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 Sso1pSec9 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.

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 Sso1pSec9 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.

Experimental Procedures

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 Xhol sites as a C-terminal His6-tagged protein. To introduce a unique cysteine residue for the specific nitroxide attachment, native cysteine 286 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 (Novagen). 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 A600 reached 0.6–0.8. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM. The cells were grown for an additional 4 h at 18 °C. Cell pellets were harvested by centrifugation at 6000 rpm for 10 min.
Partially Zipped SNARE Complex

The protein was stored at 4 °C. 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 then washed eight times with an excess of PBS buffer with 0.2% Triton X-100 to remove dithiothreitol. The protein was cleaved from the resin by thrombin (Sigma) at room temperature—

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 120 min. The protein-bound beads were washed with an excess volume of PBS buffer with 0.2% Triton X-100 for at least six rounds. When washing, 0.2% (v/v) Triton X-100 was added to Sso1pHT, whereas no detergent was added to Sn2pS. 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—

Spectra were collected at room temperature. Partially Zipped SNARE Complex—For the lipid-mixing fusion assay, two different populations of vesicles were separately prepared. Sn2p 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-benzo- xadiazol-4-yl) at a molar ratio of 62:35:1:5:1. 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 Sn2p-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 all samples were 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.

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 poly peptides become α-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 unstructured-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 a or d 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 Gibb’s free energy difference (ΔG°) 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 ΔG° = −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 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
Fig. 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.
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 disrupt coiled coil 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 mem-
brane 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 engage 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.

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