. AEBSF was added to the protein after the cleavage (2 mM final concentration). The protein was stored at -80°C with 10% glycerol.

Cysteine mutants of Sso1pHT were spin labeled before thrombin cleavage. After the cell lysate was incubated with beads and washed with PBS buffer containing 0.2% Triton X-100, DTT was added to a final concentration of 5 mM. The sample was incubated at 4°C for 40 min, and the beads were then washed six times with an excess volume of PBS buffer with 0.2% Triton X-100 to remove DTT. About 20-fold excess of (1-oxyl-2,2,5,5-tetramethylpyrroliyl-3-methyl) methanethiosulfonate spin label (MTSSL) was immediately added to the protein. The reaction mixture was left overnight at 4°C. Free MTSSL was removed by washing with excess PBS buffer with 0.2% Triton X-100. The proteins were cleaved by thrombin in cleavage buffer with 0.2% Triton X-100/1% OG. It was expected the small amount of thrombin (3 μM) would not influence the experimental data.

Labeling Sso1p and Snc2p proteins with fluorophores was described as earlier (28). Sso1pHT E185C and Sso1pHT R290C proteins were labeled with Cy3 maleimide, Snc2p P13C and Snc2p S115C were labeled with Cy5 maleimide (Amersham). The free dyes were removed from the proteins by using PD-10 desalting columns (Amersham).

The His₆-tagged protein Sec9c was expressed in E. coli Rosetta (DE3) pLysS. The cells were grown at 37°C in LB medium with glucose (2 g/liter), kanamycin (30 μg/ml), and chloramphenicol (25 μg/ml) until the A₆₀₀ reached 0.6–0.8. After IPTG (1 mM) addition, the cells were incubated further for four more hours at 16°C. The cell pellets were collected by centrifugation at 6000 r.p.m. for 10 min.

For purification, the frozen cell pellet was resuspended in lysis buffer (PBS buffer with 20 mM imidazole, 0.5% Triton X-100, 2 mM AEBSF, pH 8.0). After sonication on ice, the cell lysate was centrifuged at 15,000g for 15 min at 4°C. The supernatant was mixed with nickel-nitrilotriacetic acid-agarose beads (Qiagen) in lysis buffer. The mixture was mutated
for binding at 4°C for 120 min. After binding, the beads were washed with washing buffer (PBS buffer with 50 mM imidazole, pH 8.0). Then the protein was eluted by elution buffer (PBS buffer with 250 mM imidazole, pH 8.0). The protein can be stored at -80°C with 10% glycerol. All purified proteins were examined with 15% SDS–PAGE.

*Membrane Reconstitution.* Large unilamellar vesicles (~100 nm in diameter) of 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine (POPC) containing 15% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) were prepared in a detergent-free cleavage buffer using an extruder. The total lipid concentration was 100mM. To study the stoichiometry of Sso1p TMD oligomer, the proteins were reconstituted into vesicles by the Bio-Beads method. The proteins were mixed with vesicles at a series of protein-to-lipid molar ratios (1:400, 1:200, 1:100, 1:50). The detergent was removed by treating the sample with Bio-Beads SM2 (Bio-rad), which was directly added to the sample in the ratio of 200 mg/1 mL of the mixed solution. After 45 min of nutation, the Bio-beads were removed from the sample by centrifugation at 10000g for 1 min. The same procedure was repeated three times. Reconstitution efficiency was estimated by determining the protein concentration using EPR before and after reconstitution.

For *cis*-SNARE reconstitution, Sso1p, Snc2p and Sec9 were ratio equally mixed to form *cis*-SNARE complex in HEPES buffer (25mM HEPES, 500mM KCl, 1% OG) at room temperature for 1 hour. Preformed *cis*-SNARE complex was mixed with liposome at protein to lipid molar ratio of 1:50, and then incubated at 4°C about 1 hour. Then the liposome/protein mixture was diluted until the concentration of OG was below the critical micelle concentration. The detergent was removed through dialysis with 2 litters of dialysis buffer (25mM HEPES, 250mM KCl, 5% glycerol) at 4°C overnight. Sso1p reconstitution for all mutants at high protein to lipid molar ratio (1:50) was also performed by similar dialysis method. The reconstitution efficiency of both methods was approximately 70%. Before taking EPR spectra, all samples were concentrated by 30-kDa cutoff centrifugal filter (Millipore).

*EPR Data Collection.* EPR spectra were collected using the Bruker ESP 300 spectrometer equipped with a loop-gap resonator. The modulation amplitude was set at no greater than one-fourth of the line width. Spectra were collected at room temperature.
**Lipid Mixing Assay for Wild-type Proteins.** Sso1pHT was reconstituted into the vesicles containing POPC and DOPS (molar ratio 65:35) in a lipid/protein ratio of 200:1. Snc2p was reconstituted into the vesicles containing POPC, DOPS, NBD-PS (1,2-dioleoyl-sn-glycero-3-phosphoserine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)), and rhodamine-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)) in the molar ratio of 62:35:1.5:1.5. To measure the lipid mixing, v-SNARE (Snc2p) liposomes were mixed with t-SNAREs (Sso1pHT) liposomes and Sec9c in the ratio of 1:1:1. The final solution for each reaction contained about 1mM lipids in Hepes buffer (25mM Hepes, 100mM KCl, PH 7.4) with a total volume of 100μl. Fluorescence intensity was monitored with the excitation and emission wavelengths of 465 and 530nm, respectively. The fluorescence signal was recorded by a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100μl with a 2-mm path length. After 3600s, 10% reduced-triton was added to obtain the maximum fluorescence intensity (MFI). All of the lipids mixing experiments were carried out at 35°C. The inner leaflet mixing assay was modified from the method developed by Meers et al (29). The details of the method had been described elsewhere. After collecting the time traces of total lipid mixing and inner leaflet mixing separately, the outer leaflet mixing was calculated as \(2P_T - P_I\), where \(P_T\) is the percentage of maximum for total lipid mixing and \(P_I\) is the percentage of maximum for inner leaflet mixing.

**SNARE Assembly Assay and self Quench/Dequench Measurement for Mutant Proteins.** The reconstitution procedure is similar to that described above for wild-type proteins except that the vesicles used here contain POPC and DOPS (molar ratio 65:35). Fluorescence intensity was monitored in two channels with the excitation wavelength of 555 nm and emission wavelengths of 570 and 668 nm respectively. To measure self quench/dequench, fluorescence intensity was monitored with the excitation and emission wavelengths of 555 and 570 nm for cy3 labeled proteins and with the excitation and emission wavelengths of 625 and 668 nm for cy5 labeled proteins. N-terminal SNARE assembly was monitored by mixing N-terminal labeled Snc2p P13C, Sso1pHT E185C and Sec9. C-terminal SNARE assembly is monitored by mixing C-terminal labeled Snc2p S115C, Sso1pHT R290C and Sec9.
References


Figures and Captions

Figure 1. Primary structures of recombinant yeast SNAREs.

(a). Sso1pHT contains the amino acids 185-290 of Sso1p. This polypeptide includes both the SNARE motif and the transmembrane domain, which are represented by the cylinder and the rectangle, respectively. The amino acid sequence of the spin-labeled region is shown below the schematic diagram. Selected positions for EPR spectral subtraction are labeled by asterisks.

(b). Snc2p contains the amino acids 1-115. The molecule covers the SNARE motif and the transmembrane domain, which are represented similarly as in Sso1p by the cylinder and the rectangle, respectively.

(c). Sec9c represents amino acids 401-651 of Sec9 and contains two SNARE motif regions. The SNARE motif regions are represented by the cylinders.
Figure 2. Room-temperature EPR spectra of spin-labeled Sso1pHT mutant I273C and illustration of EPR spectral subtraction method.

(a). Room-temperature EPR spectra were collected at selected spin-labeled position of Sso1p TMD (in this case 273) at a series of increasing protein-to-lipid molar ratios: 1:400, 1:200, 1:100, 1:50.

(b). The room-temperature EPR spectrum of Spin-labeled Sso1pHT mutant can be decomposed into one sharp EPR spectrum and one broad EPR spectrum. Fraction of broad spectrum reflecting the Sso1p TMDs forming oligomers is calculated.
Figure 3. Stoichiometry of Sso1p TMD oligomer obtained through fitting and calculation of results from EPR spectral subtraction analysis.

(a). Fraction of oligomerized Sso1p TMDs was calculated from the EPR subtraction method for each position at a certain protein to lipid molar ratio. For each position selected along Sso1p TMD (269, 272, 273, 284), a plot was drawn with logarithm value of specific total Sso1p protein concentration versus fraction of oligomerized Sso1p TMDs. The data was fitted by a curve according to the formula: \[ \ln C = \frac{\ln K + \ln n + n \times \ln(1-x) - \ln x}{1-n} \]. C represents total concentration of Sso1p in the membrane, K represents the equilibrium constant of Sso1p TMD monomers and oligomers. x represents the fraction of oligomerized Sso1p TMD monomers in the membrane.

(b). Overlapping of individual fitting results: red, 269; blue, 272; pink, 273; green, 284.
Figure 4. EPR assay of cis-SNARE complex formation and Sso1p TMD oligomerization at high protein concentration.

(a). Room-temperature EPR spectra of spin-labeled Sso1pHT transmembrane mutants reconstituted at protein-to-lipid molar ratio 1:50.

(b). EPR spectra of Sso1pHT mutants after it formed cis-SNARE complex and was reconstituted into lipid vesicle at the same protein concentration as above.
Figure 5. Self quench/self dequench measurement for the four mutants individually. Fluorescence changes of Cy3 or Cy5 labeled proteins for Sso1pHT E185C(Cy3) (blue), Sso1pHT R290C(Cy3) (pink), Snc2p P13C(Cy5) (red) and Snc2p S115C(Cy5) (green).
Figure 6. Comparison of the kinetics of SNARE complex formation with that of the lipid mixing.

(red) the Snc2p115C(Cy5) self quench kinetics, (blue) the SNARE complex N-terminal assembly kinetics, (pink) the outer leaflet mixing, (black) the inner leaflet mixing, and (green) the SNARE complex C-terminal assembly kinetics.
CHAPTER 5: GENERAL CONCLUSION

It has been postulated by zippering model of SNARE assembly that SNAREs assembled from N-terminal to C-terminal of SNARE motif, generating energy for membrane fusion (1, 2). By EPR-scanning yeast trans-SNARE assembly on membrane, we identified a partially zipped SNARE complex in which the N-terminal regions of individual SNARE motifs engaged one another to form the coiled coil, whereas the C-terminal regions are separate and unstructured. The existence of partially zipped complex has been supported by several biochemical experiments (3-5). Transition from this partially zipped SNARE to fully zipped SNARE released little energy, which leaves open the possibility of multi-SNARE complex involved in this process.

Kinetically and functionally, transmembrane domain is proposed to be the executer of membrane fusion (6-10). The next step is to define its structure. Accessibility profile to solvent-exposed NiEDDA and solvent-secluded oxygen demonstrated a membrane spanned structure of Sso1p TMD. Immersion depth of each position was calculated and plotted, which showed an overall V-shaped curve with position 275 locating 19 Å from the phosphate group nearing the center of the bilayer. Approximately 21 residues of Sso1p TMD span the 27-30 Å thick acyl chain regions. With above analysis we can confidently concluded that Sso1pHT TMD is membrane spanning α-helix of some tilt with respect to membrane normal. This discovery is comparable to the α-helix of transmembrane domain of v-SNARE Snc2p (8), establishing SNAREs anchored in the membrane by membrane spanning α-helix.

t-SNARE Sso1p TMDs self associate into oligomer in the membrane. This provides clues to study high order SNARE multi-complex in the membrane. Transmembrane domain of SNARE protein is proposed to be important for SNARE complex oligomerization (11). Stoichiometry of SNARE oligomer has been estimated indirectly from biochemical and electrophysiological methods (12, 13). EPR spectral change demonstrated t-SNARE Sso1p TMD oligomer coexists with TMD monomer in equilibrium in the membrane: The broad component of EPR spectra corresponding to tertiary contact positions of intra-oligomers increases as surface density of SNARE protein rises. EPR spectral subtraction method based on the establishment of equilibrium discloses existence of Sso1p TMD oligomers of
stoichiometries ranging from 3 to 6 in the membrane. In forming *trans*-SNARE complex, the preformed Sso1p oligomer induced the self association of Snc2p TMDs in the vesicle. All these indicate Sso1p oligomer acts as a scaffold to recruit Sec9 and Snc2p to form supra *trans*-SNARE complexes prior to membrane fusion.

Several SNARE assembly intermediates were also discovered to be potentially coupled to substeps of membrane fusion. First, the Sso1p TMD oligomer has different conformations according to different surface density of SNARE protein on membrane. At low protein to lipid ratio, the TMD oligomer is partial oligomer, of which the upper half of TMD is oligomerized while lower half is flexible, unstructured; At high protein to lipid ratio, the oligomer is fully assembled along the whole length of Sso1p TMD. The implication of structural change upon the change of surface density of SNARE protein is explained as the promotion of membrane curvature change during membrane fusion (from negative to positive), which is a critical step from hemifusion to full fusion \((14)\). This is also a structural explanation why low density of SNARE protein on membrane arrested the fusion at hemifusion \((8, 15)\).

Next, EPR spectra Sso1p TMD of *cis*-SNARE complex in the membrane are overall relatively sharp in the transmembrane domains, indicating lack of full strong interaction during TMD heterodimerization between t- and v-SNARE in the final *cis*-SNARE formation. TMD heterodimerization is still required for the fusion pore formation. Kinetic and structural data suggests in the hemifusion-fusion pore transition, TMDs in SNARE may experience several tentative contacts before final full heterodimerization leads to irreversible fusion pore formation.

Last, Comparing the EPR spectra of for Sso1p TMD oligomer and *cis*-SNARE in the membrane demonstrates that Sso1p TMD no longer has close interaction between each other in the membrane once *cis*-SNARE complex forms. It is thus hypothesized that Sso1p TMD oligomer is broken apart upon the *cis*-SNARE formation on membrane, potentially eliminating fusion barrier to fusion pore enlargement.

From above analysis, my work tried to establish a structural and temporal relationship between SNARE assembly intermediates and sub-steps of membrane fusion process. It is also implied that the synergy between cytoplasmic and transmembrane domain of SNARE
proteins facilitates completion of the membrane fusion process. This study contributes a novel structural perspective to illustrate the mystery of membrane fusion mechanism.

References
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